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The Involvement of Retroviruses in Human T Cell Leukaemias and Lymphomas

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Abstract

Human T lymphotropic virus type I (HTLV-I) causes adult T cell leukaemia/lymphoma (ATL), a neoplasm of CD4+ T cells. The related virus HTLV-II has been isolated from cases of CD8+ T cell variant hairy cell leukaemia but has not been definitively associated with neoplasia. Bovine leukaemia virus (BLV), which causes B cell leukaemia and lymphoma in cattle, belongs to the same group of retroviruses. The hypothesis tested in this study was that HTLV-I, HTLV-II or related viruses are associated with T cell leukaemias and lymphomas in the United Kingdom, particularly mycosis fungoides (MF) and Sézary syndrome (SS). A combination of cell and molecular biology techniques was used in an attempt to identify retroviruses in patients with these neoplasms.

Lymphocytes were cultivated from blood, skin and lymph nodes of patients with cutaneous lymphoid infiltrates to establish continuous T cell lines that might propagate HTLV-I, HTLV-II or related retroviruses. Techniques for the establishment of cultures included stimulation with mitogens, cytokines, conditioned medium and cocultivation. Cultured cells were examined for evidence of retroviruses by electron microscopy (EM), reverse transcriptase (RT) assay and the polymerase chain reaction (PCR). No retroviruses were isolated from 158 cultures initiated from 18 patients with cutaneous T cell lymphomas (predominantly MF and SS) and three patients with cutaneous B cell lymphomas (CBCLs). Four interleukin 2-dependent CD8+ T cell lines derived from skin of three patients with MF were maintained in culture for 6 to 8 months but no viruses were detected in these cultures by EM or PCR. One Epstein-Barr virus-infected B lymphoblastoid cell line was cultured for 5 months from a skin sample of a patient with CBCL. Supernatants tested from cell cultures derived from these and 10 other patients were negative for RT activity.

PCR was used to test for LTR, gag, pol, env and pX sequences of HTLV-I or HTLV-I/II in clinical samples and cultured cells from 36 patients with cutaneous lymphoid infiltrates (predominantly MF and SS), six cases of large granular lymphocytic leukaemia and six cases of acute lymphoblastic leukaemia (ALL). Patients with ALL were tested by PCR for BLV infection since all were butchers from Cardiff with occupational exposure to cattle. There was no definitive evidence of HTLV-I, HTLV-II or BLV infection.

The HTLV-I Tax protein has transforming properties in vitro and is important in HTLV-I leukaemogenesis. A model for Tax oncogenesis is c-Myc, which activates pathways leading to either proliferation or apoptosis, depending on the availability of survival factors. It was hypothesised that Tax or other pX region proteins have anti-apoptotic properties, since several oncogenic viruses produce proteins that inhibit apoptosis. The pX genes tax, rex, p21*, p30*, p13* and p12* were cloned and expressed in Rat1 fibroblasts stably transfected with a gene encoding a chimaeric c-Myc-modified oestrogen (tamoxifen) receptor protein. The chimaeric protein translocates to the nucleus in the presence of 4-hydroxytamoxifen, inducing apoptosis when Rat1 cells are deprived of serum. pX gene products were tested for their ability to protect Rat1 cells from c-Myc-induced apoptosis in a low concentration of serum. It was demonstrated that Tax induces apoptosis in Rat1 cells deprived of serum but there was no inhibition of c-Myc or Tax-induced apoptosis by other pX region gene products.
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# Abbreviations

Abbreviations are used throughout this thesis for frequently used technical terms and nomenclature. The full name is given when a term is first used in a chapter, followed by the abbreviation in brackets. Terms are redefined when used in each subsequent chapter. Definitions are sometimes repeated within a chapter to improve clarity. In some cases where a term is used only once, for example APES, an abbreviation is given if it is the usual form recognised by the scientific community. Some terms, for example JAK/STAT, are not defined in the text if this would result in loss of clarity. Standard symbols are used in most places for the names of chemicals. Système International d'Unités (SI) and derived units are used except where alternative units are commonly accepted (Aylward and Findlay 1974).

Accepted letter codes for nucleotides and amino acids, including nomenclature for incompletely specified bases, are listed in Appendix 2. Reference to genes, mRNA transcripts and proteins of the HTLV/BLV group of viruses is for HTLV-I unless otherwise designated and the numbering of the HTLV-I genome is according to Seiki \textit{et al} (1983) (Appendix 6).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>%G+C</td>
<td>Percentage of G and C nucleotides</td>
</tr>
<tr>
<td>Ω</td>
<td>Ohm</td>
</tr>
<tr>
<td>[M']</td>
<td>Adjusted monovalent cation concentration</td>
</tr>
<tr>
<td>2ME</td>
<td>2 (β) mercaptoethanol</td>
</tr>
<tr>
<td>3H-TTP</td>
<td>Tritiated thymidine triphosphate</td>
</tr>
<tr>
<td>ADF</td>
<td>ATL-derived factor</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ALC L</td>
<td>Anaplastic large cell lymphoma</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukaemia</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATL</td>
<td>Adult T cell leukaemia/lymphoma</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix loop helix</td>
</tr>
<tr>
<td>B-LCL</td>
<td>B lymphoblastoid cell line</td>
</tr>
<tr>
<td>BLV</td>
<td>Bovine leukaemia virus</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BPV</td>
<td>Bovine papillomavirus</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bZIP</td>
<td>Basic region leucine zipper</td>
</tr>
<tr>
<td>C terminus</td>
<td>Carboxy terminus</td>
</tr>
<tr>
<td>C</td>
<td>Coulomb</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBCL</td>
<td>Cutaneous B cell lymphoma</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte-associated antigen</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leucocyte antigen/Histocompatibility locus antigen</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HRES</td>
<td>HTLV-related endogenous sequence</td>
</tr>
<tr>
<td>HRV</td>
<td>Human retrovirus</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>HT</td>
<td>Hydroxytamoxifen</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human T cell lymphotropic virus</td>
</tr>
<tr>
<td>HTLV-I</td>
<td>Human T lymphotropic virus type I</td>
</tr>
<tr>
<td>HTLV-II</td>
<td>Human T lymphotropic virus type II</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NFκB</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL2R</td>
<td>Interleukin 2 receptor</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-1-thio-β-D-galactoside</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridisation</td>
</tr>
<tr>
<td>IVDU</td>
<td>Intravenous drug user</td>
</tr>
<tr>
<td>J</td>
<td>Joules</td>
</tr>
<tr>
<td>JAK</td>
<td>Just another kinase (JANUS family kinase)</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi's sarcoma</td>
</tr>
<tr>
<td>L</td>
<td>Length of the DNA:DNA duplex in bp</td>
</tr>
<tr>
<td>Large T</td>
<td>Large tumour antigen</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LCA</td>
<td>Leucocyte common antigen</td>
</tr>
<tr>
<td>LDGL</td>
<td>Lymphoproliferative disease of large granular lymphocytes</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LGL</td>
<td>Large granular lymphocyte/lymphocytic</td>
</tr>
<tr>
<td>liqN₂</td>
<td>Liquid nitrogen</td>
</tr>
<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
</tr>
<tr>
<td>LRF</td>
<td>Leukaemia Research Fund</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MDV</td>
<td>Marek's disease virus</td>
</tr>
<tr>
<td>MEKKK</td>
<td>Mitogen-activated protein/extracellular signal-regulated kinase kinase</td>
</tr>
<tr>
<td>MF</td>
<td>Mycosis fungoides</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)-2-hydroxypropane sulphonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MSC</td>
<td>Microbiological safety cabinet</td>
</tr>
<tr>
<td>MuLV</td>
<td>Murine leukaemia virus</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N terminus</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>NaPP₄</td>
<td>Tetrasodium pyrophosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate (sodium lauryl sulphate)</td>
</tr>
<tr>
<td>SI</td>
<td>Système International d'Unités</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SnRV</td>
<td>Snakehead fish retrovirus</td>
</tr>
<tr>
<td>SP</td>
<td>Signal peptide</td>
</tr>
<tr>
<td>SRE</td>
<td>Serum response element</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>SS</td>
<td>Sézary syndrome</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium saline citrate</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SU</td>
<td>Surface</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetic acid-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-boric acid-EDTA</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TCGF</td>
<td>T cell growth factor</td>
</tr>
<tr>
<td>TCL</td>
<td>T cell lymphoma</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylthlenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIF-1</td>
<td>Tax interacting factor 1</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour infiltrating lymphocytes</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>TNE</td>
<td>Tris-NaCl-EDTA</td>
</tr>
<tr>
<td>TPA</td>
<td>12-o-tetradecanoyl phorbol-13 acetate</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRE</td>
<td>Tax response element</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Ts</td>
<td>T suppressor</td>
</tr>
<tr>
<td>TSP</td>
<td>Tropical spastic paraparesis</td>
</tr>
<tr>
<td>U3</td>
<td>3' unique region</td>
</tr>
<tr>
<td>U5</td>
<td>5' unique region</td>
</tr>
<tr>
<td>UCMC</td>
<td>Umbilical cord blood mononuclear cell</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UNG</td>
<td>Uracil N-glycosylase</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indoly-β-D-galactoside</td>
</tr>
</tbody>
</table>
Chapter 1

Literature Review: The Role of Retroviruses in Human T Cell Leukaemias and Lymphomas

1.1 Introduction

Neoplasms are abnormal proliferations of tissue, unresponsive to normal mechanisms for control of cell growth, differentiation and death. Viruses, particularly members of the Families Retroviridae, Herpesviridae, Papillomaviridae and Hepadnaviridae, have been implicated as aetiological agents in many neoplasms, including T and B cell leukaemias and lymphomas (Zur Hausen 1991). In leukaemias neoplastic cells of haematopoietic lineage circulate in the blood. Lymphomas are solid growths of neoplastic lymphoid cells. The association of human T lymphotropic virus type I (HTLV-I) with adult T cell leukaemia/lymphoma (ATL) has led to the hypothesis that HTLV-I or related retroviruses are involved in the aetiology of other T cell neoplasms (Hall 1994, Lessin et al 1994).

This thesis describes studies conducted at the Leukaemia Research Fund (LRF) Virus Centre using cell and molecular biology techniques to search for HTLV-I or related retroviruses in cutaneous T cell lymphomas (CTCLs), large granular lymphocytic (LGL) leukaemia and adult acute lymphoblastic leukaemia (ALL) in the United Kingdom (UK). It also describes experiments to investigate the role of HTLV-I pX gene products in oncogenesis by studying their effects on apoptosis in cultured cells. This chapter reviews the biology of HTLV-I and related retroviruses, diseases associated with these viruses and the mechanisms of oncogenesis of HTLV-I. The classification and pathogenesis of CTCLs and LGL leukaemia are reviewed and evidence for the involvement of HTLV-I and related viruses in their aetiology is discussed.

1.2 Retroviruses

1.2.1 Classification of retroviruses

Viruses in the family Retroviridae possess reverse transcriptase (RT), which transcribes viral RNA to DNA during the retroviral replication cycle (Baltimore 1970, Temin and Mizutani 1970). Retroviruses have been categorised according to their biological behaviour into the oncornaviruses (oncogenic RNA viruses), lentiviruses (slow viruses) and spumaviruses (foamy viruses) (Teich 1985). A more recent classification is based on the structure of the retroviral genome (Van Regenmortel et al 2000) (Table 1.1). Replication competent simple retroviruses contain gag (group antigen), pro (protease), pol (polymerase) and env (envelope) genes from 5' to 3', flanked by unique 5' (U5) and 3' (U3) sequences (Coffin et al 1997).
Table 1.1: Classification of Family *Retroviridae* (Van Regenmortel *et al* 2000).

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Simple retroviruses</strong></td>
<td></td>
</tr>
<tr>
<td><em>Alpharetrovirus</em></td>
<td>Avian leucosis virus-RSA*</td>
</tr>
<tr>
<td></td>
<td>Rous sarcoma virus (Prague C)</td>
</tr>
<tr>
<td></td>
<td>Avian myeloblastosis virus (defective)</td>
</tr>
<tr>
<td></td>
<td>Avian myelocytomatosis virus 29 (defective)</td>
</tr>
<tr>
<td><em>Betaretrovirus</em></td>
<td>Mouse mammary tumour virus*</td>
</tr>
<tr>
<td></td>
<td>Mason-Pfizer monkey virus</td>
</tr>
<tr>
<td></td>
<td>Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus)</td>
</tr>
<tr>
<td></td>
<td>Squirrel monkey retrovirus</td>
</tr>
<tr>
<td><em>Gammaretrovirus</em></td>
<td>Murine leukaemia virus*</td>
</tr>
<tr>
<td></td>
<td>Abelson murine leukaemia virus</td>
</tr>
<tr>
<td></td>
<td>Friend murine leukaemia virus</td>
</tr>
<tr>
<td></td>
<td>Moloney murine leukaemia virus</td>
</tr>
<tr>
<td></td>
<td>Feline leukaemia virus</td>
</tr>
<tr>
<td></td>
<td>Gibbon ape leukaemia virus</td>
</tr>
<tr>
<td></td>
<td>Viper retrovirus</td>
</tr>
<tr>
<td></td>
<td>Reticuloendotheliosis virus</td>
</tr>
<tr>
<td><strong>Complex retroviruses</strong></td>
<td></td>
</tr>
<tr>
<td><em>Deltaretrovirus</em></td>
<td>Bovine leukaemia virus*</td>
</tr>
<tr>
<td></td>
<td>Human T lymphotropic virus type I</td>
</tr>
<tr>
<td></td>
<td>Human T lymphotropic virus type II</td>
</tr>
<tr>
<td></td>
<td>Simian T lymphotropic virus type I</td>
</tr>
<tr>
<td></td>
<td>Simian T lymphotropic virus type II</td>
</tr>
<tr>
<td></td>
<td>Simian T lymphotropic virus type III</td>
</tr>
<tr>
<td><em>Epsilonretrovirus</em></td>
<td>Walleye dermal sarcoma virus*</td>
</tr>
<tr>
<td></td>
<td>Snakehead retrovirus (tentative)</td>
</tr>
<tr>
<td><em>Lentivirus</em></td>
<td>Human immunodeficiency virus type 1*</td>
</tr>
<tr>
<td></td>
<td>Human immunodeficiency virus type 2</td>
</tr>
<tr>
<td></td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td></td>
<td>Bovine immunodeficiency virus</td>
</tr>
<tr>
<td></td>
<td>Equine infectious anaemia virus</td>
</tr>
<tr>
<td></td>
<td>Feline immunodeficiency virus</td>
</tr>
<tr>
<td></td>
<td>Caprine arthritis encephalitis virus</td>
</tr>
<tr>
<td></td>
<td>Maedi/visna virus</td>
</tr>
<tr>
<td><em>Spumavirus</em></td>
<td>Chimpanzee (formerly human) foamy virus*</td>
</tr>
<tr>
<td></td>
<td>Bovine foamy virus</td>
</tr>
<tr>
<td></td>
<td>Feline foamy virus</td>
</tr>
<tr>
<td></td>
<td>Simian foamy virus 1</td>
</tr>
<tr>
<td></td>
<td>Simian foamy virus 3</td>
</tr>
</tbody>
</table>

* Type species
Complex retroviruses, which include the lentiviruses (Genus Lentivirus), the HTLV/bovine leukaemia virus (BLV) group of viruses (Genus Deltaretrovirus) and the foamy viruses (Genus Spumavirus), have an additional region, designated pX, which encodes regulatory proteins. Viruses in the HTLV/BLV group include HTLV-I, HTLV-II, simian T lymphotropic virus type I (STLV-I), STLV-II and BLV.


1.2.2 Structure and organisation of retroviruses

Retrovirus virions are enveloped structures approximately 100 nm in diameter (Coffin et al 1997). Proteins produced from the env gene are expressed on the envelope in the form of trimers. The conical nucleocapsid consists of two identical molecules of single stranded ribonucleic acid (RNA), three or four proteins cleaved from a polyprotein produced by the gag gene, a protease produced by the pro gene and an RT/integrase/ribonuclease (RNase) H produced by the pol gene. Each viral RNA molecule is associated with a transfer RNA (tRNA) molecule of host origin.

1.2.3 Replication cycle of retroviruses

Infection by retroviruses is initiated by fusion of the retroviral envelope with the host cell membrane allowing entry of the nucleocapsid into the cytoplasm. Double-stranded complementary deoxyribonucleic acid (cDNA) is synthesised from the two single-stranded viral RNA templates by the viral RT (Coffin et al 1997). The associated tRNA molecules act as primers for synthesis of the minus strand by reverse transcription. The 5' and 3' long terminal repeats (LTRs) of the provirus are formed by duplication of the U5 and U3 regions of the viral genome, with an intervening repeat (R) region. The cDNA translocates to the nucleus where it is integrated as a provirus into the DNA of the host cell by the viral integrase. Regulatory and structural messenger RNA (mRNA) transcripts are produced and virions are assembled from translated structural proteins and viral genomic RNA. Complete virions are released from the cell by budding through the plasma membrane to acquire a lipid envelope containing viral proteins. Most retroviruses, including the HTLVs, establish persistent, life-long infections in their hosts and production of infectious virions is not dependent on host cell lysis.
1.3 Human T lymphotropic viruses

1.3.1 Terminology

Human T lymphotropic virus type I (HTLV-I) has also been referred to as ATL-associated virus (ATLV), human T cell leukaemia virus type I and human T cell lymphoma/leukaemia virus type I (Poiesz et al 1980a, 1981, Yoshida et al 1982, Blattner et al 1983). HTLV-II is also known as human T cell lymphoma/leukaemia virus type II.

1.3.2 Propagation of human T lymphotropic viruses

1.3.2.1 Discovery of HTLV-I and HTLV-II

HTLV-I was first detected in CD4^ T cell lines established from lymph node cells and peripheral blood mononuclear cells (PBMCs) of a patient in the United States of America (USA) with a form of ATL initially diagnosed as mycosis fungoides (MF) (Gazdar et al 1980, Poiesz et al 1980a, b) (Sections 1.6.1.2 and 1.11.1.2). A second isolate was obtained from a CD4' T cell line derived from PBMCs of a patient also with a form of ATL but initially diagnosed as having Sézary syndrome (SS) (Poiesz et al 1981). Serum from patients with ATL reacted with antigens expressed by a cell line producing retrovirus-like particles established from a case of ATL in Japan (Miyoshi et al 1980, Hinuma et al 1981). Retrovirus particles, initially designated ATLV but later shown to be identical to HTLV-I, were also detected in a cell line established by cocultivation of umbilical cord blood mononuclear cells (UCMCs) with PBMCs from a patient with ATL (Miyoshi et al 1981a, b). Subsequently, HTLV-I has been identified in numerous other T cell lines established from patients with ATL (Hinuma et al 1982a, Yoshida et al 1982, Hoshino et al 1983, Markham et al 1983) and HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) (Jacobson et al 1988) (Section 1.6.3), as well as asymptomatic people (Gotoh et al 1982, Sugamura et al 1984a). HTLV-II was first isolated from a CD8' T cell line derived from a patient with a T cell variant of hairy cell leukaemia (HCL) (Saxon et al 1978b, Kalyanaraman et al 1982b) (Section 1.7.1).

1.3.2.2 Cellular tropism of HTLV-I and HTLV-II

HTLV-I establishes productive infection of activated CD4^ T cells of both Th helper (Th) 1 and 2 phenotypes in vitro and in vivo (Meri et al 1984, Richardson et al 1990, Fan et al 1992, Macchi et al 1993) (Section 1.10.1.3). CD8' T cells and B cells are also susceptible to infection but have lower levels of viral transcriptional activity (Longo et al 1984, Mann et al 1984, Franchini et al 1985, Koyanagi et al 1993). Natural killer (NK) cells can be infected with HTLV-I following activation but do not produce infectious virions (Macchi et al 1987, Lo et al 1992). A wide range of other cell types is susceptible to infection with HTLV-I, including monocytes, macrophages, microglial cells, dendritic cells, fibroblasts, epithelial cells including enterocytes and mammary epithelial cells, synoviocytes, endothelial cells and neurons, but the role of these cells in maintaining viral infection in vivo or in transmitting HTLV-I is uncertain (Hoxie et al 1984, Kitajima et al 1991, Fan et al 1992, Hoffman et al 1992, Macalonia et al 1992, Zacharopoulos et al 1992, Ali et al 1993, De Revel et al 1993,
Koyanagi et al 1993, Sakai et al 1993, Lehky et al 1994, Southern and Southern 1998). The receptor for HTLV-I has not been identified but appears to be expressed by many types of cells (Gavalchin et al 1995). T cells from PBMCs, UCMCs or bone marrow can be infected with HTLV-I following cocultivation with lethally irradiated HTLV-I-infected cells, whereas the infectivity of cell-free virus is low (Miyoshi et al 1981c, Yamamoto et al 1982b, De Rossi et al 1985, Fan et al 1992). CD8+ T cells are the main cells infected by HTLV-II, although CD4+ T cells, B cells and other lymphoid cells are also susceptible to infection (Rosenblatt et al 1988b, Ijichi et al 1992, Lal et al 1995).

1.3.2.3 Transformation of T cells by HTLV-I

In vitro transformation is the conversion of cells with a limited life span into immortalised cells (cells that are able to grow indefinitely) with an altered phenotype and often a reduced requirement for growth factors such as those present in serum. Upon transformation in culture, normally adherent cells such as fibroblasts exhibit focus formation (piling up due to loss of contact inhibition) and colony formation in soft agar or suspension cultures (due to loss of anchorage dependence). Transformed cells may also be tumorigenic in athymic (nude) mice or other animal systems. T cells are considered to be transformed when they are able to proliferate indefinitely in the absence of exogenous interleukin (IL) 2 (Grassmann et al 1994).

Normal human T cells can be transformed in vitro following infection with HTLV-I and HTLV-II (Miyoshi et al 1981c, Chen et al 1983, Markham et al 1983, Popovic et al 1983, Merl et al 1984, Aboud et al 1987, Graziano et al 1987). T cells undergo polyclonal expansion 1 to 7 weeks following cocultivation of recipient PBMCs or UCMCs with HTLV-I-infected cells (Lando et al 1983, Kimata and Ratner 1991). Eventually there is selective outgrowth of one or a few dominant clones, usually CD4+ T cells although some may be CD8+, with oligoclonal integration of HTLV-I provirus (De Rossi et al 1985, Kimata and Ratner 1991). The efficiency of infection and transformation is increased if T cells have been activated but HTLV-I infection per se activates T cells (Merl et al 1984, Martin and Southern 1996, Höllsberg 1999) (Section 1.8.8). HTLV-I-infected T cell clones derived from PBMCs have variable expression of HTLV-I mRNA; T cells with transcriptionally silent proviruses do not spontaneously proliferate (Richardson et al 1997). HTLV-I-infected T cell lines often have defective proviruses with preferential retention of the pX region (Kobayashi et al 1984, Bhat et al 1993) (Section 1.3.3).

Infection of T cells with HTLV-I is associated with increased expression of IL2 and IL2 receptor (IL2R), as well as alterations in expression and function of many other cellular constituents (Section 1.8). HTLV-I-infected T cell lines may be IL2-dependent (immortalised) or IL2-independent (transformed) (Arima et al 1986, Katoh et al 1986, Arima et al 1987). HTLV-I-transformed T cells form colonies in soft agar in the absence of exogenous IL2 (Duc Dodon and Gazzolo 1987). In most in vitro models of HTLV-I infection of T cells, there is an initial phase of IL2-dependent growth following which the cells become independent of IL2, usually after 6 to 12 months (Markham et al 1983, Yssel et al 1989, Höllsberg et al 1992, Rohwer et al 1994). These results are consistent with a role for IL2 in proliferation of T cells in the early stages of HTLV-I infection and transformation, although some HTLV-I-infected T
cell lines do not produce IL2 and do not require exogenous IL2 for survival (Markham et al. 1983, Arya et al. 1984, Volkman et al. 1985). Maintenance of IL2 production in vitro may depend on the presence of macrophages in primary cultures of PBMCs from ATL patients (Arima et al. 1992). The roles of the HTLV-I Tax protein and IL2 in transformation of T cells by HTLV-I are discussed in Section 1.8. The use of cell culture for isolation and diagnosis of infection with HTLV-I and HTLV-II is discussed in Section 1.4.1.

**1.3.3 Genomic structure of HTLV-I**

The 9,068 nucleotide HTLV-I genome is organised from 5' to 3' into U5, gag, pol, env, pX and U3 regions (Seiki et al. 1983, Gallo et al. 1988, Myers et al. 1991) (Figure 1.1). The genomic structures of HTLV-II, STLV-I, STLV-II and BLV are similar (Sagata et al. 1985, Shimotohno et al. 1985b, Watanabe et al. 1985). HTLV-I gag encodes the group antigens p19\(^{\text{Gag}}\), p24\(^{\text{Gag}}\) and p15\(^{\text{Gag}}\), which are capsid proteins, gag-pol encodes a protease, p14\(^{\text{Pol}}\), pol encodes the RT/integrase/RNase H p95\(^{\text{Pol}}\) and env encodes the envelope glycoproteins gp46\(^{\text{Env}}\) and gp21\(^{\text{Env}}\). The pX region encodes regulatory proteins (Section 1.3.4).

**1.3.4 HTLV-I pX gene expression**

The proteins p40\(^{\text{N}}\) (Tax), p27\(^{\text{II}}\) (Rex), p21\(^{\text{II}}\), p30\(^{\text{II}}\) (Tof), p13\(^{\text{II}}\) and p12\(^{\text{I}}\) are encoded by the four main open reading frames (ORFs I, II, III and IV) in the pX region of HTLV-I (Kiyokawa et al. 1984, Lee et al. 1984, Slamon et al. 1984, Kiyokawa et al. 1985b, Shimotohno et al. 1985a, Ciminale et al. 1992, Korallnik et al. 1992) (Appendix 1). Tax (transactivator) and Rex (regulator of expression) are the major proteins expressed from pX, whereas p21\(^{\text{II}}\), Tof, p13\(^{\text{II}}\) and p12\(^{\text{I}}\) are minor products derived by translation from alternatively spliced transcripts (Kiyokawa et al. 1985b, Seiki et al. 1985, Berneman et al. 1992a, Ciminale et al. 1992, Korallnik et al. 1992). mRNAs expressed from the pX region derive their initiation codons by splicing with short transcripts from the gag-env region using several donor and acceptor sites (Figure 1.1). The 2 kb doubly spliced taxirex mRNA that encodes Tax, Rex and p24\(^{\text{I}}\) consists of a non-coding exon (exon 1) from the 5' LTR (HTLV-I 1 to 119), a small first coding exon (exon 2) overlapping the 3' end of pol and the 5' end of env (containing the AUG initiation codons and using splice acceptor sites at HTLV-I 4641, 4685 or 4701 and a single splice donor site at HTLV-I 4831) and a large second coding exon (exon 3) in the distal portion of the pX region with a splice acceptor site at HTLV-I 4641 (Seiki et al. 1983, Kiyokawa et al. 1985b, Seiki et al. 1985, Wachsman et al. 1985, Nagashima et al. 1986). Tof is encoded by a doubly spliced mRNA consisting of the same exon 1 from the 5' LTR, exon 2 from HTLV-I 4641 to 4831 and exon 3 from the splice acceptor site HTLV-I 4678 in ORF II of the pX region (Ciminale et al. 1992, Korallnik et al. 1992). p13\(^{\text{II}}\) is encoded by a singly spliced mRNA consisting of exon 1 spliced to exon 2 at HTLV-I 4685 in ORF II of the pX region (Berneman et al. 1992b). p12\(^{\text{I}}\) can be encoded by singly or doubly spliced pX ORF I mRNA transcripts (Korallnik et al. 1992). The singly spliced mRNA consists of exon 1 spliced to exon 2 at HTLV-I 6383 in pX ORF I. The doubly spliced mRNA consists of exon 1 spliced to exon 2 (HTLV-I 4641 to 4831), with exon 3 starting from the splice acceptor site at HTLV-I 6383 in pX ORF I, as for the singly spliced mRNA. The initiation codons for both of the mRNA transcripts encoding p12\(^{\text{I}}\) are located in pX ORF I.
Figure 1.1: Human T lymphotropic virus type I genome organisation and proteins (Koralnik et al 1992, Coffin et al 1997).
HTLV-II, STLV-I and BLV also make use of complex splicing for expression of pX products (Alexandersen et al 1993, Orita et al 1993b). Tax and Rex are encoded by the pX region of HTLV-II (Rosenblatt et al 1988a). Tax\text{BLV} and Rex\text{BLV} are produced by BLV (Rice et al 1987) (Section 1.9.2.1).

In HTLV-I-infected T cells Tax and p13\text{III} are predominantly localised in the nuclear matrix, Rex and Tof in the nucleolus, p21\text{III} in the cytoplasm and p12\text{I} in the endoplasmic reticulum and Golgi apparatus (Goh et al 1985, Slamon et al 1988, Nosaka et al 1989, Koralnik et al 1993).

A 152 amino acid pX product p17\text{I} (Rof) has been identified in cells transfected with a molecular clone of HTLV-I, but has not been detected in naturally infected cells (Ciminale et al 1992). RNA transcripts arising from several ORFs on the negative strand of HTLV-I have been detected in infected cells but their significance is unknown (Larocca et al 1989, Chou et al 1995). The pX ORFs I and II that encode Tof, p13\text{I} and p12\text{I} are not necessary for HTLV-I replication and immortalisation in cultured cells but may be necessary for efficient replication in vivo (Derse et al 1997, Collins et al 1998, Robek et al 1998, Albrecht et al 2000, Bartoe et al 2000).

1.3.5 Structure and function of HTLV-I pX gene products

1.3.5.1 Tax (p40\text{IV})

Tax is a 353 amino acid phosphoprotein translated from ORF IV of the doubly spliced tax/rex mRNA transcript (Kiyokawa et al 1984, Lee et al 1984, Slamon et al 1984, Kiyokawa et al 1985a, Seiki et al 1985) (Section 1.3.4) (Appendix 1). Its main functions are to activate transcription from the HTLV-I LTR and control expression of cellular genes (Sodroski et al 1984, Cann et al 1985). A zinc finger motif located in the amino (N) terminal region is responsible for dimerisation and localisation of Tax to the nucleus (Smith and Greene 1992). Separate regions in the N terminus mediate transactivation through the nuclear factor κB (NFκB) and cyclic adenosine monophosphate (cAMP) response element binding (CREB)/activating transcription factor (ATF) pathways (Wachsman et al 1987, Smith and Greene 1990, Semmes and Jeang 1992, Adya and Glam 1995). The carboxy (C) terminal region also interacts with transcription factors. The role of Tax in HTLV-I leukaemogenesis is discussed in Section 1.8.

1.3.5.2 Rex (p27\text{III})

Rex is a 189 amino acid phosphoprotein expressed from a different reading frame (ORF III) of the same doubly spliced mRNA as Tax (Nagashima et al 1986, Adachi et al 1992a) (Appendix 1). A domain at amino acids 57 to 69 allows Rex to form multimers (Welchcellbraun et al 1992). An N terminal nucleolar localisation signal (NLS) directs Rex to the nucleolus (Siomi et al 1988, Nosaka et al 1989). The NLS also binds to the HTLV-I Rex response element (RxRE), a sequence of approximately 254 base pairs (bp) that forms a stable stem loop structure in U3 of the full-length viral mRNA transcript, inhibiting production
of the doubly spliced mRNA transcripts encoding Rex and Tax (Hidaka et al. 1988, Ballaun et al. 1991, Unge et al. 1991, Gröne et al. 1994). A leucine motif/activation domain at amino acids 82 to 93 of Rex acts as a nuclear export signal (NES) by interacting with cellular proteins that export mRNA from the nucleus, allowing Rex to shuttle between the nucleus and the cytoplasm (Bogerd et al. 1996, Kubota et al. 1996a, Palmeni and Malim 1996). Rex promotes transport of unspliced (gag-pol) and singly spliced (env) viral mRNA transcripts from the nucleus to the cytoplasm instead of doubly spliced (tax/rex) mRNA (Inoue et al. 1987, Hidaka et al. 1988, Nosaka et al. 1989). It thus acts as a switch from early expression of regulatory proteins (Tax, Rex) to late expression of structural proteins (Gag, Pol, Env). Virions are not produced in the absence of Rex. The function of Rex is modulated by phosphorylation and may be influenced by expression of p21 (Adachi et al. 1992a, Kubota et al. 1996a) (Section 1.3.5.3).

1.3.5.3 p21

The 111 amino acid p21 is translated from an internal ribosomal entry site of ORF III of the doubly spliced tax/rex mRNA, as well as from an alternative singly spliced ORF III mRNA transcript (Kiyokawa et al. 1985b, Nagashima et al. 1986, Furukawa et al. 1991, Orita et al. 1991, Kubota et al. 1996a) (Appendix 1). It represents a truncated form of Rex (amino acids 79 to 189) that lacks the NLS but retains the NES and may compete with Rex for cellular proteins that export mRNA (Kubota et al. 1996a). This leads to retention of unspliced viral mRNAs in the nucleus. p21 could thus have a role in reducing the expression of structural proteins, maintaining latency and minimising the host immune response. Although p21 mRNA has been detected by RT-PCR in PBMCs of HTLV-I-infected people, Orita et al. (1993a) associated p21 expression with defective proviral genomes in HTLV-I-infected T cell lines and its function in vivo has not been confirmed.

1.3.5.4 Tof (p30)

Tof is a protein of 241 amino acids derived from a doubly spliced ORF II mRNA transcript (Ciminale et al. 1992, Koralnik et al. 1993) (Section 1.3.4) (Appendix 1). It has serine, arginine and threonine-rich regions with sequence similarity to activation domains of the POU family of transcription factors and interacts with CREB binding protein (CBP)/p300 (Ciminale et al. 1992, Zhang et al. 2001) (Section 1.8.2.3). Two arginine-rich regions act as a NLS by allowing Tof to bind to the RxRE and point towards a role for this protein in regulation of mRNA expression (D'Agostino et al. 1997).

1.3.5.5 p13

p13 is a protein of 87 amino acids translated from an internal initiation codon in pX ORF II and derived from singly spliced and doubly spliced mRNAs (Berneman et al. 1992a, Ciminale et al. 1992, Koralnik et al. 1992) (Section 1.3.4) (Appendix 1). p13 interacts with farnesyl pyrophosphate synthetase but its function is unknown (Lefèbvre et al. 2002).
1.3.5.6 p12

p12, expressed from singly or doubly spliced pX ORF I mRNA transcripts (Section 1.3.4), is a 99 amino acid hydrophobic protein with two predicted transmembrane domains, between which is a proline-rich region (Ciminale et al. 1992, Koralnik et al. 1992, 1993) (Appendix 1). It forms dimers and localises in the endoplasmic reticulum and Golgi apparatus in association with calreticulin and calnexin (Koralnik et al. 1993, Trovato et al. 1999, Ding et al. 2004). In these sites p12 binds to major histocompatibility (MHC) class I molecules, preventing their association with β2 microglobulin and enhancing MHC-I degradation by the proteosome complex (Johnson et al. 2001). This may prevent surface expression of MHC-I and interfere with presentation of antigens, allowing HTLV-I to escape immune recognition. p12 also binds to the 16 kDa vacuolar H+ adenosine triphosphatase (ATPase) and the β and γ chains of IL2R (Franchini et al. 1993, Koralnik et al. 1995, Mulloy et al. 1996). A role for p12 in oncogenesis may be to stimulate proliferation of T cells by binding to molecules involved in cell signalling pathways (Section 1.8.4).

1.3.6 Control of HTLV-I gene expression


Each TRE-1 can be further divided into three elements (A, B and C) with different binding specificities for a variety of transcription factors (Montagne et al. 1990, Yoshimura et al. 1990, Tsujimoto et al. 1991, Muchardt et al. 1992, Morita et al. 1993, Nyunoya et al. 1993). Most of these factors bind to TRE-1 in the absence of Tax but their affinity for the HTLV-I LTR and thus transcriptional activation of the viral promoter is augmented by dimersisation in the presence of Tax (Matthews et al. 1992, Zhao and Giam 1992, Armstrong et al. 1993, Franklin et al. 1993) (Section 1.8.2). Alternatively, Franklin and Nyborg (1995) have suggested that CREB/ATF proteins anchor Tax to the promoter site, allowing Tax to induce transcription from its own C terminal activation domain.

Repressive sequences in the U5 and R regions may also be involved in control of HTLV-I gene expression (Kashanchi et al. 1994, Xu et al. 1994, Okumura et al. 1997). Other mechanisms of transcriptional control include differential methylation of the LTR, gag-pol-env

Whereas expression of HTLV-I mRNA and protein in leukaemic cells of ATL patients is low and selective (Section 1.6.1.6), there is upregulation of expression of HTLV-I genes when PBMCs from HTLV-I-infected people, including those with ATL, are cultivated in vitro (Poiesz et al 1980a, Hinuma et al 1982a, Hoshino et al 1983, Salahuddin et al 1983, Clarke et al 1984, Setoyama et al 1992). Expression of HTLV-I genes is also induced following activation with mitogens or through induction of the cellular stress (heat shock protein) response (Andrews et al 1995, Lin et al 1998). The effects of Tax on expression of cellular genes and mechanisms of regulation of these genes are described in Sections 1.8.1 and 1.8.2.

As discussed in Section 1.3.5.2, Rex and possibly p21! are involved in post-transcriptional control of HTLV-I gene expression, increasing the expression of structural proteins by regulating mRNA processing and thus directing the production of virions (Inoue et al 1987, Hidaka et al 1988, Nosaka et al 1989). The roles of Tof, p139 and p120 in the control of HTLV-I gene expression are uncertain.

1.4 Detection of infection with human T lymphotropic viruses

The sensitivity and specificity of diagnostic tests for the detection of infection with HTLVs are critical in establishing associations between HTLV-I, HTLV-II or related viruses and disease entities. This section summarises the virological, serological and molecular assays commonly used for diagnosis of infection with HTLVs. Serological tests are used in the clinical diagnosis of HTLV infection.

1.4.1 Virus isolation

HTLV-I and HTLV-II are usually isolated by cultivating PBMCs from infected people and promoting the selective outgrowth of immortalised CD4+ or CD8+ T cell lines, respectively (Poiesz et al 1980a, Yoshida et al 1982, Rosenblatt et al 1986) (Section 1.3.2). Conditioned medium (CM) or purified cytokine preparations, especially those containing IL2, are used to stimulate the proliferation of CD4+ T cells for isolation of HTLV-I (Chapter 2). UCMCs are used as recipient cells that become infected with HTLV-I and proliferate to form T cell lines when cocultivated with donor PBMCs from infected people (Miyoshi et al 1981a, b, Hjelle et al 1992b).

Virus particles in cultured T cells may be difficult to distinguish by electron microscopy (EM) from non-virus particles such as cell membrane blebs. Detection of RT activity in supernatants from infected cell lines is of diagnostic value, but HTLV-I or HTLV-II-specific RT activity has to be distinguished from that produced by other exogenous and endogenous retroviruses and from non-specific RT activity. Stronger evidence for the presence of a retrovirus is obtained by density gradient centrifugation and association of RT activity with a
particular density of particle. Virus-specific antigens can be demonstrated in infected cell
cultures by immunofluorescence (IF) and other immunological techniques and viral genomes
and transcripts can be detected by molecular methods (Section 1.4.3).

1.4.2 Serological assays

Strong humoral immune responses are mounted against HTLV-I Env (gp21\textsuperscript{Env}, gp46\textsuperscript{Env}), Gag
(p19\textsuperscript{Gag}, p24\textsuperscript{Gag}) and Tax (p40\textsuperscript{Tax}) antigens, whereas antibodies against Rex (p27\textsuperscript{Rex}/p21\textsuperscript{Rex}, Tof
(p30\textsuperscript{Tof}) and p12\textsuperscript{Tof} are detected at lower titres or in only a small proportion of HTLV-I-infected
Dekaban \textit{et al.} 2000). Immunological assays used for screening serum samples for
antibodies against HTLV-I and HTLV-II include the enzyme-linked immunosorbent assay
(ELISA) and particle agglutination (PA) assay (Beilke 1992). Supplemental tests for
serological confirmation of HTLV-I and HTLV-II infection include the western blot (WB)
ELISA and radioimmunoprecipitation assay (RIPA) (Aboulafia \textit{et al.} 1992). Criteria for HTLV-
I/II seropositivity are repeated reactivity in the ELISA and reactivity against proteins from two
genes, for example HTLV-I p19\textsuperscript{Env} and p24\textsuperscript{Gag}, by WB ELISA or RIPA. Often both
supplemental tests are required for comprehensive detection of reactivity against HTLVs, as
reactivity tends to be stronger against HTLV Env proteins by WB ELISA and stronger
against Gag proteins by RIPA. The delay between infection and seroconversion can result in
false negative results (see Section 1.5.1.1). In people who become infected with HTLV-I
following blood transfusions, antibodies against p24\textsuperscript{Gag} are detectable earlier than antibodies
against p19\textsuperscript{Env}, resulting in indeterminate seroreactivity in testing conducted during
seroconversion (Manns \textit{et al.} 1991). A further problem with interpretation of serological
seroreactivity and geographical clustering of seroindeterminate results have been suggested
as evidence for the presence of variant HTLVs (Maruyama \textit{et al.} 1989, Nerurkar \textit{et al.} 1992,
reported in 4% of intravenous drug users (IVDUs) negative for HTLV-I in standard
serological assays (Ehrlich \textit{et al.} 1989a) (Section 1.11.1.5). The sensitivity and specificity of
the tests used and the method of sampling can affect estimates of prevalence and incidence
of infection (Section 1.5.2).

1.4.3 Molecular assays

Integrated HTLV-I and HTLV-II proviral DNA can be detected in infected patients by the
polymerase chain reaction (PCR), Southern blot (SB) hybridisation and \textit{in situ} hybridisation
(ISH) (Beilke 1992) (Chapter 3). Viral RNA genomes and mRNA transcripts are detected by
RT-PCR, northern blot hybridisation and DNA-RNA ISH. Selection of probes and control of
the stringency of hybridisation is important in optimising the sensitivity and specificity of
reactions in SB and northern blot hybridisation. PCR is highly sensitive but this sensitivity
also introduces the risk of false positive results due to contamination. Most studies
comparing PCR with serological assays have found few virus-positive antibody-negative
1.5 Epidemiology of human T lymphotropic viruses

1.5.1 Transmission

1.5.1.1 HTLV-I

HTLV-I is a cell-associated virus and transmission occurs more efficiently by transfer of intact infected T cells than by cell-free fluids (Fan et al 1992). Transmission occurs through sexual intercourse, blood transfusions and other transfers of cellular blood products, sharing of needles by IVDUs and ingestion of breast milk by babies (Okochi et al 1984, Hino et al 1985, Kajiyama et al 1986, Nakano et al 1986, Glaser et al 1988, Chen et al 1989, Lee et al 1989, Khabsaz et al 1990, Kaplan et al 1996). Sexual and lactational transmission are the main modes of infection in endemic regions, whereas in non-endemic regions intravenous drug use accounts for a substantial proportion of transmission; transplacental and perinatal transmission are less important (Blattner et al 1983) (Section 1.5.2.1). Since the main vehicles for infectivity during sexual intercourse are lymphocytes in semen, the rate of transmission is higher from male to female than from female to male (Kajiyama et al 1986). The proportion of people seroconverting after transfusion with HTLV-I infected blood constituents is 35 to 60% and occurs 20 to 90 days following transfusion (Manns et al 1991).

1.5.1.2 HTLV-II

The main modes of transmission of HTLV-II appear to be through sharing of needles by IVDUs and, prior to the introduction of screening tests, through blood transfusions (Robert-Guroff et al 1986, Lee et al 1989). HTLV-II is also transmitted by sexual intercourse and through breast milk, as for HTLV-I, and these appear to be the main modes of transmission in endemic populations (Hjelle et al 1990).

1.5.2 Geographical distribution and high risk groups

1.5.2.1 HTLV-I

HTLV-I is distributed throughout the world, with endemic foci defined by geographical regions and ethnic groups. The virus is endemic (greater than 2% prevalence) in southern Japan, the Caribbean, central America, north eastern South America, central and southern Africa, Melanesia and Polynesia (Hinuma et al 1982b, Levine et al 1988a) (Figure 1.2). Areas in which the prevalence of HTLV-I is less than 2% (non-endemic regions) include North America, Great Britain, Europe, the Mediterranean and the Middle East. The seroprevalences of infection reported among randomly selected donors in endemic regions tested by RIPA were 12% in Japan and 3.5% in the West Indies, compared to 1.5% in non-endemic regions (Blattner et al 1983). The seroprevalence of HTLV-I in Japan ranges from 0 to 35%, endemic regions being the islands of Kyushu, Shikoku and the Ryukyu chain, including Okinawa.
Figure 1.2: World map showing distribution of human T lymphotropic virus type I endemic regions (shaded areas).
HTLV-I seroprevalences of up to 14% have been detected in some populations in the Caribbean (Levine et al. 1988a). Countries with high prevalences of HTLV-I seropositivity in this region include Jamaica, Trinidad and Tobago, Martinique, Guadeloupe, Barbados and Haiti. Countries of South America with foci of high prevalences of seropositivity for HTLV-I include Colombia, Brazil, Venezuela, Surinam and Guyana. HTLV-I is prevalent (3.7 to 21.0% seropositivity by RIPA) in Africans from the Ivory Coast, Ghana, Nigeria, Zaire, Kenya, Tanzania and South Africa (Saxinger et al. 1984, Biggar et al. 1985, 1993). There is serological and virological evidence of infection with HTLV-I and related viruses in indigenous populations of Papua New Guinea, the south western Pacific islands and Australia (Kazura et al. 1987, Asher et al. 1988, May et al. 1990, Yanagihara et al. 1991). Foci of endemicity have also been reported in Italy (Manzari et al. 1985).

In the UK the prevalence of HTLV-I infection is low, although certain ethnic groups derived from HTLV-I endemic regions have high prevalences of infection (Greaves et al. 1984, Tedder et al. 1984, Mowbray et al. 1989, Banatvala et al. 1990, Brennan et al. 1990, Salker et al. 1990, Tosswill et al. 1990, Simms et al. 1994, Hale et al. 1997). In women attending antenatal clinics in England, antibodies were detected against HTLV-I by ELISA in 0.05% and against HTLV-II less than 0.01% (Ades et al. 2000). The seroprevalence of HTLV-I in Afro-Caribbean (0.97%) and African (0.26%) women was higher than in Caucasian women (0.03%). In north London 5.2% of IVDUs had antibodies against HTLV-I/II, whereas no antibodies against HTLV-I/II were detected in IVDUs in Scotland (The HTLV European Research Network 1996, McIntyre et al. 2001).

1.5.2.2 HTLV-II

HTLV-II is prevalent in some groups of American Indians and in IVDUs (Reeves et al. 1990, Maloney et al. 1992, Black et al. 1994). Endemic foci are located predominantly in South America. The prevalence is high in American Indians in Florida, New Mexico, Arizona and Panama. Cayapo and Kraho Indians of the Amazon Basin in Brazil have HTLV-II seroprevalences of 12 to 33%, whereas other tribes in the same region have low prevalences of infection (Maloney et al. 1992). High prevalences of HTLV-II infection (11 to 18%) were reported among IVDUs in the USA using serological and molecular assays (Robert-Guroff et al. 1986, Ehrlich et al. 1989b, Lee et al. 1989, Kwok et al. 1990). Prevalences of HTLV-II infection in IVDUs were 2.6% in the UK and 1.5% in Italy (Tedder et al. 1984, Zella et al. 1990).

1.5.3 Molecular epidemiology

1.5.3.1 Subtypes of human T lymphotropic viruses

HTLV-I and HTLV-II have approximately 60% nucleotide sequence identity (Seiki et al. 1983, Shimotohno et al. 1985b, Kwok et al. 1988b, Lee et al. 1993). HTLV-I has been classified into Cosmopolitan (Ia), Central African (Ib), Melanesian (Ic) and Pigmy (Id) subtypes (Mahieux et al. 1997). The prototype HTLV-Ia strain is the Japanese isolate HTLV-IaTRK-1 and Cosmopolitan isolates from Japan, the Caribbean, North and South America and Africa have greater than or equal to 97% nucleotide sequence identity across the full-length genome. Melanesian
strains have 92% identity with HTLV-IATK-1 (Gessain et al 1993). Three subtypes of HTLV-II have been identified: IIA, IIB and IIC (Switzer et al 1995, Biggar et al 1996).

1.5.3.2 Origins and global spread

STLV-I, which is closely related to HTLV-I, is present in Old World primates in Africa and Japan and it has been hypothesised that HTLV-I and STLV-I originated in Africa (Gallo et al 1983b, Gessain et al 1992b). The viruses appear to have evolved in non-human primates with multiple independent events of transmission to humans accounting for different subtypes of HTLV-I (Koralnik et al 1994, Liu et al 1996). HTLV-I may have been spread to the Americas by infected Africans in more recent times. In Japan, HTLV-I appears to have been introduced to the Jomon people between 300 and 100 BC, resulting in high prevalences of infection in their descendents, the Ainu and Ryukuans (Vidal et al 1994). The Melanesian subtype (HTLV-Ic) appears to have evolved in relative isolation from other strains (Gessain et al 1993). Foci of HTLV-I A endemicity have been identified in American Indians, consistent with introduction of HTLV-II to the Americas at least 15,000 years ago (Biggar et al 1996). However, it is uncertain whether STLV-II is present in New World monkeys (Chen et al 1994). HTLV-IIB has been identified in isolated populations of Pygmies in Africa and thus HTLV-II appears to be an ancient virus (Gessain et al 1995).

1.6 Diseases associated with HTLV-I

HTLV-I is aetiologicaly associated with ATL and HAM/TSP and has been implicated in other neoplastic or immune-mediated diseases, which are described in this section (Table 1.2). Evidence for the involvement of HTLV-I or related viruses in MF/SS, other CTCLs and LGL leukaemia is discussed in Section 1.11.

1.6.1 Adult T cell leukaemia/lymphoma

Adult T cell leukaemia/lymphoma (ATL) is an aggressive T cell neoplasm of adults caused by HTLV-I. It was first described in Japan and defined on the basis of distinctive clinicopathological features and geographical distribution (Uchiyama et al 1977, Hanaoka et al 1979, Shimoyama et al 1991).

1.6.1.1 Epidemiology

Cases of ATL are clustered in regions reflecting the distribution of HTLV-I infection, particularly south western Japan and the Caribbean (Blattner et al 1982, 1983, Hinuma et al 1982b, Robert-Guroff et al 1982) (Section 1.5.2.1). Sporadic cases occur in other parts of the world where HTLV-I infection is uncommon; many of these cases are in Japanese or Afro-Caribbean migrants from endemic regions (Greaves et al 1984, Wyld et al 1990, Pawson et al 1998a and b, Levine et al 1999). Over their life span, 1 to 5% of people infected with HTLV-I develop ATL (Kondo et al 1989, Murphy et al 1989, Tokudome et al 1989, Tajima et al 1990).
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<tr>
<td>Adult T cell leukaemia/lymphoma*</td>
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<tr>
<td>HTLV-I-associated myelopathy/tropical spastic paraparesis*</td>
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<tr>
<td>HTLV-I-associated myositis</td>
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<td>HTLV-I-associated uveitis</td>
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<td>HTLV-I-associated arthritis</td>
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<td>HTLV-I-associated pneumonitis</td>
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<td>Infective dermatitis</td>
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<td>B cell chronic lymphocytic leukaemia</td>
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<td>Acute and chronic myeloid leukaemia</td>
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<td>Mycosis fungoides/Sézary syndrome</td>
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<td>Lymphoproliferative disease of large granular lymphocytes</td>
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* Aetiological role accepted
In Japan, 0.25 to 3.90 cases/100,000 people/year are diagnosed, although in some areas the rate may be substantially higher. The latent period between exposure to HTLV-I and occurrence of ATL is usually 20 years or more. Most ATL cases present from 25 years of age and the peak occurrence is at 50 to 55 years of age, although much younger cases have been reported (Murphy et al 1989, Tajima et al 1990, Pombo de Oliveira et al 2002). Exposure to HTLV-I early in life, particularly through breast milk, is associated with a higher risk of development of ATL (Murphy et al 1989).

1.6.1.2 Clinical and pathological features

ATL usually presents as an acute form with leukaemia and neoplastic T cells infiltrating the lymph nodes, skin, liver, spleen and bone marrow (Uchiyama et al 1977, Blayney et al 1983b, Jaffe et al 1984, Shimoyama et al 1991). This form is characterised clinically by enlargement of lymph nodes, nodular skin lesions, hypercalcaemia and lytic bone lesions. Among the less common variant ATL syndromes is a chronic form with erythema but without hypercalcaemia or hepatosplenomegaly. A smouldering form with an indolent course involves the skin and is typified clinically by erythema (Yamaguchi et al 1983). A lymphomatous form characterised by lymphadenopathy without leukaemia includes primary T cell lymphomas (TCLs) involving lymph nodes (Ohshima et al 1998b). A variety of extranodal locations, including the gastrointestinal tract, may also be sites of primary lymphomas in ATL. Infection with secondary pathogens in ATL is associated with immunosuppression (White et al 1995). Spontaneous remission of ATL is rare and the disease usually has an aggressive clinical course (Shimamoto et al 1993). In patients with leukaemia, the circulating neoplastic lymphocytes are known as "flower" cells because of their lobulated nuclei (Jaffe et al 1984). The neoplastic infiltrate in solid tumours consists of a mixture of small and large pleomorphic lymphocytes with lobulated nuclei, sometimes interspersed with multinucleated cells.

1.6.1.3 Immunology

The neoplastic cells in ATL are usually CD4+ T cells that are CD7−, CD8+ and CD25+ (IL2Ra+, Tac+) (Waldmann et al 1984, Kamihira et al 1992). In addition to CD25, markers of activation expressed by ATL cells include CD30, CD71, MHC-II (HLA-DR), Ki67 and the lymphocyte common antigen (LCA) isoform CD45RA (Shirono et al 1989). In functional studies, ATL cells exhibit T suppressor (Ts) activity (Tatsumi et al 1980), whereas the pattern of cytokine secretion is variable (Tendler et al 1994, Yamada et al 1996). CD8+ and CD4+ cytotoxic T lymphocytes (CTLs) against HTLV-I gp21Env, gp46Env, p19Gag, p24Gag, Tax and Rex antigens can be induced in vitro from PBMCs of patients with ATL, as well as asymptomatic carriers (Katahira et al 1995). Fresh ATL cells have low susceptibility to lysis by Tax-specific CTLs due to reduced HTLV-I expression in vivo (Kozuru et al 1989, Kannagi et al 1993). NK cells are able to lyse HTLV-I-infected T cells that express viral antigens and evasion of NK cell-mediated destruction may be important in survival of neoplastic T cells (Feuer et al 1995, Stewart et al 1996). Plotnick et al (1994) noted that IL2-dependent HTLV-I-infected T cell lines are resistant to NK cell-mediated lysis, whereas IL2-independent cell lines are susceptible. An immunosuppressive effect of HTLV-I gp21Env has been demonstrated but its relevance to the pathogenesis of neoplasia is uncertain (Cianciolo et al 1984).
1.6.1.4 Molecular and genetic characteristics


Although clonal numerical and structural chromosomal abnormalities are found in neoplastic T cells in a high proportion of patients, no common defects have been identified (Kamada et al 1992, Ohshima et al 1998a). The TCR α locus may be involved in ATL cases, as well as HTLV-I negative T cell leukaemias, with breakpoints at chromosome 14q11 (Sadamori et al 1986). As with many neoplasms, p53 mutations are frequent in leukaemic cells from ATL patients (Nagai et al 1991, Sugito et al 1991, Cesman et al 1992, Sakashita et al 1992, Yamato et al 1993) (Section 1.8.5). Deletions are present in 10 to 56% of HTLV-I proviruses in neoplastic cells but there is preferential retention of the pX region (Manzari et al 1983, Konishi et al 1984, Kortez et al 1991, Ohshima et al 1991, Sakurai et al 1992a, Chou et al 1995, Tamiya et al 1996). Mutations in pX ORF II preventing expression of Tsf and p15II have been identified in ATL cells in vivo indicating that these pX proteins may not be essential for leukaemogenesis (Chou et al 1995).

The sites of integration of HTLV-I proviruses differ between patients with ATL and appear to be non-specific (Seiki et al 1984, Chou et al 1996, Ohshima et al 1998a). In some cases integration of HTLV-I adjacent to cellular genes such as IL2R has been demonstrated, but insertional mutagenesis is not considered to be the principal mode of oncogenesis (Okamoto et al 1986, Macera et al 1992, Nakamura et al 1994, Kubota et al 1996b, Chi et al 1997) (Section 1.8). A small number of patients with ATL-like leukaemia or lymphoma have anti-HTLV-I serum antibodies but no evidence of monoclonal integration of proviral DNA, suggesting that HTLV-I could induce leukaemia indirectly or by a hit-and-run mechanism (Yoshida et al 1989b, Fujiwara et al 2001). The rare occurrence of HTLV-I-negative cases of ATL-like neoplasia also supports the hypothesis that other molecular and cytogenetic events are important in the pathogenesis of ATL (Shimoyama et al 1987). ATL and HAM/TSP do not appear to be caused by different strains or molecular variants of HTLV-I, as the two diseases may develop in the same person or in members of the same family (Kawai et al 1989, Uozumi et al 1991, Major et al 1993, Tamiya et al 1995). Sequence variations appear to be related to geographical origin of HTLV-I types rather than pathogenicity (Daenke et al 1990, Kinoshita et al 1991, Komurian et al 1991). It has also been shown that the HTLV-I LTR directs expression in a wide range of tissues independent of the source of virus (Gonzalez-Dunia et al 1993).

1.6.1.5 Viral aetiology

The viral aetiology of ATL was established following the isolation of HTLV-I and its association with T cell leukaemias and lymphomas in Japan, the Caribbean and the USA (Blattner et al 1982, Hinuma et al 1982b, Blattner et al 1983, Blayney et al 1983a). Patients with ATL are almost invariably infected with HTLV-I and have serum antibodies against the virus (Posner et al 1981, Kalyanaraman et al 1982a, Robert-Guroff et al 1982, Galic et al 1983a). Infection precedes the development of ATL and HTLV-I genomes can be detected in
leukaemic cells. Thus, although ATL develops in only a small proportion of HTLV-I-infected people after a long latent period, the evidence for an aetiological association is compelling. A small number of patients with ATL are negative in standard serological assays but have molecular evidence of HTLV-I infection; in some cases this may be due to the presence of defective HTLV-I (Korber et al 1991, Ohshima et al 1991, Kubota et al 1995). ATL-like neoplasia has also been observed in a small number of patients without serological or molecular evidence of HTLV-I infection, but these cases appear to be exceptional (Shimoyama et al 1986).

1.6.1.6 Pathogenesis

HTLV-I does not possess a classical viral oncogene with homology to a cellular gene, as carried by acutely transforming simple retroviruses, and does not appear to induce neoplasia by insertional mutagenesis (Section 1.6.1.4). Instead, there is strong evidence that proteins encoded by the pX region are responsible for oncogenicity (Section 1.3.5). Tax and Rex are essential for replication of HTLV-I (Chen et al 1985) and Tax is the main HTLV-I-encoded protein implicated in leukaemogenesis (Nerenberg et al 1987, Tanaka et al 1990, Grassmann et al 1992) (Sections 1.8.1, 1.8.2 and 1.8.3). There is also evidence that p12 has transforming properties (Section 1.8.4), but the oncogenic roles of Tof, p21 and p13 are uncertain (Chou et al 1995, Derse et al 1997, Robek et al 1998).

Most leukaemic cells from ATL patients do not express viral antigens detectable by methods of low sensitivity such as IF or WB immunoassay but viral antigens are readily detected following in vitro cultivation of PBMCs from these patients due to growth of HTLV-I in non-leukaemic cells (Poiesz et al 1980a, Hinuma et al 1982a, Hoshino et al 1983, Salahuddin et al 1983). Similarly, HTLV-I mRNA cannot be detected in most leukaemic cells from ATL patients by northern blot hybridisation, whereas transcripts are readily detected by this method in cultured PBMCs (Clarke et al 1984, Franchini et al 1984) and ATL cells infiltrating skin (Setoyama et al 1992). Virus particles are produced in abundance in most HTLV-I-infected T cell lines, including ATL cells adapted to grow in culture (most of which are not derived from the leukaemic clone), whereas virus particles are rarely produced by ATL cells in vivo (Yoshida et al 1982, Vital et al 1993). A low level of expression of HTLV-I mRNA and proteins in ATL patients could be a manifestation of viral latency due to regulation by viral or cellular gene products or by the immune system of the host (Section 1.3.6). Serum antibodies against HTLV-I suppress viral antigen expression in infected T cells in culture (Tochikura et al 1985). HTLV-I p24 can be detected more frequently in the serum of patients with acute or advanced ATL than in HTLV-I carriers or patients with chronic ATL (Ishibashi et al 1987). The host immune response may suppress populations of infected cells that express viral antigens until advanced stages of disease when the immune response is weakened. The increase in mRNA and protein expression in cultivated cells may result from activation of latent HTLV-I provirus in ATL cells. However, most HTLV-I-transformed cell lines that grow in culture from PBMCs of ATL patients are not derived from leukaemic clones (Yoshida et al 1982). Salahuddin et al (1983) observed limited expression of HTLV-I antigens in UCMCs following HTLV-I infection. These results indicate that a high level of expression of viral antigens is not required for maintenance of the transformed phenotype in patients with ATL.
Using more sensitive RT-PCR, HTLV-I genomic, env and pX mRNA transcripts, including those encoding Tax, Rex, Tof, p21\(^\text{II}\), p13\(^\text{II}\) and p12\(^\text{I}\), can be detected in uncultured PBMCs of most patients with ATL, as well as unaffected HTLV-I carriers, but levels are 10\(^5\) to 10\(^3\) times less than that in HTLV-I-infected T cell lines (Kinoshita et al 1989, Berneman et al 1992b, Cininale et al 1992, Koralnik et al 1992). HTLV-I tax/rex mRNA transcripts were detected in 0.001\% of PBMCs in five of six ATL patients by RT-PCR (Kinoshita et al 1989). Setoyama et al (1994) detected tax/rex mRNA by the less sensitive ISH in 1 to 90\% of leukaemic cells in peripheral blood of four patients with ATL and 1 to 50\% of leukaemic cells infiltrating skin lesions in 10 patients. Oshidima et al (1996) detected tax/rex mRNA by RT-PCR-ISH in the cytoplasm of 10\% of PBMCs containing HTLV-I proviruses from ATL patients. Mutations in pX ORF II preventing expression of Tof and p13\(^\text{I}\) have been identified in ATL cells in vivo (Chou et al 1995).

In asymptomatic people infected with HTLV-I there is polyclonal integration of provirus in CD4\(^+\) T cells (Yamaguchi et al 1988). With progression to ATL the proportion of circulating lymphocytes infected with HTLV-I increases and oligoclonal or monoclonal integration of provirus becomes detectable (Yoshida et al 1984, Kinoshita et al 1985, Leclercq et al 1998). HTLV-I-infected CD4\(^+\) T cell clones may persist for many years in carriers (Etoh et al 1997). In the preclinical stages of ATL there may be leucocytosis or the presence of abnormal lymphocytes in the peripheral blood (Kinoshita et al 1985, Ikeda et al 1993). Some patients experience phases of smouldering or chronic ATL before the onset of acute ATL (Shimoyama et al 1991). Upregulation of IL2 and IL2R\(\alpha\) (CD25) by Tax, generating an autocrine loop, is thought to be one of the mechanisms by which HTLV-I stimulates proliferation of T cells early in the course of infection (Arima et al 1986, Maeda et al 1987, Maruyama et al 1987, Siekevitz et al 1987) (Section 1.8.1.1). Many other biochemical pathways are altered in HTLV-I-infected T cells and could contribute to abnormal pre-neoplastic proliferation or maintenance of the neoplastic phenotype. Accumulation of mutations in proliferating T cells leads to the emergence of neoplastic clones (Fukuhara et al 1983, Sanada et al 1985, 1986, Mortreux et al 2001). The roles of Tax, p12\(^\text{I}\), p53, c-Myc, apoptosis and Env in HTLV-I leukaemogenesis are discussed in Section 1.8.

### 1.6.2 HTLV-I and non-T cell neoplasia

Serological studies have associated HTLV-I with B cell, myeloid and other non-T cell neoplasia but an aetiological role is unconfirmed (Blomberg and Fäldt 1985, Asou et al 1986).

#### 1.6.2.1 B cell neoplasia

A patient with B cell chronic lymphocytic leukaemia (CLL) had HTLV-I infection of T cells; leukaemic B cells were negative for HTLV-I but expressed immunoglobulins reactive with HTLV-I Gag and Env proteins (Mann et al 1987). HTLV-I infection of T cells may have resulted in chronic antigenic stimulation of B cells, promoting oncogenic events leading to leukaemia. HTLV tax sequences have also been amplified by PCR from PBMCs of other patients with B cell CLL, B cell HCL and B cell non-Hodgkin lymphoma, but there is insufficient evidence for an aetiological role in these neoplasms (Cardoso et al 1996).
1.6.2.2 Myeloid neoplasia

HTLV-I/II seroreactivity has been detected in patients with acute and chronic myeloid leukaemia (Kalyanaraman et al 1982a, Möstl et al 1992). HTLV-I-related antigens, RT activity and retrovirus-like particles visible by EM were detected by Xu et al (1996a) in leukaemic PBMCs from patients with acute myeloid leukaemia. DNA from PBMCs of an HTLV-I seropositive patient with chronic myeloid leukaemia hybridised to a probe for the HTLV-I 3' LTR, but not to probes for other regions, suggesting the presence of variant or defective HTLV-I (Wong-Staal et al 1983). However, there is insufficient evidence to incriminate HTLV-I in the aetiology of myeloid neoplasia.

1.6.3 HTLV-I-associated myelopathy/tropical spastic paraparesis

HTLV-I has been aetiologically linked with HTLV-I-associated myelopathy (HAM) and tropical spastic paraparesis (TSP), which are considered to be identical syndromes (Gessain et al 1985, Bartholomew et al 1986, Osame et al 1986, Roman and Osame et al 1988).

1.6.3.1 Epidemiology

HAM/TSP occurs in areas endemic for HTLV-I (Levine et al 1988a) (Section 1.5.2.1). Sporadic cases have been reported from non-endemic areas, mainly in immigrants from HTLV-I endemic areas (Sheremata et al 1992). HAM/TSP develops in approximately 0.25% of people infected with HTLV-I over a lifetime of 75 years and the incidence in Japan is 3.1 cases/100,000 HTLV-I infected people/year (Kaplan et al 1990). Recipients of blood transfusions appear to be at higher risk of developing HAM/TSP, but infection by sexual intercourse is also a risk factor (Kramer et al 1995). The latent period between exposure to HTLV-I and the development of disease is shorter for HAM/TSP than for ATL (Osame et al 1986).

1.6.3.2 Clinical and pathological features

HAM/TSP is a chronic demyelinating disorder of the central nervous system (CNS) with a slowly progressive course characterised by weakness, spastic paraparesis or paraplegia (Gessain et al 1985, Osame et al 1986, Nakagawa et al 1995). Atypical neurodegenerative disorders not matching the case definition for HAM/TSP have also been associated with HTLV-I (Kazadi et al 1990, Hyer et al 1991).

1.6.3.3 Molecular and genetic characteristics

Integration of HTLV-I provirus in PBMCs of HAM/TSP patients is usually polyclonal or oligoclonal (Greenberg et al 1989b, Gessain et al 1990b, Furukawa et al 1992). HTLV-I infects 3 to 30% of PBMCs and 15 to 18% of circulating T cells in HAM/TSP patients; most infected cells have a single integrated provirus (Richardson et al 1997).
1.6.3.4 Viral aetiology

Anti-HTLV-I antibodies are present in the serum and cerebrospinal fluid (CSF) of patients with HAM/TSP, supporting the viral aetiology of this disease (Gessain et al 1985, Osame et al 1987). HTLV-I sequences can be detected in PBMCs, CSF and CNS tissue (Bangham et al 1988, Bhagavati et al 1988, Iannone et al 1992) and HTLV-I-infected T cell lines can be generated from PBMCs and CSF (Jacobson et al 1988).

1.6.3.5 Immunology and pathogenesis

HAM/TSP is thought to be an immune-mediated demyelinating disorder of the CNS with pathological changes induced by CTLs. Acute lesions in the CNS are infiltrated with activated CD4+ T cells, followed by accumulation of CD8+ T cells and macrophages in the chronic stages (Umehara et al 1993). A strong humoral immune response is mounted against HTLV-I Env, Gag and Tax antigens (Dekaban et al 1994). CTL responses are directed predominantly against Tax (Jacobson et al 1990, Parker et al 1994). Most HTLV-I-infected T cells are transcriptionally silent in HAM/TSP patients; expression of tax/rex mRNA has been demonstrated in 0.001 to 1% of PBMCs (Beilke et al 1991, Gessain et al 1991).

The quantity of provirus in the peripheral blood is usually higher in HAM/TSP patients than in HTLV-I-infected people without neurological disease and, paradoxically, the level and frequency of expression of tax in infected PBMCs may be higher than in ATL (Yoshida et al 1989a, Gessain et al 1990b, Furukawa et al 1995). HAM/TSP patients may have a defective immune response to HTLV-I infection, permitting increased replication and expression of viral antigens coupled with development of autoimmunity. Certain MHC-I alleles, particularly HLA-A*02, are associated with reduced proviral load and reduced risk of HAM/TSP (Jeffery et al 1999). HTLV-I tax mRNA has also been detected by ISH in the CNS of HAM/TSP patients (Lehky et al 1995).

1.6.4 Other HTLV-I-associated immune-mediated diseases

HTLV-I has been associated with myositis, uveitis, arthritis, pneumonitis and several other apparently immune-mediated diseases. Attempts have been made to implicate HTLV-I in Sjögren's syndrome, multiple sclerosis (MS), systemic lupus erythematosus (SLE) and rheumatoid arthritis but the evidence for an association with these diseases is unconvincing.

1.6.4.1 HTLV-I-associated myositis

In Jamaica 11 of 13 patients with polymyositis had antibodies against HTLV-I (Morgan et al 1989). HTLV-I sequences have been detected in muscle tissue of patients with polymyositis and antigens were localised immunohistochemically to mononuclear inflammatory cells (Sherman et al 1995). In contrast, only one of 54 Japanese patients with polymyositis was seropositive for HTLV-I (Nishikai et al 1991). Virus could not be detected by immunohistochemistry or ISH in muscle fibres of a series of HTLV-I-infected patients in Japan with polymyositis (Higuchi et al 1992). Thus, other factors may be involved in the pathogenesis of myositis in patients with HTLV-I infection in the Caribbean.
1.6.4.2 HTLV-I-associated uveitis

Antibodies against HTLV-I were detected in a higher proportion of patients with idiopathic uveitis (35%) in Japan than patients with uveitis of defined aetiology (10%) or other ocular diseases (16%), compared to a seroprevalence of 11.5% in the local population (Mochizuki et al 1992). HTLV-I proviral sequences can be detected in the inflammatory exudate in the anterior chamber of patients with HTLV-I-associated uveitis.

1.6.4.3 HTLV-I-associated arthritis

HTLV-I is associated with arthritis and polyarthritis in endemic regions (Nishioka et al 1989, Sato et al 1991). Antibodies against HTLV-I are present and virus has been identified in the synovium and infiltrating T cells from joints of affected patients (Kitajima et al 1991, Eguchi et al 1992b). In contrast, no HTLV-I sequences were detected by PCR in one study of rheumatoid arthritis in a non-endemic region (Di Giovine et al 1994).

1.6.4.4 HTLV-I-associated pneumonitis

HTLV-I-associated pneumonitis, characterised by T lymphocytic alveolitis, has usually been observed in patients with other HTLV-I-related diseases (Sugimoto et al 1987, Vernant et al 1988, Higashiyama et al 1994). Therefore, HTLV-I-associated pneumonitis may not be a distinct clinicopathological entity.

1.6.4.5 Infective dermatitis

Infective dermatitis, a chronic dermatitis associated with Staphylococcus aureus or Streptococcus spp infection, has been associated with HTLV-I infection (LaGrenade et al 1990). This may be a consequence of immunosuppression due to HTLV-I; similarly an increase in parasitic and bacterial diseases has been epidemiologically linked to HTLV-I infection (Nakada et al 1987, Murphy et al 1997). A lymphadenopathy characterised by infiltration of lymph nodes with polyclonal CD4+ T cells has also been attributed to immune dysfunction in HTLV-I-infected people (Ohshima et al 1992).

1.6.4.6 HTLV-I and other immune-mediated diseases

Serological and virological markers of HTLV-I infection have been identified in patients with several other immune-mediated diseases, including Sjögren's syndrome (Eguchi et al 1992a, Terada et al 1994), MS (Koprowski et al 1985), SLE (Olsen et al 1987, Lipka et al 1996), autoimmune thyroiditis (Kawai et al 1992), autoimmune thrombocytopenia (Dixon et al 1989) and chronic fatigue syndrome (DeFreitas et al 1991, Honda et al 1993), but evidence for an aetiological role in these conditions remains inconclusive.

Sjögren's syndrome and MS have been of particular interest because of their possible association with endogenous retroviruses that cross-react antigenically with HTLV-I. An apparent HTLV-I p19Seg seroreactivity in some cases of Sjögren's syndrome may be due to a cross-reactive antigen expressed in the salivary gland from the endogenous retroviral
sequence HRES-1 (Banki et al. 1992, Shattles et al. 1992). Several groups have detected low copy numbers of HTLV-I tax sequences by PCR in labial salivary gland samples from some patients with Sjögren's syndrome (Mizokami et al. 1998, Mariette et al. 2000). It will be necessary to verify the methodology used in these studies and determine the pathogenetic role of the tax sequences before an aetiological link can be accepted.

HTLV-I sequences and antibodies have been identified in MS patients (Koprowski et al. 1985, Greenberg et al. 1989a, Reddy et al. 1989) but an extensive series of follow-up studies failed to confirm the involvement of HTLV-I or related viruses in this disease (Ehrlich et al. 1991). mRNA transcripts of MS-associated retroviral element (MSRV), which belongs to the human endogenous retrovirus (HERV) family HERV-W, can be detected in retrovirus-like particles from cultured PBMCs of patients with MS, as well as in normal human placenta (Blond et al. 1999). HERV-H sequences are also expressed in Epstein-Barr virus (EBV)-infected B lymphoblastoid cell lines (B-LCLs) established from MS patients (Haahr et al. 1994, Christensen et al. 2000). Further independent studies are necessary to determine if retroviruses are involved in MS.

1.7 Diseases associated with HTLV-II

The role of HTLV-II in the aetiology of disease is uncertain. The virus has been associated with CD8+ T cell lymphocytosis and leukaemia and chronic neurodegenerative disease resembling HAM/TSP but is not a cause of ATL (Kiyokawa et al. 1991) (Table 1.3). Kaplan et al. (1991) identified a syndrome of severe skin disease, eosinophilia, and dermatopathic lymphadenopathy in patients with HTLV-II complicating HIV-1 infection. There is also evidence that HTLV-II is involved in immune-mediated diseases; HTLV-II pol and tax sequences were amplified from PBMCs of 52% of patients with Hashimoto's thyroiditis and 12% of patients with Graves' disease compared to 2% of controls and 1% of blood donors in Japan, although all were seronegative for HTLV-II (Yokoi et al. 1995). Independent confirmation of this study is required. Evidence for the involvement of HTLV-II in MF/SS and LGL leukaemia is discussed in Section 1.11.

1.7.1 CD8+ T cell lymphocytosis and leukaemia

Early isolates of HTLV-II were obtained from patients with T cell variants of HCL (atypical CD8+ T cell HCL) (Kalyanaraman et al. 1982b, Rosenblatt et al. 1986) and CD8+ T cell lymphocytosis in association with B cell HCL (Rosenblatt et al. 1988b). Sohn et al. (1986) detected antibodies against HTLV-II/III in two patients with T cell variants of HCL. Subsequent molecular and seroepidemiological studies have not established an association between HTLV-II and T or B cell forms of HCL (Rosenblatt et al. 1987, Lion et al. 1988, Hjelle et al. 1991a).
Table 1.3 Proposed association of human T lymphotropic virus type II with human diseases.

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<th>Disease</th>
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<tr>
<td>CD8$^+$ T cell lymphocytosis and leukaemia</td>
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<td>Chronic neurodegenerative disease</td>
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<td>Dermatopathic lymphadenopathy</td>
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<td>Autoimmune thyroiditis</td>
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<td>Mycosis fungoides</td>
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<td>Lymphoproliferative disease of large granular lymphocytes</td>
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1.7.2 Chronic neurodegenerative disease

Infection with HTLV-II has been identified in patients with chronic neurodegenerative diseases resembling HAM/TSP (Hjelle et al 1992, Harrington et al 1993, Jacobson et al 1993, Murphy et al 1993, Sheremata et al 1993). The virus has been isolated from PBMCs and detected by PCR in PBMCs but not CSF of patients with neurological disorders clinically indistinguishable from HAM/TSP (Jacobson et al 1993, Murphy et al 1993). HTLV-II infection has also been demonstrated serologically and by PCR in four patients with tropical ataxic neuropathy, a neurological disorder characterised by ataxia, altered mental state, optic atrophy, deafness and peripheral neuropathy and described in Africa and the Caribbean (Sheremata et al 1993). However, HTLV-II does not appear to be a common cause of chronic neurodegenerative disease.

1.8 Molecular mechanisms of HTLV-I leukaemogenesis

Tax is the main HTLV-I-encoded protein implicated in the pathogenesis of ATL (Franchini 1995, Franklin and Nyborg 1995) (Section 1.6.1.6). This section discusses the mechanisms by which Tax modulates expression of cellular genes in HTLV-I-infected cells, the transforming properties of Tax, p12\(^{t}\) and Env and the roles of p53, c-Myc and apoptosis in HTLV-I oncogenesis.

1.8.1 Effect of Tax on gene expression in HTLV-I-infected cells

The expression of a wide variety of cellular genes is altered following infection by HTLV-I and this effect is mediated predominantly by Tax (Sahahuddin et al 1984, Miyatake et al 1988, Kelly et al 1992, Yoshida et al 1995) (Table 1.4). Differential regulation of cellular genes by Tax is hypothesised to be the main mechanism by which HTLV-I transforms T cells \textit{in vitro} and initiates the oncogenic events leading to leukaemia \textit{in vivo}. Many of the cellular genes transactivated by Tax are involved in activation, proliferation and differentiation of T cells, consistent with this protein having a direct leukaemogenic role in ATL (Wano et al 1988, Marriott et al 1991, Höllsberg 1999).

1.8.1.1 Cytokines and cytokine receptors

The high affinity IL2R is composed of \(\alpha\) (CD25), common \(\beta\) (CD122, \(\beta_c\)) and common \(\gamma\) (CD132, \(\gamma_c\)) chains (Taniguchi et al 1995). The \(\beta_c\) and \(\gamma_c\) chains transduce signals resulting from binding of IL2 at the cell surface but their affinity for IL2 is increased substantially by inclusion of the \(\alpha\) chain in the receptor complex. In activated T cells transduction of signals through the IL2R normally activates the Src family protein tyrosine kinases (PTKs) Lck and Fyn (Mills et al 1993). These in turn activate Ras, leading to up-regulation of the cellular oncogenes \textit{fos} and \textit{jun}. The IL2 and IL2R\(\alpha\) genes are up-regulated by Tax and this effect is more pronounced in T cells than other cell types (Krönke et al 1985, Greene et al 1986, Inoue et al 1986, Waldmann 1986, Manyama et al 1987, Siekevitz et al 1987).
Table 1.4: Alteration of cellular gene expression by HTLV-I Tax.

<table>
<thead>
<tr>
<th>Genes up-regulated through the NFκB pathway</th>
<th>Genes up-regulated through CREB/ATF/AP1 pathways</th>
<th>Genes up-regulated through other pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukins 1α, 2, 3, 4, 6, 8, 10 and 15</td>
<td>Interleukin 5</td>
<td>Interleukin 1β (NF-IL6, Sp1)</td>
</tr>
<tr>
<td>Interleukin 2 receptor α and interleukin 15 receptor</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
<td>Interleukin 2 (NFκB, NF-AT)</td>
</tr>
<tr>
<td>Tumour necrosis factors α (cachectin) and β (lymphotoxin)</td>
<td>Transforming growth factor β</td>
<td>Interleukin 4 (NFκB, NF-AT, NF-IL6)</td>
</tr>
<tr>
<td>Granulocyte-macrophage colony stimulating factor</td>
<td>Parathyroid hormone-related protein</td>
<td>Interleukin 5 (GATA4, AP1)</td>
</tr>
<tr>
<td>Granulocyte colony stimulating factor</td>
<td>β and α globin</td>
<td>Interleukin 2 receptor α (NFκB, SRF)</td>
</tr>
<tr>
<td>CD106 (vascular cell adhesion molecule 1)</td>
<td>Egr1 and Egr2</td>
<td>Platelet-derived growth factor receptor (Sis) (Sp1, Egr1)</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>Fos and Fra1</td>
<td>Parathyroid hormone-related protein (CREB/ATF, Sp1, EBS)</td>
</tr>
<tr>
<td></td>
<td>Nur77</td>
<td>Egr1 and Egr2 (CREB/ATF, SRE, EBS)</td>
</tr>
<tr>
<td></td>
<td>Bcl-xL</td>
<td>Fos (CREB/ATF, SRF, SIE, octanucleotide direct repeat)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fra1 (Rb, AP1)</td>
</tr>
</tbody>
</table>

**Genes down-regulated by Tax through CREB/ATF pathway**

- Cyclins A and D3
- DNA polymerase α

**Genes down-regulated by Tax via bHLH proteins**

- Lck
- p53
- Neurofibromin (NF1)
- Bax
IL2 and IL2Rα are constitutively expressed in many T cell lines transformed by HTLV-I, although expression is not always maintained and HTLV-I-infected T cell lines may be IL2-dependent or IL2-independent (Arya et al 1984, Wano et al 1984, Arima et al 1987, Kelly et al 1992) (Section 1.3.2.3). Expression of IL2Rα is increased on the surface of PBMCs of HTLV-I-infected people in association with expression of tax/rex mRNA (Okayama et al 1997). A Tax-mediated autocrine mechanism involving upregulation of IL2Rα expression and increased secretion of IL2 may thus be involved in maintenance of proliferation of T cells infected with HTLV-I in vivo (Arima et al 1986, Maeda et al 1987, Maruyama et al 1987, Siskevitz et al 1987).

However, other pathways are almost certainly involved in stimulation of T cell growth by HTLV-I. Lck and Fyn are not necessary for IL2-mediated signal transduction in HTLV-I-infected cells, indicating the presence of alternative signalling pathways, for example activation of JAK/STAT through binding of p12δ to χ (Mills et al 1992, Franchini et al 1993, Migone et al 1995) (Section 1.8.4). Lck is expressed in IL2-dependent but not IL2-independent HTLV-I-infected T cell lines, consistent with activation of alternative pathways in the process of transformation (Koga et al 1989) (Section 1.8.1.5). Human T cells expressing Tax proliferate in response to IL2 but can also be stimulated by anti-CD3 monoclonal antibodies (MAbs) through an IL2-independent pathway (Akagi and Shimotohno 1993) (Section 1.8.8). Many HTLV-I-infected T cells in short-term cultures from ATL patients do not proliferate in response to IL2, despite expressing IL2Rα and β (Uchiyama et al 1985, Katoh et al 1986, Noma et al 1989, Höllsberg et al 1992). Furthermore, the IL2-dependence of T cell lines transformed by HTLV-I is unrelated to the level of expression of Tax (Katoh et al 1986, Arima et al 1987) (Section 1.3.2.3). The χ component of the IL2R is shared with the receptors for IL4, IL7, IL9, IL13 and IL15 (Giri et al 1994, Kawahara et al 1994, Taniguchi et al 1995) and could be responsible for proliferation of ATL cells in response to exogenous IL2, IL4, IL7 and IL15 (Uchiyama et al 1988, Umadome et al 1988, Mori et al 1993b, Persaud et al 1995, Yamada et al 1998) (Section 2.2.1.2). The β component of the IL2R is shared with the IL15 receptor.

The main mechanism by which Tax induces expression of IL2 and IL2Rα is through activation of the NFκB pathway (Ballard et al 1988, Leung and Nabel 1988, Ruben et al 1988, Hoyos et al 1989, Crenon et al 1993) (Section 1.8.2.1). The IL2 gene enhancer also has a response element for CD28RC, which is activated by members of the NF of activated T cells (NF-AT) family, themselves up-regulated by Tax in HTLV-I-infected T cells (Curtiss et al 1996, Good et al 1996). As well as NFκB elements, the enhancer of the IL2Rα gene has a serum response element (SRE) transactivated by serum response factor (SRF) (Cross et al 1987) (Section 1.8.2.3).

Levels of the interleukins IL1α and β, IL3, IL5, IL6, IL8, IL10 and IL15 are also increased in HTLV-I-infected T cells and this increase is mediated by Tax (Miyatake et al 1988, Sawada et al 1992, Muraoka et al 1993, Wolin et al 1993, Mori et al 1994c, Yamashita et al 1994, Baba et al 1996). IL1α and β and the IL1α receptor are expressed by HTLV-I-infected T cell lines from ATL patients and the cells respond to IL1α and β, consistent with a model of self-stimulation similar to the IL2-IL2R autocrine loop (Wano et al 1987, Yamashita et al 1987,
Shirakawa et al. 1989, Mori et al. 1994c). Tax up-regulates the IL1β gene promoter by interacting with the transcription factors NF-IL6 and Spi1 (Tsukada et al. 1997).

In experimental models Tax increases expression of IL4 in T cells through NFκB, NF-AT and NF-IL6 sites in the IL4 promoter (Miyatake et al. 1988, Li-Weber et al. 2001). ATL cells have increased expression of the IL4 receptor and have a proliferative response to IL4 (Uchiyama et al. 1988, Umadome et al. 1988, Mori et al. 1993b, 1996). However, Kodaka et al. (1989) and Mori et al. (1994b) were unable to demonstrate IL4 mRNA expression in leukaemic cells from ATL patients. Tax acts in synergism with GATA4 and API in transactivation of the IL5 gene and HTLV-I-infected T cell lines constitutively express this cytokine (Yamagata et al. 1997). T cells do not normally produce IL6 but production of this cytokine is induced following infection with HTLV-I (Sawada et al. 1990, Lal and Rudolph 1991, Villiger et al. 1991). Tax transactivates the IL6 and IL8 promoters through NFκB binding sites (Muraoka et al. 1993, Mori et al. 1994d, Yamashita et al. 1994, Mori et al. 1995a).

IL10, IL15 and the IL15 receptor are also constitutively produced by ATL cells and HTLV-I-infected T cell lines through activation of the NFκB pathway (Azimi et al. 1998, Mori and Prager 1998, Mariner et al. 2001). IL15 may substitute for IL2 in activation of the JAK/STAT pathway in HTLV-I-infected T cell lines (Barnford et al. 1994, Burton et al. 1994, Migone et al. 1995, Azimi et al. 1998, Yamada et al. 1998). ATL cells and HTLV-I-infected T cell lines express IL9 mRNA but this expression is independent of Tax and ATL cells do not proliferate in response to exogenous IL9 (Matsushita et al. 1997).

Tumour necrosis factors (TNF) α and β are similarly increased in HTLV-I-infected T cells by Tax through the NFκB pathway (Tschachler et al. 1989, Paul et al. 1990, Albrecht et al. 1992, Nakajima et al. 1993). Macrophage inflammatory protein 1α is constitutively expressed in HTLV-I-infected T cells and induced by Tax (Kelly et al. 1992, Baba et al. 1996). HTLV-I-infected T cell lines also produce interferon (IFN) γ (Sugamura et al. 1983). Levels of expression of granulocyte-macrophage colony stimulating factor (GMCSF), granulocyte colony stimulating factor (GCSF), macrophage activating factor and transforming growth factor (TGF) β are increased in HTLV-I-infected T cells by Tax (Jones et al. 1988,Niitsu et al. 1988, Wano et al. 1988, Nimer et al. 1989, Kim et al. 1990, Sawada et al. 1992, Himes et al. 1993). Transactivation of the GMCSF promoter by Tax is mediated through a number of transcription sites including those binding NFκB and CREB/ATF (Nimer 1991, Himes et al. 1993). NFκB-binding sites in the GCSF promoter are responsible for Tax-mediated transactivation of this gene (Himes et al. 1993). Expression of platelet-derived growth factor (PDGF) β and its receptor are increased in HTLV-I-infected T cells and the PDGFβ receptor (c-sis) promoter is up-regulated by interaction of Tax with the zinc finger transcription factors Spi and Egr1 (Goustin et al. 1990, Trejo et al. 1997). The PDGFβ receptor mediates transformation by the bovine papillomavirus type 1 (BPV-1) E5 protein and could be one of the targets of HTLV-I p12 (Nilson and DiMaio 1993) (Section 1.8.4). HTLV-I-infected T cell lines also have increased expression of insulin-like growth factor (IGF) receptor type I and proliferate in response to IGF-I (Lal et al. 1993).
1.8.1.2 Cell surface molecules

As well as the cytokine and growth factor receptors listed in Section 1.8.1.1, cell surface molecules up-regulated by Tax in HTLV-I-infected T cells include CD9, CD11, CD23 (Fcɛ receptor II), CD21 (complement receptor 2), CD40, CD71 (transferrin receptor), CD80 (B7-1), MHC-I and GD2 ganglioside (Mann et al 1983, Nutman et al 1987, Vidal et al 1988, Yssel et al 1989, Valle et al 1990, Furukawa et al 1993, McNearney et al 1993, Dezzutti et al 1995, Ohtsubo et al 1997). CD54 (intercellular adhesion molecule 1, ICAM1) is expressed on uncultured leukaemic cells from ATL patients and levels of CD54 mRNA are increased in HTLV-I-infected T cell lines derived by transformation of cocultivated PBMCs or UCMCs (Fukudome et al 1992, Dezzutti et al 1995). However, CD54 and its ligand CD11 (Section 1.10.1.5) may be down-regulated in HTLV-I-infected T cell lines derived directly from leukaemic cells of ATL patients (Fukudome et al 1992, Tanaka et al 1995). Thus, although Tax induces the CD54 promoter, its role in the regulation of CD54 expression in vivo is uncertain (Mori et al 1994a, Tanaka et al 1995). CD62 ligand (CD62L) is expressed on ATL cells and its promoter is transactivated by Tax, whereas this leucocyte adhesion molecule is down-regulated when normal T cells are activated (Tatewaki et al 1995). CD106 (vascular cell adhesion molecule 1) is expressed at high levels on HTLV-I-infected T cells and transactivation of the CD106 gene by Tax is mediated through NfκB-binding sites (Valentin et al 2001). Tax induces CD134 (OX40) and OX40 ligand, the latter belonging to the TNF family, in HTLV-I-infected cells (Baum et al 1994, Higashimura et al 1996). Integrins α5β1 and α4β1, which act as fibronectin receptors, are up-regulated on HTLV-I-infected T cells (Dhawan et al 1993). CD151 (SFA-1, PETA-3), which associates with integrin α5β1 and contributes to fibronectin adhesion, is also expressed on HTLV-I-transformed T cells and the CD151 gene is transactivated by Tax (Hasegawa et al 1998).

1.8.1.3 Oncogenes, transcription factors, signalling pathways and cell cycle regulators

The activities of a number of molecules involved in signal transduction pathways, including the PTKs Lyn and Fyn, protein kinase A and protein kinase C (PKC), are increased by Tax in HTLV-I-infected T cells (Koga et al. 1989, Yamanashi et al. 1989, Kadison et al. 1993, Uchiumi et al. 1992, Lindholm et al. 1986, Lemasson et al. 1997, Weil et al. 1999). Calpain II, a cysteine protease that activates PKC, is increased by Tax in HTLV-I-infected T cells (Adachi et al. 1992b). In contrast, the activity of Lck is decreased (Koga et al. 1989, Lemasson et al. 1996) (Section 1.8.1.5). The JAK/STAT pathway is constitutively activated by tyrosine phosphorylation in uncultured leukaemic cells from ATL patients, IL2-independent HTLV-I-transformed T cell lines and Tax-expressing rodent fibroblast cell lines (Franchini et al. 1993, Migone et al. 1995, Xu et al. 1996b, Takemoto et al. 1997). This pathway may mediate transduction of signals through IL2R via p12’ (Section 1.8.4). HTLV-II is able to transform T cells independently of JAK/STAT activation (Mulloy et al. 1998b).

Tax interacts with the cyclin-dependent kinase (CDK) inhibitor p16^INK4a, releasing its repression of CDK4 and permitting the cell cycle to continue past the G1/S arrest points (Suzuki et al. 1996, Low et al. 1997, Schmitt et al. 1998). Deletions in p16^INK4a, which is a tumour suppressor gene, have been reported in patients with ATL (Ogawa et al. 1994, Hatta et al. 1995). Hofmann et al. (2001) demonstrated methylation of p16^INK4a and other genes involved in cell cycle control in a proportion of ATL patients. Tax also associates with cyclin D (Neuveut et al. 1998) and the mitotic checkpoint protein MAD1 (Jin et al. 1998).

1.8.1.4 Other proteins up-regulated by Tax

The parathyroid hormone-related protein (PTHRP) gene is transactivated by Tax through the CREB/ATF pathway and interactions with the transcription factors Ets1 and Sp1 (Ikeda et al. 1993, Dittmer et al. 1997). Elevated production of PTHRP by HTLV-I-infected cells may contribute to increased resorption of bone and retention of calcium by the kidneys in ATL patients (Fukumoto et al. 1989, Watanabe et al. 1990, Ejima et al. 1993). ATL-derived factor (ADF)/thioredoxin-like protein is produced by HTLV-I-infected T cell lines but not uninfected T cell lines and the thioredoxin gene is transactivated by Tax (Wakasugi et al. 1990, Masutani et al. 1996). ADF induces IL2R and synergises with IL1α and β and IL2 in stimulating the growth of T cells, possibly through its effect on redox status (Wakasugi et al. 1990). HTLV-I-infected T cell lines, as well as activated T cells, produce oncostatin M, a factor that stimulates growth of fibroblasts, but inhibits growth of melanoma cells (Nair et al. 1992). The lymphocyte chemoattractant stromal cell-derived factor 1 and its receptor CXCR4 are preferentially expressed in HTLV-I-infected T cells and in T cells expressing Tax (Arai et al. 1998b). Levels of expression of the cytoskeletal proteins vimentin and cytokeratin 7 are increased by Tax in HTLV-I-infected cells through the NFκB pathway (Lilienbaum et al. 1990). Tax also transactivates several globin genes (Fox et al. 1989).

1.8.1.5 Proteins down-regulated by Tax

Tax down-regulates expression of Lck, the tumour suppressor proteins p53 and neurofibromin (NF1) and the apoptosis accelerator Bax (Uittenbogaard et al. 1995, Feigenbaum et al. 1996, Brauweiler et al. 1997, Lemasson et al. 1997). Repression of these genes, mediated by the basic helix-loop-helix (bHLH) family of transcription factors
(Ultenbogaard et al 1995), may promote survival and proliferation of HTLV-I-infected T cells, contributing to oncogenesis. Although Lck and JAK/STAT are activated in some T cell neoplasms (Yu et al 1997) and IL2-dependent HTLV-I-infected T cell lines (Section 1.8.1.1), Lck is a regulator of apoptosis and therefore the ability of HTLV-I-infected cells to downregulate and bypass Lck may promote survival (Belka et al 2003). Repression of β polymerase by Tax may also contribute to oncogenicity by inhibiting repair of DNA, allowing mutations to accumulate (Jeang et al 1990). An increased frequency of random mutations has been observed in HTLV-I-infected and Tax-transfected cells (Majone et al 1993, Saggioro et al 1994b, Miyake et al 1999). Tax represses cyclin A, cyclin D3 and DNA polymerase α by acting through CREB/ATF binding sites in the enhancers of these genes (Kibler and Jeang 2001). Expression of the TCR-CD3 complex is down-regulated by Tax in HTLV-I-infected T cell lines and leukaemic cells from patients with ATL (De Waal Malefyt et al 1990, Matsuda et al 1994).

1.8.1.6 Clinical relevance of alterations in cellular gene expression

It is difficult to ascribe specific clinicopathological correlates to the wide variety of cellular changes associated with HTLV-I infection in patients with ATL (Section 1.6.1.2). Overexpression of receptor activator of NFκB (RANK) ligand (Nosaka et al 2002) and increased production of IL1α, TNFβ and PTHRP by HTLV-I-infected T cells may induce hypercalcaemia and lytic bone lesions (Motokura et al 1989, Ishibashi et al 1991, Mori et al 1994c). Upregulation of IL3, IL5 and GMCSF could contribute to granulocytosis and eosinophilia (Prin et al 1988). However, the clinical significance of many of the alterations in gene expression caused by Tax in HTLV-I-infected T cells is unknown. Since the level of expression of viral gene products in ATL cells in vivo may be low (Section 1.6.1.6), the effects of HTLV-I and Tax on cellular function in vivo cannot necessarily be extrapolated to ATL patients. Furthermore, it has been shown that clonally related ATL cells derived from the same patient may have different patterns of cytokine expression (Noma et al 1989).

1.8.2 Mechanisms of regulation of cellular genes by HTLV-I Tax

Dysregulation of cellular functions by Tax is mediated through a variety of cellular transcription factors that attach to specific DNA sequences and activate or suppress cascades of genes. Tax does not bind directly to DNA but interacts with proteins in the NFκB and CREB/ATF families, increasing transcriptional activity by enhancing their dimerisation or by facilitating their translocation to the nucleus following dissociation or degradation of cytoplasmic inhibitors (Wagner and Green 1993, Kanno et al 1994, Baranger et al 1995, Franklin and Nyborg 1995, Yoshida et al 1995). Tax also activates transcription through pathways other than NFκB and CREB/ATF, such as SRF. The NFκB, CREB/ATF and SRF pathways appear to be important in transformation of cells by Tax (Smith and Greene 1991, Yamaoka et al 1996, Matsumoto et al 1997, Rosin et al 1998, Robek and Ratner 1999) (Section 1.8.3).
Members of the NFκB family are transcription factors that bind to consensus DNA sequences in the enhancers of many cellular genes. NFκB proteins include NFκB1 (p50) derived from the precursor p105, NFκB2 (p52) derived from the precursor Lyt-10 (p100), Rel (p65), RelA (p55) and RelB (p52B) (Baeuerle and Henkel 1994). The NFκB pathway is involved in proliferation and differentiation of lymphocytes and is activated in T cells following stimulation through the TCR and by cytokines, mitogens and viral infection. HTLV-I-infected T cells constitutively express NFκB proteins and Tax specifically increases the functional activity of NFκB1, NFκB2, Rel and RelA in T cells (Lindholm et al 1990, Arima et al 1991, Li et al 1993, Lanoix et al 1994, Sun et al 1994). Elevated expression of NFκB1/p105 and relA mRNA has been demonstrated in leukaemic cells from ATL patients (Inoue et al 1998).

Cellular genes with NFκB-binding sites that are up-regulated by Tax include IL1α, IL2, IL2Rα, IL6, IL15, IL15R, TNFα and β, GMCSF and GCSF (Section 1.8.1).

There are at least three mechanisms by which Tax activates the NFκB pathway. The most important of these is by promoting release of NFκB dimers from inhibitors (IκBs) in the cytoplasm, allowing NFκB proteins to translocate to the nucleus and transcribe cellular genes (Karin 1999). IκBs include IκBα, β, γ, ε (identical to the C terminal 607 amino acids of p105) and Bcl-3; Lyt-10 and p105 also have IκB-like activity. Tax binds to MEKK1, resulting in activation of IκB kinases (Chu et al 1998, Geleziunas et al 1998, Yin et al 1998). IκB kinases phosphorylate IκBs, facilitating their degradation by the proteosome complex (Sun et al 1994, Kanno et al 1995, Lacoste et al 1995, Maggirwar et al 1995, Good and Sun 1996). Released from IκBs in the cytoplasm, NFκB proteins translocate to the nucleus, where they activate cellular genes. Tax also binds to ankyrin repeat motifs in IκBs and this interaction further contributes to translocation of NFκB proteins to the nucleus by facilitating competitive dissociation of NFκB-IκB complexes (Watanabe et al 1993, Hirai et al 1994, Kanno et al 1994, Muñoz et al 1994, Suzuki et al 1995, Petropoulos et al 1996, Petropoulos and Hiscott 1996).

Furthermore, Tax mediates transactivation of NFκB-binding sites by interacting with ankyrin repeat motifs in the Rel homology domains of NFκB proteins, promoting the formation of NFκB dimers, which bind to DNA (Lacoste et al 1991, Armstrong et al 1993, Suzuki et al 1994, Hiscott et al 1995, Petropoulos et al 1996). NFκB-binding activity induced by Tax is thus initially due to translocation of NFκB dimers (especially NFκB1-RelA) from the cytoplasm to the nucleus and is later supplemented by complexes containing newly synthesised proteins as a result of transcriptional activation of NFκB promoters by Tax in a positive feedback loop (Li et al 1993, Kanno et al 1994, Sun et al 1994). Conversely, sequestration of Tax in the cytoplasm by association with Lyt-10 (and to a lesser extent p105) could form part of a negative feedback loop leading to viral latency following activation of NFκB in HTLV-I-infected T cells (Béraud et al 1994, Pepin et al 1994) (Section 1.3.6).
1.8.2.2 CREB/ATF

Proteins in the CREB/ATF and AP1 families are basic region leucine zipper (bZIP) proteins that bind to CREB response elements (CREs) in the enhancers of many genes (Lemaigre et al 1993, Karin et al 1997). CREB, ATF1 and ATF2 up-regulate expression, whereas CRE modulator (CREM) down-regulates expression. The mechanism by which Tax activates transcription through CREB/ATF and AP1 factors is by interaction of Tax dimers with the basic regions of these proteins, promoting dimerisation through their leucine zippers, which enhances their ability to bind to DNA (Zhao and Giam 1992, Wagner and Green 1993, Baranger et al 1995, Perini et al 1995, Kwok et al 1996). Cellular genes responsive to transactivation by Tax through CREB/ATF-binding sites include egr1, egr2, fos, GMCSF, PTHRP, Nur77, cyclin A and DNA polymerase α (Section 1.8.1).

1.8.2.3 Other mechanisms of activation by Tax

Tax interacts with SRF and enhances its binding to CArG elements in the SREs of egr1, egr2, fos and IL2Rα (Fujii et al 1992, Armstrong et al 1993, Suzuki et al 1993b) (Section 1.8.1). Other mediators of transcriptional activation by Tax include CBP/p300 (Kwok et al 1996, Bex et al 1998, Van Orden et al 1999), HEB1 (Béraud et al 1991), TATA box-binding protein (TBP) (Caron et al 1993), TFIIA (Clemens et al 1996) and NF-YB (Pise-Masison et al 1997). Tax mutants that selectively activate the CREB/ATF pathway localise with CBP, whereas Tax mutants that selectively activate the NFκB pathway localise with p300 (Bex et al 1998). Tof also binds to CBP/p300 (Zhang et al 2001) (Section 1.3.5.4). Repressive effects of Tax on cellular genes are mediated through CREB/ATF and bHLH pathways (Section 1.8.1.5). The enhancers of many genes have more than one site responsive to transactivation by Tax.

1.8.3 Transforming properties of Tax

Tax is able to transform established rodent fibroblast cell lines, immortalise primary rat embryo fibroblasts (REFs) and immortalise human T cells (Grassmann et al 1989, Pozzatti et al 1990, Tanaka et al 1990, Smith and Greene 1991, Grassmann et al 1992). Tax cooperates with Ras in the transformation of primary REFs, but is unable to transform them when expressed alone (Pozzatti et al 1990). Miyazaki et al (1996) demonstrated cooperation of Tax with c-Myc and Lck in the transformation of a B cell line to IL3-independence. Participation of Lck and Ras in Tax-mediated transformation in this model is consistent with involvement of the IL2R signalling pathway (Section 1.8.1.1). Primary human T cells immortalised with Herpesvirus saimiri or retroviral vectors expressing Tax remained dependent on IL2 and had increased expression of IL2Rα (Grassmann et al 1989, Akagi et al 1995). Mutational inactivation or deletion of tax from transformed cells resulted in reversion of the transformed phenotype (Sakurai et al 1992b, Yamaoka et al 1992). An established rodent fibroblast cell line (Rat2 cells) transformed by Tax lost its transformed phenotype when fused with non-transformed human fibroblasts, even though expression of functional Tax was maintained in the hybrid cells, indicating that transformation by Tax is dependent on additional oncogenic events, particularly inactivation of tumour suppressor
Expression of Tax is maintained in T cell lines transformed by HTLV-I even though such cell lines may not express structural genes (Sodroski et al. 1985). Tax2 is essential for transformation of human T cells by HTLV-II (Ross et al. 1996, Endo et al. 2002).

As discussed in Section 1.8.2, the NFκB, CREB/ATF and SRF pathways are involved in Tax-mediated transformation. Tax mutants that stimulate the CREB/ATF pathway but not the NFκB pathway are able to transform Rat2 cells (Smith and Greene 1991, Rosin et al. 1998). In contrast, Yamaoka et al. (1998) demonstrated that activation of NFκB was necessary for Tax-induced transformation of another established rodent fibroblast cell line (Rat1 cells). An IL2-dependent murine T cell line (CTLL-2) lost its dependence on IL2 for growth when transfected with a plasmid expressing Tax and this effect was mediated through the NFκB pathway rather than the CREB/ATF pathway (Iwanaga et al. 1999). The same cell line required expression of Tax that transactivated both pathways in order to form colonies in soft agar in the absence of IL2 (Iwanaga et al. 1999). Colony formation by tax-transfected Rat1 cells in soft agar was dependent on the NFκB pathway, whereas focus formation by primary REFs in cooperation with Ras was dependent on the SRF pathway (Matsumoto et al. 1994, 1997). Immortalisation of primary human T cells (indefinite growth in the presence of IL2) was dependent on activation of the NFκB pathway by Tax but did not require an active CREB/ATF pathway (Robek and Ratner 1999). Similarly, activation of the NFκB pathway in tax-transfected human PBMCs conferred growth responsiveness to IL2, whereas clonal expansion of CD4+ T cells from this population was associated with activation of the CREB/ATF and SRF pathways (Akagi et al. 1997a). In HTLV-I-infected T cells the IL2-dependent phase of growth is associated with activation of the NFκB pathway, whereas loss of dependency on IL2 is associated with activation of the CREB/ATF, SRF and JAK/STAT pathways (Migone et al. 1995) (Section 1.3.2.3). These results suggest that different signalling pathways are involved in distinct facets of the transformation process mediated by Tax.

1.8.4 Role of p12' in HTLV-I oncogenesis

p12' has oncogenic properties and may play a role in HTLV-I leukaemogenesis. Although unable to induce transformed foci when transfected alone, p12' enhances transformation of murine C127 fibroblasts when co-transfected with BPV-1 E5, a 44 amino acid oncoprotein implicated in the development of papillomas induced by BPV-1 in cattle (Schlegel et al. 1986, Franchini et al. 1993). Transformation of C127 cells by E5 is mediated by the PDGFβ receptor (Section 1.8.1.1); E5 transforms NIH 3T3 cells by interacting with the epidermal growth factor (EGF) receptor (Martin et al. 1989, Nilson and DiMaio 1993). p12' and E5 have structural similarities, both being hydrophobic proteins that localise in the endoplasmic reticulum and Golgi apparatus (Koralnik et al. 1993) (Section 1.3.5.6). The second of two putative transmembrane regions of p12' (amino acids 39 to 62) has 59% amino acid identity with the single transmembrane region of E5 (amino acids 6 to 28), a common amino acid motif LFLL and a conserved Q residue (Franchini et al. 1993) (Appendices 1 and 2). p12' and E5 both associate with 16 kDa vacuolar H+ ATPase, a proton pump responsible for acidification of vesicles (Goldstein et al. 1991) (Section 1.3.5.6). However, the sites of
interaction of p12' and E5 with 16 kDa vacuolar H^+ ATPase are different and the binding domain in p12' is not the same as the domain that mediates its ability to potentiate transformation (Koralnik et al. 1995). BPV-1 E5 enhances the binding of 16 kDa vacuolar H^+ ATPase to the PDGFβ receptor and this may be the mechanism by which the PDGFβ receptor is constitutively activated in transformed C127 cells (Goldstein et al. 1992). It is unknown whether p12' has a similar mechanism of action.

p12' binds to the cytoplasmic portions of the β_c and γ_c chains of the IL2R (Franchini et al. 1993, Mulloy et al. 1996) (Section 1.3.5.6). A proline-rich region between the two transmembrane domains of p12' is involved in binding to IL2Rβ and also mediates interaction with 16 kDa vacuolar H^+ ATPase (Mulloy et al. 1996). p12' binds to an acidic region in the cytoplasmic domain of IL2Rβ, which is the site of interaction with Lck and involved in recruitment of JAK1 to IL2Rβc and JAK3 to IL2Rγc following IL2 stimulation, resulting in activation of STAT5 (Migone et al. 1995, Nicot et al. 2001). p12' may thus bypass the requirement of proliferating T cells for IL2. A Tax-p12'-based model for HTLV-I-induced transformation of T cells is for Tax to activate the IL2-IL2R autocrine loop early in the course of infection, with p12' maintaining proliferation or preventing apoptosis later in infection through ligand-independent stimulation of the IL2R signalling pathway.

### 1.8.5 Role of p53 in HTLV-I oncogenesis

p53 is a tumour suppressor protein that induces cell cycle arrest and apoptosis in response to DNA damage (Liebermann et al. 1995) (Section 1.8.7). Mutations resulting in inactivation of p53 are found in many neoplasms and have been identified in cases of ATL (Holstein et al. 1991, Newcomb 1995) (Section 1.6.1.4). Leukaemic T cells in nine (42%) of 21 cases of ATL had significant mutations in p53 (Nagai et al. 1991, Sugito et al. 1991, Ceserano et al. 1992, Sakashita et al. 1992, Yamato et al. 1993). Nucleotide sequence changes identified were predicted to lead to amino acid substitution (seven cases), deletion (one) or premature termination (one) in the encoded protein. There was a corresponding loss of the opposite p53 allele (loss of heterozygosity) in four cases. Mutations in p53 were more frequent in acute than chronic ATL (Nagai et al. 1991, Sugito et al. 1991, Sakashita et al. 1992, Nishimura et al. 1995).

p53 and other tumour suppressor proteins are frequent targets of viral oncogenes (Hoppe-Seyler and Butz 1995). p53 is increased in HTLV-I-infected T cell lines but the protein is inactivated by phosphorylation and this effect is mediated by Tax (Koeffler et al. 1986, Reid et al. 1993, Pise-Masison et al. 2000). T cell lines immortalised by Tax also have increased expression of p53 (Akagi et al. 1997b). The increase in p53 is due to stabilisation of the protein (increased half life) rather than an increase in the amount of mRNA, since Tax down-regulates p53 through binding of bHLH transcription factors to an E box element in the p53 enhancer (Lübbert et al. 1989, Reid et al. 1993, Uittenbogaard et al. 1994) (Section 1.8.1.5). Tax does not bind to p53 but induces phosphorylation of this protein through the NFκB pathway (Pise-Masison et al. 2000). Phosphorylation blocks the interaction of transcription factors such as TFIIID and MDM2 with the N terminal activation domain of p53. As a consequence, p53-mediated responses to cell damage are impaired in HTLV-I-infected cell

1.8.6 Role of c-Myc in HTLV-I oncogenesis

The Myc family of proteins, c-Myc, N-Myc and L-Myc, are nuclear phosphoproteins with bZIP and bHLH domains that act as transcription factors with an important role in cell proliferation (Marcu et al 1992, Lüscher and Larsson 1999). c-Myc dimerises with Max and is involved in transformation, cell cycle progression and apoptosis (Helkkila et al 1987, Dang et al 1989, Kato et al 1990, Evan et al 1992, Amati et al 1993). It cooperates with Ras in transformation (Land et al 1983, Strasser et al 1990). Activation of c-Myc appears to prime cells for either proliferation or death by apoptosis, depending on whether conditions are permissive for survival (Evan et al 1992) (Section 1.8.7). Survival signals that allow c-Myc to induce proliferation in different contexts include cytokines present in serum such as IL2, IL3, IGF-I and PDGFβ and expression of apoptosis inhibitors such as Bcl-2 (Fanidi et al 1992, Harrington et al 1994a). c-myc is activated in a wide variety of neoplasms, including human, murine, feline and avian leukaemias and sarcomas (Cory et al 1984).

HTLV-I appears to have a variety of disparate effects on c-Myc expression and function. No mutations in c-myc were detected in leukaemic T cells from 10 ATL patients (Cesarman et al 1992) and c-myc mRNA could be detected by ISH in 25% of leukaemic cells (Moriuchi et al 1988). The level of c-myc mRNA was increased in an HTLV-I-infected T cell line examined by Koizumi et al (1989). Duyao et al (1992) demonstrated transactivation of c-myc by Tax through NFκB-response elements, whereas in a study by Fuji et al (1988) Tax failed to activate transcription from the c-myc promoter. Similarly, there was no upregulation of c-Myc in a T cell line transfected with tax (Nagata et al 1989). Inhibition of c-myc expression in HTLV-I-infected T cell lines was correlated with inhibition of growth (Koizumi et al 1989, Fujita and Shiku 1993). Tax inhibits c-Myc function, including its transforming properties in Rat1 cells, through a post-translational mechanism involving the CREB/ATF pathway (Semmes et al 1996). These differences probably reflect the complex pattern of regulation of expression and the diverse functions of c-Myc in cells. Experiments investigating the effects of HTLV-I pX genes on apoptosis induced by c-Myc in rodent fibroblasts under conditions of serum deprivation are described in Chapter 4.

1.8.7 Role of apoptosis in HTLV-I oncogenesis

1.8.7.1 Apoptosis and oncogenesis

Apoptosis is a mechanism of programmed cell death in response to pathological or physiological stimuli (Kerr et al 1972). In the process of oncogenesis, cells have a
requirement to switch on genes that promote growth, for example the oncogene \textit{c-myc} (Section 1.8.6). However, \textit{c-myc}-induced proliferation is automatically coupled to activation of an apoptosis pathway, allowing cells to respond to potentially oncogenic events by committing suicide (Evan \textit{et al} 1992, Evan and Littlewood 1993). Therefore, additional events that prevent apoptosis in response to aberrant cell proliferation are required for oncogenesis, for example by activating the anti-apoptotic gene \textit{bcl-2} or inactivating the tumour suppressor gene \textit{p53} (Bissonnette \textit{et al} 1992, Fanidi \textit{et al} 1992, Harrington \textit{et al} 1994b, Pan \textit{et al} 1997). Tax up-regulates the anti-apoptotic protein \textit{Bcl-xL} and down-regulates the tumour suppressor proteins \textit{p53} and \textit{NF1} and the pro-apoptotic protein \textit{Bax} (Uittenbogaard \textit{et al} 1995, Feigenbaum \textit{et al} 1996, Brauweiler \textit{et al} 1997, Mori \textit{et al} 2001) (Sections 1.8.1.3 and 1.8.1.5). Apoptosis induced by expression of \textit{c-Myc} is mediated by \textit{p53} (Hermeking and Eick 1994) (Section 1.8.6).

1.8.7.2 Survival signals

Suppression of apoptosis is important in maintaining survival of normal cells under physiological conditions. Cells that survive in the presence of growth factors, such as \textit{IL2} or \textit{IGF-I}, may undergo apoptosis when these growth factors are withdrawn, for example by cultivation in low concentrations of serum (Evan \textit{et al} 1992, Harrington \textit{et al} 1994a, Kulkarni and McCulloch 1994, Preston \textit{et al} 1994). \textit{IL2}-dependent \textit{T} cells undergo apoptosis when \textit{IL2} is withdrawn (Duke and Cohen 1986). \textit{IL2}, \textit{IL4} and \textit{IL7} prevent apoptosis of quiescent \textit{T} cells in response to \textit{γ}-irradiation (Boise \textit{et al} 1995). Similarly, apoptosis is the mechanism of death in \textit{IL3}- and \textit{IL6}-dependent haematopoietic cells deprived of these cytokines (Colotta \textit{et al} 1992, Gottlieb \textit{et al} 1994). A potential mechanism of \textit{HTLV-I} leukaemogenesis would be to prevent apoptosis in \textit{T} cells. However, \textit{PBMCs} from patients with \textit{ATL} undergo apoptosis when cultivated in serum-free medium, similar to \textit{PBMCs} from normal people (Debatin \textit{et al} 1993). This effect is only partially inhibited by \textit{IL2}, indicating that other growth factors in serum are required to prevent apoptosis of \textit{ATL} cells and normal \textit{PBMCs} (Tsuda \textit{et al} 1993). The growth of \textit{IL2}-dependent \textit{HTLV-I}-infected \textit{T} cell lines is arrested in early \textit{G1} (\textit{G0}) after withdrawal of \textit{IL2}, due to inhibition of the cyclin E-CDK2 complex by \textit{p27Kip1}, whereas cell cycle progression is maintained in \textit{IL2}-independent \textit{T} cell lines upon serum starvation (Cereseto \textit{et al} 1999).

1.8.7.3 \textit{CD95}-mediated apoptosis

The \textit{CD95} (\textit{Fas}, \textit{Apo-1})-\textit{CD95 ligand} (\textit{CD95L}) system is one of the pathways mediating activation-induced death in \textit{T} cells (Owen-Schaub \textit{et al} 1992, Klas \textit{et al} 1993, Wang \textit{et al} 1994, Alderson \textit{et al} 1995, Brunner \textit{et al} 1995, Dhein \textit{et al} 1995, Ju \textit{et al} 1995). Binding of \textit{CD95L} to \textit{CD95} by cell-cell contact activates an apoptosis cascade through interleukin 1β-converting enzyme (\textit{ICE})-like proteases (Enari \textit{et al} 1995, Los \textit{et al} 1995). \textit{CD95} is expressed by leukaemic cells in most cases of \textit{ATL} and by \textit{HTLV-I}-infected \textit{T} cell lines derived from \textit{ATL} patients, but the level of expression is variable (Debatin \textit{et al} 1990, 1993, Kotani \textit{et al} 1994, Sugahara \textit{et al} 1997, Tamiya \textit{et al} 1998). \textit{CD95} was expressed on leukaemic cells of 46 (98%) of 47 \textit{ATL} cases examined by Tamiya \textit{et al} (1998). \textit{HTLV-I}-infected \textit{T} cell lines, short-term cultured \textit{PBMCs} and fresh neoplastic cells from most \textit{ATL} patients, all of which expressed \textit{CD95}, were susceptible to apoptosis induced by anti-\textit{CD95}...
MAbs (Debatin et al 1990, 1993, Kotani et al 1994). In two CD95 ATL cases, leukaemic cells had mutations in both alleles of the CD95 gene and were resistant to apoptosis induced by anti-CD95 MAb (Tamiya et al 1998, Maeda et al 1999). Resistance to CD95-mediated apoptosis in other HTLV-I-infected T cell lines (especially long-term cell lines established by cocultivation and not derived from leukaemic clones) was associated with expression of Tax and/or Fas-associated phosphatase 1 (FAP1), which acts as a negative regulator of the CD95 apoptosis pathway (Copeland et al 1994, Sato et al 1995, Arai et al 1998a).

Expression of Tax reduced the susceptibility of uninfected CD4+ T cell lines to CD95-mediated apoptosis, provided the cells were cultivated in 5 to 10% serum (Copeland et al 1994, Chlichlia et al 1995, Chen et al 1997, Chlichlia et al 1997, Arai et al 1998a). The effect of duration of cultivation on expression of Tax by T cells could account for differences in sensitivity to CD95-mediated apoptosis. Tax up-regulates CD95L on T cells through NFkB and CREB/ATF pathways and these cells have an increased rate of apoptosis through the CD95-CD95L autocrine cell suicide pathway (Chlichlia et al 1995, Chen et al 1997, Chlichlia et al 1997). CD95-CD95L interactions are involved in physiological regulation of T cell responses, but it is uncertain whether this system plays a significant role in ATL (Alderson et al 1995, Dhein et al 1995).

1.8.7.4 Tax and apoptosis

The response of cells expressing Tax to withdrawal of growth factors is similar to that of cells expressing c-Myc. Established (immortalised) rodent fibroblast cell lines undergo growth arrest in G1 but survive when serum is withdrawn. Expression of c-Myc in the presence of serum leads to uncontrolled proliferation (transformation), whereas expression of c-Myc under conditions of serum deprivation leads to a reduction in the rate of population increase because continued proliferation is balanced by an increased rate of apoptosis (Evan et al 1992) (Sections 1.8.6 and 1.8.7.1). Rat1 cells transformed by Tax, as well as REFs and 3Y1 rat fibroblasts expressing Tax, undergo apoptosis when deprived of serum (Sakurai et al 1992b). This effect is blocked by Bcl-2 (Yamada et al 1994). The proportion of Rat1 cells expressing Tax that remain in S phase is maintained upon serum deprivation, indicating that Tax promotes cell cycle progression in the absence of external growth factors (Yamada et al 1994). Fujita and Shiku (1995) also observed that Rat1 cells transformed by tax underwent apoptosis following deprivation of serum, although the degree of apoptosis was less than that observed in Rat1 cells transformed by c-myc or fos. In a manner similar to c-Myc, Tax may thus activate pathways leading to either proliferation or apoptosis, depending on the availability of survival signals (Section 1.8.7.2). Appropriate survival signals would include growth factors such as those present in serum or anti-apoptotic factors such as Bcl-2. Conversely, since Tax may up-regulate c-myc (via NFκB) there is a possibility that c-Myc could mediate the apoptotic effect of Tax (Koizumi et al 1989, Fujita and Shiku 1993) (Section 1.4.6). Fos also induces apoptosis under conditions of serum deprivation and up-regulation of fos (via CREB/ATF or SRF) could be another indirect mechanism by which Tax activates this pathway (Alexandre and Verrier 1991, Colotta et al 1992, Smeyne et al 1993, Preston et al 1996), p21<sup>WAF1/CIP1</sup>, which induces apoptosis independent of p53 activity, is also up-regulated by Tax via NFκB in HTLV-I-infected and Tax-transformed T cell lines (Akagi et al 1996, Cereseto et al 1996, Gartenhaus et al 1996, Parker et al 1996, Low et al 1997).
The roles of the CREB/ATF and NFκB pathways in Tax-mediated apoptosis are uncertain. Iwanaga et al. (1999) provided evidence that the CREB/ATF pathway mediated Tax-induced apoptosis, whereas Tax inhibited apoptosis through the NFκB pathway. Tax reduced the rate of apoptosis of the IL2-dependent murine T cell line CTLL-2 in the absence of IL2 and this effect was maintained by Tax mutants defective for activation of the CREB/ATF pathway, whereas Tax mutants with an active CREB/ATF pathway, but defective NFκB pathway, accelerated the rate of apoptosis (Iwanaga et al. 1999). In contrast, Rivera-Walsh et al. (2001) showed that Tax-induced apoptosis was mediated by TNF-related apoptosis-inducing ligand (TRAIL) acting through the NFκB pathway and that Tax induces expression of TRAIL mRNA. An understanding of the pathways involved in Tax-induced apoptosis would allow the importance of this mechanism in HTLV-I leukaemogenesis to be better defined.

1.8.8 HTLV-I envelope

Proliferation of T cells is stimulated following infection with HTLV-I and also by contact with inactivated virions (Gazzolo and Duc Dodon 1987, Martin and Southern 1995, Höllsberg 1999). Cytokine-receptor loops such as IL2-IL2R appear to be important components in maintaining HTLV-I-induced T cell proliferation (Section 1.8.1.1) but initial activation of HTLV-I-infected T cells may be induced by viral gp46Env and through cell-cell contact involving CD2-lymphocyte function-associated antigen (LFA) 3, CD54-LFA1 and CD3-TCR interactions (Wucherpfennig et al. 1992, Akagi and Shimotohno 1993, Kimata et al. 1993, Cassé et al. 1994, Kimata et al. 1994a). Activation of T cells increases their permissiveness for HTLV-I infection (Section 1.3.2.2). The C terminus of HTLV-I p21Env has sequence similarity to the EGF receptor but there is no evidence that it plays a role in virus-induced proliferation of T cells (Lai 1991).

1.9 Animal models of HTLV-I leukaemogenesis

Animal models used in attempts to understand the pathogenesis of ATL, HAM/TSP and other HTLV-I-associated diseases include tax-transgenic mice, natural infections of cattle with BLV, natural infections of primates with STLV-I and experimental infections of mice, rats, rabbits and primates with HTLV-I.

1.9.1 HTLV-I tax-transgenic mice

Mice transgenic for tax have developed LGL leukaemia, fibroproliferative lesions, epithelial neoplasms, immune-mediated diseases and skeletal abnormalities (Hinrichs et al. 1987, Nerenberg et al. 1987, Furuta et al. 1989, Green et al. 1989, Iwakura et al. 1991, Ruddle et al. 1993, Grossman et al. 1995). However, T cell neoplasms similar to ATL (Section 1.6.1) have not been demonstrated and in some tax-transgenic models there is no increase in the frequency of neoplasia (Furuta et al. 1989). The observation of LGL leukaemia in tax-transgenic mice is most significant, since HTLV-I has been associated with this lymphoproliferative disorder in humans (Sections 1.10.2 and 1.11.2). In this model, mice transgenic for tax under the control of the lymphocyte-specific granzyme B enhancer, targeting expression to T, NK and lymphokine-activated killer (LAK) cells, developed LGL
leukaemia and NK cell tumours of the extremities and lymph nodes (Grossman et al. 1995). Transgenic mice expressing tax in a wide variety of tissues from the HTLV-I LTR, as well as mice with tax expression targeted to T cells by Thy1.2 or CD3-ε, frequently develop fibroproliferative lesions at sites of trauma, mainly on the extremities (Hinrichs et al. 1987, Nerenberg et al. 1987, Coscoy et al. 1998, Hall et al. 1998). The lesions are associated with peripheral nerves but can be distinguished from neurofibromatosis. Increased frequencies of other mesenchymal tumours (fibrosarcomas and lipomas), adrenal adenomas, adenocarcinomas of the mammary glands, salivary glands and lungs and fibroproliferative lesions of the iris and ciliary body have also been observed in HTLV-I LTR-tax-transgenic mice (Green et al. 1992a, Iwakura et al. 1994). Rats transgenic for the HTLV-I pX region developed mammary adenocarcinomas (Yamada et al. 1995). Proliferating cells from LGL lymphomas, fibroproliferative lesions and epithelial neoplasms in transgenic mice have high levels of expression of Tax, supporting a role for this protein in oncogenesis. However, only LGL leukaemia has been associated with HTLV-I infection in humans.

Mice and rats transgenic for tax are also predisposed to development of immune-mediated diseases, similar to conditions associated with HTLV-I infection in humans (Section 1.6.4). Arthritis developed in mice transgenic for the pX region of HTLV-I under control of the HTLV-I LTR or the metallothionein promoter (Iwakura et al. 1981, Yamamoto et al. 1983, Kira et al. 1997, Saggioro et al. 1997). Autoantibodies and CTL responses against Tax and Env in joints were demonstrated (Iwakura et al. 1995, Fujisawa et al. 1998). A condition resembling Sjögren's syndrome, with proliferation of ductal epithelial cells in salivary, lachrymal and Harderian glands in association with infiltration of lymphocytes and plasmacytes, has also been observed in HTLV-I LTR-tax-transgenic mice (Green et al. 1989, Bieberich et al. 1993) (Section 1.6.4.6). Lymph nodes adjacent to affected salivary glands were hyperplastic and occasionally had metastases of salivary ductal epithelium (Green et al. 1989). Rats transgenic for the HTLV-I LTR-env-pX region developed arthritis, polyarteritis, polymyositis, myocarditis, sialoadenitis and thymic atrophy, consistent with an immune-mediated multisystem disease process (Yamazaki et al. 1995). Thymic atrophy and immunosuppression in tax-transgenic mice is associated with growth retardation and increased mortality from bacterial and parasitic diseases (Hinrichs et al. 1987, Nerenberg et al. 1987, Furuta et al. 1989, Iwakura et al. 1991, Bieberich et al. 1993, Hall et al. 1998). Skeletal abnormalities in HTLV-I LTR-tax-transgenic mice were characterised by remodelling of bone and myelofibrosis due to increased activity of osteoclasts and osteoblasts (Ruddle et al. 1993, Saggioro et al. 1997).

CD3⁺ CD4⁺ CD8⁺ TCLs of the abdominal lymph nodes and CNS neoplasms resembling neuroblastomas developed rapidly in 100% of mice co-transgenic for HTLV-I LTR-tax and Ig-c-myc (Benvenisty et al. 1992). However, mice transgenic for c-myc alone under the influence of the HTLV-I LTR also developed CD4⁺ CD8⁺ T cell or B cell lymphomas in the mesenteric lymph nodes. Since in these experiments c-myc expression was driven by the HTLV-I LTR, the activity of which is up-regulated in the presence of Tax, it was not possible to determine whether Tax cooperated with c-Myc independently of its action on the HTLV-I LTR.
1.9.2 Natural animal models of HTLV-I infection

1.9.2.1 Bovine leukaemia virus


BLV is structurally similar to HTLV-I and contains genes in the pX region that express TaxBLV and RexB LV, as well as accessory proteins R' and G' (Sagata et al 1985, Rosen et al 1986, Willems et al 1987, Jensen et al 1991, Haas et al 1992, Alexandersen et al 1983, Willems et al 1994). Kettmann et al (1982) found that defective BLV proviruses were frequent in EBL, with preferential retention of the pX region, similar to HTLV-I in ATL. Other studies have reported conservation of complete BLV provirus with infrequent occurrence of deletions (Tajima et al 1998). TaxBLV immortalises B cells and cooperates with Ras in transformation of primary REFs, although it may not be necessary for maintenance of the transformed phenotype (Willems et al 1990, 1992, Twizere et al 2000). G' also cooperates with Ras in transforming primary REFS and inducing tumours in athymic mice (Kerkhofs et al 1998).

1.9.2.2 Simian T lymphotropic viruses

STLV-I has been serologically associated with lymphomas in a variety of species of non-human primates (Homma et al 1984). ATL-like leukaemia with monoclonal integration of STLV-I has been described in African green monkeys (Cercopithecus aethiops) (Tsujimoto et al 1987). In captive colonies of baboons (Papio hamadryas) there is a high prevalence of STLV-I infection and most animals with TCLs are seropositive for STLV-I (Moné et al 1992, Voevodin et al 1996). STLV-II does not appear to be associated with T cell neoplasia in monkeys.

1.9.3 Experimental animal models of HTLV-I infection

Animals susceptible to experimental infection with HTLV-I include rabbits, rats, mice and primates (Miyoshi et al 1985, Suga et al 1991, Kazanji et al 1997b, Fang et al 1998). HTLV-I infects and transforms rabbit and non-human primate T cells, as well as a variety of different cell types from these and other species (Miyoshi et al 1982, 1983). Genetic background appears to be an important determinant of susceptibility to infection and disease (Kazanji et al 1997a). Among several strains of rats infected experimentally with HTLV-I, only those
derived from the WKAH strain develop ATL-like or HAM/TSP-like disease (Ishiguro et al 1992, Oka et al 1992, Kushida et al 1994). Mesenchymal tumours and polyarthritis have also been observed in experimentally infected rats (Kira et al 1997) (Sections 1.6.4.3 and 1.9.1). Rabbits infected with HTLV-I develop ATL-like T cell leukaemia and CTCL (Seto et al 1988, Simpson et al 1996). A rhesus macaque (Macaca mulatta) developed polyarthritis, uveitis and polymyositis following infection with HTLV-I (Beilke et al 1996) (Section 1.6.4). However, HTLV-I infection of non-human primates is not a useful model for ATL, since neoplasia is likely to be an infrequent and delayed consequence of experimental exposure (Kazanjian 2000). CD4+ TCLs of human origin can be established in severe combined immunodeficiency (SCID) mice by inoculation of PBMCs from ATL patients (Feuer et al 1993, Kondo et al 1993).

1.10 T cell and natural killer cell neoplasms

The International Lymphoma Study Group Revised European-American Lymphoma (REAL) classification is used in this thesis for terminology relating to T cell and natural killer (NK) cell neoplasms (Harris et al 1994), with reference to Willemze et al (1997) for specific classification of cutaneous T cell lymphomas (CTCLs) (Table 1.5). HTLV-I and related retroviruses have been associated with ATL (Sections 1.6.1 and 1.11), CTCLs (particularly MF/SS, cutaneous CD30+ ALCL and angiocentric TCL) and LGL leukaemia. CTCLs are neoplasms of T cells that primarily involve the skin. The epidemiology, clinicopathological features and pathogenesis of these neoplasms will be described in this section. The biology of acute lymphoblastic leukaemia (ALL), small plaque parapsoriasis, Langerhans cell histiocytosis (LCH) and cutaneous B cell lymphoma (CBCL) will also be described. These entities are included in the differential diagnosis of ATL and CTCL and were among cases from which samples were received for laboratory examinations described in Chapters 2 and 3.

1.10.1 Mycosis fungoides/Sézary syndrome

Alibert (1806) introduced the term mycosis fungoides (MF) to describe a mushroom-shaped tumour of the skin that developed following a rash. MF is now established as a distinct clinicopathological entity. Sézary and Bouvrain (1938) described the syndrome of erythroderma, leukaemia and lymphadenopathy that has become known as Sézary syndrome (SS) and is now considered to be the leukaemic form of MF (Edelson 1980).

1.10.1.1 Epidemiology

MF/SS occurs sporadically and there is limited evidence for case clustering or areas of endemcity. The incidence of reported cases of MF/SS per 100,000 population per year is 0.13 to 0.14 in Europe and Australia and 0.29 in the USA (Weinstock and Horm 1988); 0.9 cases per 100,000 per year were reported in a more intensive, localised study in Minnesota, USA (Chuang et al 1990). About 15% of cases of MF/SS are classified as SS. The median age of onset of skin lesions is 51 years; only a small proportion of cases appear during childhood or in young adults (Zackheim et al 1997). The male:female adjusted ratio is 2:1 to 3:1 (Weinstock and Horm 1988, Chuang et al 1990). A trend to increasing incidence of
reported cases has been observed (Weinstock and Reynes 1999). MF and other lymphoid neoplasms have occasionally been observed in relatives of patients with MF/SS (Greene et al 1982). Cohen et al (1980) identified an increased risk for MF in people employed in the manufacturing or construction industries, especially in petrochemical, textile, metallurgical and mechanical occupations, whereas other studies have not confirmed these associations (Tuyp et al 1987, Whittemore et al 1989).

1.10.1.2 Clinical and pathological features

MF is a slowly growing neoplasm, usually affecting adults, characterised by infiltration of the skin with neoplastic T cells exhibiting marked epidermotropism. In the early stages of MF neoplastic T cells infiltrate the epidermis with a band pattern (lichenoid reaction, vacuolar interface dermatitis), sometimes forming intraepidermal clusters (Pautrier’s microabscesses) (Shapiro and Pinto 1994). Patches, plaques and nodules (tumours) are formed as the disease progresses and there is involvement of lymph nodes and viscera in the advanced stages (Vonderheide et al 1994). SS is a leukaemia characterised by erythroderma, lymphadenopathy and leucocytosis, with neoplastic T cells circulating in the peripheral blood (Matutes et al 1990). Neoplastic lymphocytes infiltrate the skin in SS but may have less marked epidermotropism than in MF (Trotter et al 1997, Kamarashev et al 1998). Clinical erythroderma without a histologically distinct neoplastic infiltrate often precedes MF (Bakels et al 1991). A rare “tumeur d’emblée” form of MF presents as tumours with no apparent premalignant or plaque stages and has a rapidly progressive course (Blasik et al 1982). Other variants of MF include spongiotic/vesicular, bullous, follicular/mucinous, pustular, granulomatous and verrucous forms, as well as cases with altered pigmentation of the skin (LeBoit 1991).

The neoplastic cells infiltrating the skin in MF, circulating in the peripheral blood in SS and infiltrating lymph nodes in advanced forms of the disease are T cells that are similar morphologically and immunologically to those of MF/Sézary cells (Zucker-Franklin et al 1974). These are round cells with highly convoluted (cerebriform) nuclear contours (Lutzner et al 1971). Transformation of neoplastic T cells to a large cell morphology characteristic of anaplastic large cell lymphoma (ALCL) occurs in some late stage cases of MF and SS (Salhany et al 1988, Wood et al 1993) (Section 1.10.3).

1.10.1.3 Immunology

Table 1.5: Classification of T cell and natural killer cell neoplasms (Harris et al 1994).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Neoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Precursor T cell neoplasm</td>
<td>Precursor T-lymphoblastic lymphoma/leukaemia</td>
</tr>
<tr>
<td>II. Peripheral T cell and natural killer cell neoplasms</td>
<td>T cell chronic lymphocytic leukaemia/prolymphocytic leukaemia</td>
</tr>
<tr>
<td></td>
<td>Large granular lymphocytic leukaemia</td>
</tr>
<tr>
<td></td>
<td>Large granular lymphocytic leukaemia, T cell type</td>
</tr>
<tr>
<td></td>
<td>Large granular lymphocytic leukaemia, NK cell type</td>
</tr>
<tr>
<td>3. Mycosis fungoides/Sézary syndrome</td>
<td>Large granular lymphocytic leukaemia, NK cell type</td>
</tr>
<tr>
<td>4. Peripheral T cell lymphomas, unspecified</td>
<td>Medium-sized cell peripheral T cell lymphoma*</td>
</tr>
<tr>
<td></td>
<td>Mixed medium-sized and large cell peripheral T cell lymphoma*</td>
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<tr>
<td></td>
<td>Large cell peripheral T cell lymphoma*</td>
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<tr>
<td></td>
<td>Lymphoepithelioid cell (Lennert’s lymphoma)*</td>
</tr>
<tr>
<td></td>
<td>Hepatosplenic γ6 T cell lymphoma*</td>
</tr>
<tr>
<td></td>
<td>Subcutaneous panniculitic T cell lymphoma*</td>
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<tr>
<td>5. Angioimmunoblastic T cell lymphoma</td>
<td>Medium-sized cell peripheral T cell lymphoma*</td>
</tr>
<tr>
<td>6. Angiocentric T cell lymphoma</td>
<td>Mixed medium-sized and large cell peripheral T cell lymphoma*</td>
</tr>
<tr>
<td>7. Intestinal T cell lymphoma (+/- enteropathy associated)</td>
<td>Large cell peripheral T cell lymphoma*</td>
</tr>
<tr>
<td>8. Adult T cell lymphoma/leukaemia, HTLV-I*</td>
<td>Lymphoepithelioid cell (Lennert’s lymphoma)*</td>
</tr>
<tr>
<td>9. Anaplastic large cell lymphoma, CD30*</td>
<td>Hepatosplenic γ6 T cell lymphoma*</td>
</tr>
<tr>
<td></td>
<td>Anaplastic large cell lymphoma, T cell type</td>
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<tr>
<td>10. Anaplastic large cell lymphoma, Null cell type</td>
<td>Anaplastic large cell lymphoma, Null cell type</td>
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<tr>
<td></td>
<td>Anaplastic large cell lymphoma, Hodgkin’s-like*</td>
</tr>
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</table>

* Provisional entities
It has been suggested that the cytokine profile of neoplastic T cells is predominantly Th1 in MF and Th2 in SS (Saed et al. 1994, Lee et al. 1999), but other studies support a Th2 profile in both MF and SS (Vowels et al. 1992, Tendler et al. 1994, Vowels et al. 1994, Dummer et al. 1996). Naïve (Th0) CD4^ T cells are induced by IL12 to differentiate into Th1 cells, which produce IFNγ, or by IL4 to differentiate into Th2 cells, which produce IL4, IL5 and IL13 (Abbas et al. 1996). Since these cells have different roles in the regulation of immune responses, hypotheses as to the pathogenesis of MF/SS are restricted by uncertainty about the functional categorisation of the neoplastic cells.

Immunological abnormalities observed in patients with advanced MF/SS include decreased responsiveness of T cells to antigens and mitogens, decreased NK cell activity, decreased LAK cell activity, eosinophilia and increased serum IgE and IgA (Wood et al. 1990b). In SS there is a marked reduction in the number of non-neoplastic T cells in the peripheral circulation (Heald et al. 1994) and an associated immune deficiency may be responsible for secondary infections in MF/SS patients (Axelrod et al. 1992). An MHC-I-restricted cell-mediated immune response against neoplastic T cells has been observed in MF/SS patients (Berger et al. 1996). An infiltrate of activated cytotoxic CD8^ and CD4^ T cells accompanies the neoplastic CD4^ infiltrate in the early stages of MF/SS but decreases with advancement of disease, as does the proportion of activated CD8^ T cells in the peripheral blood (Wood et al. 1994a, Asadullah et al. 1997). Some authors have reported restricted use of TCR Vβ epitopes by neoplastic T cell clones in MF, suggesting that exposure to a common antigen, such as a bacterial superantigen or persistent viral antigen, could contribute to chronic T cell activation and proliferation in MF (Potoczna et al. 1996), but others have shown that a variety of Vβ genes are used (Longley et al. 1995, Bigler et al. 1996).

1.10.1.4 Molecular and genetic characteristics

The neoplastic character of MF/SS has been confirmed by the detection of clonal TCR β or γ gene rearrangements in skin, PBMCs and lymph nodes (Weiss et al. 1985b, Whittaker et al. 1991). Clonality in MF/SS has also been demonstrated by cytogenetic studies but no consistent karyotypic abnormalities have been identified (Eidelberg et al. 1979, Nowell et al. 1982, Johnson et al. 1985). Mutations, rearrangements or deletions involving lyt-10, p53 or tal1 have been observed in some cases of MF/SS but their pathogenetic role is uncertain (Thakur et al. 1994, Neri et al. 1995). Mutations in p53 develop during neoplastic progression, being found in one third of patients with tumour stage MF but not in patients with plaque stage MF (McGregor et al. 1999).

1.10.1.5 Pathogenesis

In models of the development of MF/SS a multistage progression of oncogenic events has been hypothesised (Lessin et al. 1994). Acute to chronic dermatitis with recruitment of a polyclonal T cell infiltrate often precedes the development of MF/SS (Sigurdsson et al. 1997). The next stage is cutaneous lymphoid hyperplasia, with formation of preneoplastic patches in the skin containing polyclonal lymphocytes exhibiting nuclear atypia (Lange Wantzin et al. 1988). Primary neoplasia in the form of plaques is initially localised to the skin, then tumours
develop in association with erythroderma and there is spread to lymph nodes, viscera and peripheral blood (Bunn et al. 1980, Tobback and Edelson 1985). Transformation of MF to ALCL is marked by development of aggressive disease (Wood et al. 1993). MF/Sézary cells circulate between cutaneous and extracutaneous sites and systemic dissemination of neoplastic T cell clones can be demonstrated early in the development of the disease (Miller et al. 1980, Veelken et al. 1995, Muché et al. 1997). MF/Sézary cells proliferate most rapidly in lymph nodes, with a lower rate of proliferation in the skin and limited proliferation in the peripheral blood (Shackney and Schuette 1983, Tu et al. 1994). As disease progresses the rate of proliferation increases, while the proportion of cells undergoing apoptosis decreases (Kikuchi and Nishikawa 1997). Expression of c-Myc, Ras, Jun, Fos, PCNA and p53 increases with advancing stages of MF (Kanavaros et al. 1994, Tu et al. 1994, Beylot-Barry et al. 1995, Lauritzen et al. 1995, Qin et al. 1999).

Selective expression of adhesion molecules is responsible for the tropism of subsets of T cells for the skin, lymph nodes and other tissues and may explain the distribution of cellular infiltrates at different stages of MF/SS (Miller et al. 1980, Borowitz et al. 1993, Campbell and Butcher 2002). CD54, induced on keratinocytes and dermal endothelial cells by T cell-derived IFNγ and TNFα, binds to CD11a/CD18 (LFA1) expressed by T cells, including those in MF (Veijsgaard et al. 1989, Imayama et al. 1992, Uccini et al. 1993). This interaction appears to be necessary but not sufficient for epidermotropic behaviour. Loss of CD54 expression by keratinocytes due to reduced IFNγ expression by neoplastic T cells may permit systemic dissemination in SS (Nickoloff et al. 1989). CD62E (E-selectin, endothelial leucocyte adhesion molecule 1, ELAM1), which is expressed by dermal endothelial cells, particularly in inflamed skin, is one of the ligands for cutaneous lymphocyte-associated antigen (CLA), a carbohydrate epitope induced on CD62P (P-selectin) glycoprotein ligand 1 (PSGL1) in T cells, including those infiltrating the skin in MF (Fuhlbrigge et al. 1997, Tietz et al. 1998). CLA, which acts as a skin homing receptor, is expressed on CTCLs but is absent from nodal TCLs (Picker et al. 1990, Noorduyn et al. 1992). Similarly, integrins α3β1, α5β1 and α5β7, which act as extracellular matrix receptors, are expressed by epidermotropic T cells in MF but are absent from circulating MF/Sézary cells in SS (Savoia et al. 1992, Simonitsch et al. 1994). Most cases of MF lack expression of CD62L (L-selectin, peripheral lymph node homing receptor), whereas expression of CD62L has been associated with the involvement of lymph nodes by CTCL (Wood et al. 1990a, Borowitz et al. 1993). MHC-II, the ligand for CD4, is expressed by keratinocytes and other cells in lesional skin in MF/SS, but is not correlated with the presence of epidermal lymphocytes (Wood et al. 1994b). IFNγ- and TNFα-inducible proteins (IPs) IP9, IP10 and monokine induced by IFNγ (MIG), which are chemotactic for CD4+ T cells, are expressed by epidermal keratinocytes in MF/SS lesions and may also contribute to the epidermotropism of MF/Sézary cells (Dallani et al. 1998, Tensen et al. 1998).

Abnormal production of cytokines by MF/Sézary cells, keratinocytes or antigen presenting cells (APCs) may contribute to the proliferation and distribution of neoplastic T cells in MF/SS. Increased expression of IL1, IL5, IL6, GMCSF, IFNγ, TNFα, TGFα and EGF have been detected in MF (Kupper et al. 1988, Lawlor et al. 1990, Hansen et al. 1991, Vowels et al. 1992, Tendler et al. 1994, Dummer et al. 1996, Asadullah et al. 1998, Dallani et al. 1998,
Courville et al. (1999). IL4, IL10 and IL15 expression may increase with progression of MF (Vowels et al. 1994, Asadullah et al. 1996a, 2000). The response of MF/Sézary cells to cytokines is discussed in Section 2. Little is known about the expression of receptors for these cytokines. Neoplastic T cells in MF express both CD28 and its ligand CD80, providing the opportunity for self-costimulation (Nickoloff et al. 1994). Rook et al. (1993) hypothesised that IFNγ production by tumour infiltrating lymphocytes (TILs) responding to the neoplastic infiltrate exceeds IL4 production by MF/Sézary cells in the early stages of MF, decreasing the rate of expansion of the population of neoplastic cells and promoting epidermotropism through induction of CD54 on keratinocytes. Eventually the population of neoplastic cells reaches a size at which IL4 production counteracts the production and biological activity of IFNγ, leading to reduced expression of CD54 by keratinocytes, loss of epidermotropism with systemic dissemination of neoplastic cells and reduced anti-neoplasm immunity.

Rowden and Lewis (1976) proposed that persistent antigenic stimulation was responsible for the abnormal T cell proliferation in MF/SS. APCs, including Langerhans cells and interdigitating cells, are present among the neoplastic T cell infiltrate (Tjernlund 1982). Langerhans cells have been shown to contribute to epidermotropism of T cells (Shiohara et al. 1988). It has been hypothesised that retroviral infection of Langerhans cells leads to chemotaxis or retention of T cells in the epidermis (MacKie 1981, Slater et al. 1985, Lessin et al. 1994). Chronic stimulation of this population would eventually lead to the selective outgrowth of a neoplastic T cell clone. Alterations in the morphology of Langerhans cells and increased interactions between Langerhans cells and MF/Sézary cells have been described in MF (Rowden et al. 1979, Füllbrandt et al. 1983). In contrast, Bani et al. (1990) observed that MF/Sézary cells were frequently associated with interdigitating cells in the epidermis, but rarely made contact with Langerhans cells. However, most evidence incriminating retroviruses in CTCLs is focussed on infection of neoplastic T cells by HTLV-I or related viruses and a direct involvement of such viruses in oncogenesis (Dosaka et al. 1991, Hall et al. 1991) (Section 1.11). In addition, the density of Langerhans cells is increased in the dermis and epidermis in plaque stage MF, but may decrease with progression to tumour stage lesions, indicating that Langerhans cells could play a role in suppression of CTCLs rather than facilitating progression (Igisu et al. 1983, Meissner et al. 1993).

1.10.2 Large granular lymphocytic leukaemia

Large granular lymphocytic (LGL) leukaemia is defined as a clonal increase in the number of circulating large granular lymphocytes (LGLs) and may be of T cell or NK cell phenotype (Loughran et al. 1985, Chan et al. 1986, Loughran 1993). A chronic proliferation of LGLs that cannot be proven to be clonal is referred to as lymphoproliferative disease of LGLs (LDLGLs) (Pandolfi et al. 1990, Scott et al. 1993, Semenzato et al. 1997). Synonyms for LGL leukaemia include chronic T cell lymphocytosis with neutropenia, T cell CLL and Ty lymphocytosis/lymphoproliferative disorder (Brouet et al. 1975, Aisenberg et al. 1981, Reynolds and Foon 1984). Hypotheses regarding the aetiology of CD3⁺ and CD3⁻ LGL leukaemia have included infection by retroviruses, activation by antigens, stimulation by cytokines and accumulation of mutations (Loughran 1993). Evidence for the involvement of viruses in LGL leukaemia is discussed in Section 1.11.
1.10.2.1 Large granular lymphocytic leukaemia, T cell type

T cell LGL leukaemia is a clonal disorder of CD3^ cells with LGL morphology and is associated with chronic neutropaenia, anaemia, splenomegaly, recurrent bacterial infections and rheumatoid arthritis or other autoimmune diseases (Loughran et al 1985, Loughran 1993, Scott et al 1993). Neoplastic lymphocytes have abundant, pale blue cytoplasm, azurophilic granules, an eccentric nucleus with moderately condensed chromatin and an inconspicuous nucleolus. The immunophenotype is usually CD2^, CD3^, CD4^, CD7^, CD8^, CD16^, CD25^ and CD56^, although CD4^ CD8^ and CD4^ CD8^dim^ phenotypes have been observed (Pandolfi et al 1991, Richards et al 1992). Most cases are TCR αβ^ but TCR γδ^ expression has been reported (Foroni et al 1988, Loughran et al 1988a). Clonality has been demonstrated by cytogenetic studies (Loughran et al 1985, McKenna et al 1985) and detection of clonal rearrangements of the TCR β or γ genes (Pellicci et al 1987, Pandolfi et al 1991, Scott et al 1993).

1.10.2.2 Large granular lymphocytic leukaemia, natural killer cell type

The clinical features of NK cell LGL leukaemia, a clonal disorder of CD3^ CD56^ cells, are hepatomegaly, splenomegaly, anaemia and thrombocytopenia, along with fever, night sweats and/or weight loss (B symptoms) (Sheridan et al 1988, Sivakumaran et al 1996). The immunophenotype is usually CD2^, CD3^, CD4^, CD8^, CD16^, CD56^ and CD57^, NK activity has been demonstrated in leukaemic cells (Loughran et al 1987). There is no expression or rearrangement of the TCR or immunoglobulin genes (McKenna et al 1985, Loughran et al 1988a, Biondi et al 1989). Clonality has been confirmed in some cases by demonstration of clonal cytogenetic abnormalities or clonal X-linked inactivation of the phosphoglycerate kinase gene (Sheridan et al 1988, Taniwaki et al 1990, Nash et al 1993, Kelly et al 1994). Cases of CD3^ CD56^ LDLGLs in which clonality cannot be demonstrated usually have a less aggressive clinical course.

1.10.3 CD30^ anaplastic large cell lymphoma

CD30^ anaplastic large cell lymphomas (ALCLs) are aggressive solitary or multiple tumours that in the primary systemic form have a bimodal age distribution and involve lymph nodes, skin, bone or soft tissues (Agnarsson and Kadin 1988, Kaudewitz et al 1989, Paulli et al 1995). Primary cutaneous CD30^ ALCLs, although indolent, limited to the skin and occasionally spontaneously regressing, paradoxically are often difficult to treat (Beljaards et al 1993, Paulli et al 1995, Willemze et al 1997). Secondary CD30^ ALCLs appear to be derived following transformation of MF, other TCLs or Hodgkin's disease (Salhany et al 1988, Wood et al 1993) (Section 1.10.1.2). In a patient with MF and cutaneous ALCL, CD30^ T cells in spontaneously regressing tumours were derived from the same clone as MF/Sézary cells in patch stage MF lesions (Woodrow et al 1996). CD30^ ALCLs are composed of large pleomorphic lymphoid cells with abundant cytoplasm and horseshoe-shaped or wreath-like nuclei with prominent nucleoli, along with multinucleated Reed-Sternberg-like cells. Inflammatory leucocytes may infiltrate the involved tissue. CD30 is a marker of activation and CD30^ ALCLs variably express other activation markers such as
CD25, MHC-II and CDw70 (Kaudewitz et al. 1989). The origin of the neoplastic cell is uncertain, since CD30⁺ ALCLs have been classified as having T cell, B cell or immature phenotype. Most cases of primary cutaneous CD30⁺ ALCL appear to be of T cell origin; 95% or more are CD4⁺ and 5% or less are CD8⁺, with variable expression of pan T cell antigens (CD2, CD3, CD5) (Kaudewitz et al. 1989, Beljaards et al. 1993, Willemze et al. 1997). Primary systemic CD30⁺ ALCLs often express epithelial membrane antigen (EMA) and are negative for CLA, whereas most cases of primary cutaneous CD30⁺ ALCL are CLA⁺ and EMA⁻ (De Bruin et al. 1993). Clonal rearrangements of TCR genes are found in 50 to 60% of primary systemic CD30⁺ ALCLs; most cases of primary cutaneous CD30⁺ ALCL also have clonal TCR gene rearrangements (Herbst et al. 1989, Banerjee et al. 1991). Many T cell and null cell primary systemic CD30⁺ ALCLs have the chromosomal translocation t(2;5)(p23;q35), which conjoins the nucleophosmin (NPM) and anaplastic lymphoma kinase (ALK) genes (Morris et al. 1994). This translocation does not appear to be present in most primary cutaneous CD30⁺ ALCLs (De Coteau et al. 1996, Li et al. 1997). The role of EBV in the aetiology of CD30⁺ ALCL is described in Section 1.11.3.1 and evidence for the involvement of HTLV-I in this entity is described in Section 1.11.1.7.

1.10.4 Angiocentric T cell lymphoma

Angiocentric TCLs are extranodal T cell neoplasms associated with blood vessels that frequently involve the nasopharyngeal region, lung and skin (Lipford et al. 1988, Kanavaros et al. 1993, Kato et al. 1999). The nasopharyngeal/sinonasal form is also known as lethal midline granuloma or polymorphic reticulosis (Gaulard et al. 1988, Strickler et al. 1994). Blood vessels are surrounded and invaded by pleomorphic neoplastic lymphocytes, sometimes with a predominance of large cells, and frequently there is an associated inflammatory infiltrate, along with ischaemic necrosis. The immunophenotype is CD2⁺, with variable expression of CD3, CD4, CD5, CD7 and CD8, indicating a T cell origin; however the neoplasms can also be CD3⁻ CD56⁺, consistent with a NK cell phenotype. TCR β gene rearrangements have been detected in angiocentric TCLs of T cell type (Gaulard et al. 1988). There are usually no clonal rearrangements or expression of TCR or immunoglobulin genes in the NK cell type, but clonality has been confirmed by karyotypic studies (Emile et al. 1996, Tien et al. 1997). EBV has been aetiologically associated with the nasopharyngeal/sinonasal form of angiocentric TCL (Section 1.11.3.1) and some groups have associated limited numbers of cases of angiocentric TCL with HTLV-I infection (Section 1.11.1.7). The neoplastic infiltrate in cutaneous ATL may have an angiocentric distribution with necrotising vasculitis (Haynes et al. 1983, Manabe et al. 1988).

Lymphomatoid granulomatosis is also an angiocentric immunoproliferative condition that may involve the lungs or skin and in most cases appears to be a form of angiocentric TCL (James et al. 1981, Kessler et al. 1981, Gaulard et al. 1988, Whittaker et al. 1988). However, some pulmonary cases express B cell antigens, have clonal immunoglobulin gene rearrangements and contain EBV genomes, so represent proliferations of EBV-infected B cells (Nicholson et al. 1996).
1.10.5 Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemias (ALLs) are aggressive clonal proliferations of T or B cell lineage lymphoid cells with heterogeneous morphological, immunophenotypic and cytogenetic characteristics (Harris et al. 1994, Copelan and McGuire 1995, Kersey 1997). Neoplastic lymphoid cells circulate in the blood and infiltrate tissues. ALL is over-represented as a neoplasm of childhood, with a peak in incidence at 2 to 5 years of age. The incidence in children under 15 years of age in the USA is approximately 30 cases/million/year (Young et al. 1986). The incidence in adults is one third of this figure but exhibits a second peak at 50 years of age followed by an increasing incidence with age. In paediatric and adult ALL, approximately 85% of cases are of precursor-B (pre-B) or B cell type and 15% of T cell type (Copelan and McGuire 1995, Kersey 1997, Khalidi et al. 1999). B cell ALLs express surface CD19 and include B-precursor (early pre-B) ALL (expressing cytoplasmic CD22 and nuclear terminal deoxynucleotidyl transferase), common ALL (expressing surface CD10), pre-B ALL (expressing cytoplasmic immunoglobulin heavy chains) and B-ALL (expressing surface immunoglobulin) (Janossy et al. 1989, Copelan and McGuire 1995) (Table 1.6). T cell ALLs have cytoplasmic expression of CD3 and include precursor-T (pre-T) ALL (CD7+) and T-ALL (CD7+, CD2+). Null ALL predominates in infants (less than 1 year of age), common ALL is predominant in children 1 to 7 years of age and T cell ALL is proportionally high in adolescents and young adults (McKinney et al. 1993).

Clonality in ALL has been demonstrated by cytogenetic analysis, X-linked inactivation of glucose-6-phosphate dehydrogenase and analysis of immunoglobulin and TCR gene rearrangements (Williams et al. 1985, Dow et al. 1985, Khalidi et al. 1999). The earlier stage B and T cell ALLs have partial or incomplete rearrangements of immunoglobulin receptor and TCR genes, respectively. Up to 5% of children and approximately 30% of adults with ALL have a Philadelphia chromosome (shortened chromosome 22) due to a t(9;22)(q34;q11) translocation that results in production of abnormal proteins, p190br^AB^ or p210br^AB^, with increased PTK activity (Chan et al. 1987, Kurzrock et al. 1987, Faderl et al. 1998). Most Philadelphia positive cases have the phenotype of B cell ALL, but appear to be derived from a pluripotential stem cell; mixed lineage leukaemia may occur (Secker-Walker et al. 1991, Copelan and McGuire 1995, Schenk et al. 1998). A wide range of other numerical and structural chromosomal abnormalities have been observed (Faderl et al. 1998, Kersey 1997). Translocations frequently generate oncogenic fusion proteins or result in deregulated expression of transcription factors. These include immunoglobulin H, κ or λ-α-myc and TEL-AML1 in B cell ALL and TCR β-TAL1 or TCR α-α-myc in T cell ALL (Croce and Nowell 1985, Nakamura et al. 1993, Kersey 1997, Wiemels and Greaves 1999). The translocation t(4;11)(q21;q23) conjoining MLL and AF-4 occurs in approximately 50% of infants with ALL, as well as in 2% of older children and 3 to 6% of adults (Pui et al. 1991, Biondi et al. 2000). Some cases of childhood ALL are initiated in utero (Wiemels et al. 1999). Patients with ataxia telangiectasia have an increased risk of developing T cell ALL (Toledano and Lange 1980).

Table 1.6: Immunophenotypes of acute lymphoblastic leukaemias
(Copelan and McGuire 1995).

<table>
<thead>
<tr>
<th>Leukaemia type</th>
<th>Surface markers</th>
<th>Cytoplasmic markers</th>
<th>Nuclear markers</th>
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<tbody>
<tr>
<td><strong>B cell acute lymphoblastic leukaemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Pre-B ALL</td>
<td>CD19</td>
<td>CD22</td>
<td>TdT</td>
</tr>
<tr>
<td>Common ALL</td>
<td>CD19, CD10</td>
<td>CD22</td>
<td>TdT</td>
</tr>
<tr>
<td>Pre-B ALL</td>
<td>CD19</td>
<td>Ig</td>
<td>TdT</td>
</tr>
<tr>
<td>B-ALL</td>
<td>CD19, CD22, Ig</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>T cell acute lymphoblastic leukaemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-T</td>
<td>CD7</td>
<td>CD3</td>
<td>TdT</td>
</tr>
<tr>
<td>T-ALL</td>
<td>CD1, CD2, CD3,</td>
<td></td>
<td>TdT</td>
</tr>
<tr>
<td></td>
<td>CD4, CD7, CD8</td>
<td></td>
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</tr>
</tbody>
</table>

ALL: Acute lymphoblastic leukaemia
CD: Cluster of differentiation
Ig: Immunoglobulin
TdT: Terminal deoxynucleotidyl transferase
Pre: Precursor
Studies using PCR with specific, consensus or degenerate primers have so far failed to implicate herpesviruses, polyomaviruses or parvovirus B19 as the cause of common ALL in children (Luka et al 1991, Gentile et al 1999, Heegaard et al 1999, MacKenzie et al 1999, Smith et al 1999, MacKenzie et al 2001). ATL can be distinguished from T cell ALL on the basis of clinicopathological features, particularly the presence of hypercalcaemia and lytic bone lesions, and the presence of HTLV-I infection.

1.10.6 Small plaque parapsoriasis

Small plaque parapsoriasis is a chronic dermatosis characterized by patches on the trunk and extremities that often resolve spontaneously (Harris et al 1994, Haefner et al 1995). Histopathological examination reveals a mild superficial perivascular lymphocytic infiltrate composed of CD4+ T cells admixed with CD8+ T cells, Langerhans cells/indeterminate cells and macrophages, associated with mild spongiosis and parakeratosis. Both dominant clonal and polyclonal T cell populations have been demonstrated (Haefner et al 1995).

1.10.7 Langerhans cell histiocytosis

Langerhans cell histiocytosis (LCH, histiocytosis X) is characterised by cutaneous infiltrations of Langerhans cells that contain Birbeck granules, express S-100 and CD1a and may have aberrant expression of CD2, CD3, CD54 and CD58 (LFA-3), consistent with an activated state (Ben-Ezra and Kao 1993, Hage et al 1993, De Graaf et al 1994, Emile et al 1994). As well as cells with histiocytic morphology, lesions contain giant cells, macrophages, neutrophils, eosinophils, lymphocytes, plasma cells and occasional mast cells. Using PCR and ISH, 56 cases of LCH in the USA were negative for HTLV-I, HTLV-II, HIV-1, EBV, herpes simplex virus (HSV) type 1, HHV-6, cytomegalovirus, adenovirus and parvovirus (McClain et al 1994). Infiltrating histiocytic cells lack TCR gene rearrangements but clonality has been determined by X-linked inactivation studies of the androgen receptor gene and with X-linked polymorphic DNA probes, consistent with LCH being a neoplastic proliferation (Willman et al 1994, Yu et al 1994, Yu and Chu 1995).

1.10.8 Cutaneous B cell lymphoma

Cutaneous B cell lymphomas (CBCLs) are described here because they represent one of the differential diagnoses of CTCL, being characterised by infiltrations of neoplastic B cells in the skin (Harris et al 1994, Rijaarsdam and Willemze 1994). They frequently have a follicular pattern with formation of nodules but neoplastic B cells may be distributed diffusely through the dermis and occasionally exhibit epidermotropism. Immunoglobulin gene rearrangements can be detected in most cases (Child et al 2001). A small proportion of cases have rearrangements in bcl-1, bcl-2, bcl-8 and lyt-10 genes (Neri et al 1995).

Herpesviruses have been excluded as likely aetiological agents in primary CBCL in immunocompetent patients (Dupin et al 1997, Nagore et al 2000), although CBCLs in acquired immune deficiency syndrome (AIDS) patients may be associated with EBV (Beylot-Barry et al 1999). An association between Lyme disease and CBCL has been hypothesised (Cerroni et al 1997); Borrelia burgdorferi sequences were detected by PCR in DNA from
seven (35%) of 20 lesions in Scotland (Goodlad et al 2000). However, further studies are required to confirm this relationship. Evidence for the involvement of HTLV-I in B cell lymphomas is discussed in Section 1.6.2.1.

1.11 Viral aetiology of cutaneous T cell lymphomas and large granular lymphocytic leukaemia

The possibility that HTLV-I or related retroviruses are involved in the aetiology and pathogenesis of CTCLs and LGL leukaemia is the main theme of this thesis. This section discusses the evidence for retroviral involvement in the aetiology of MF/SS, cutaneous CD30⁺ ALCL, angiocentric TCL and LGL leukaemia.

1.11.1 Human T lymphotropic viruses and cutaneous T cell lymphomas

1.11.1.1 Epidemiology

The sporadic occurrence and other epidemiological features of MF/SS do not immediately support an infectious aetiology (Section 1.10.1.1). ATL and HTLV-I have distinct distributions by geographical area and population groups, whereas cases of MF/SS do not exhibit clustering and are mostly reported from Europe and the USA, which are not HTLV-I endemic regions (Weinstock and Horm 1988). Hjelle et al (1991b) found no difference in the incidences of MF between three ethnic groups in the USA with differences in the prevalence of HTLV-II infection. There is little evidence that MF/SS is transmissible (Stratton 1948, Grossman and Duvic 1993).

1.11.1.2 Clinicopathological features

The similarity of clinicopathological features of MF/SS and some forms of ATL is the main basis for attempts to search for HTLV-I-related viruses in CTCLs. As discussed in Section 1.6.1.5, HTLV-I is the cause of ATL. MF/SS and ATL are both CD4⁺ T cell neoplasms that can involve the skin, lymph nodes, viscera and peripheral blood (Nagatani et al 1990). Cutaneous involvement occurs in 43 to 72% of patients with ATL and usually takes the form of rapid onset, generalised papules with erythema (Chan et al 1985, Gibbs et al 1987, Maeda and Takahashi 1989, Johno et al 1992). Smouldering ATL is a distinct cutaneous form that usually develops slowly, with systemic involvement and leukaemia being a late event, similar to most cases of progressive MF (Yamaguchi et al 1983, Jaffe et al 1984, Chan et al 1985, Aral et al 1991, Shimoyama et al 1991). The acute and lymphomatous forms of ATL are aggressive diseases; likewise the rare "tumeur d'emblée" form of MF has no premalignant phase and has a rapidly progressive course (Blasik et al 1982). The hypercalcaemia and lytic bone marrow lesions characteristic of ATL are not observed in MF/SS (Nagatani et al 1990).

Whereas MF/SS is epidermotropic, the cutaneous infiltrate in acute ATL primarily involves the dermis and subcutaneous tissues, often consists of highly pleiomorphic cells with few reactive cells and is sometimes associated with vascular lesions (Hasul et al 1987, Maeda and Takahashi 1989, Nagatani et al 1990). However, the pattern of lymphocyte infiltration in
ATL and MF can be very similar and Pautrier's microabscesses can be observed in both conditions (Nagatani et al 1990, Johno et al 1992). Neoplastic T cells in ATL have moderately indented (lobulated) nuclei, whereas MF/Sézary cells have highly indented (cerebriform) nuclei (Hasui et al 1987, Maeda and Takahashi 1989, Nagatani et al 1990). The neoplastic cells in both conditions are usually CD4^CD7^- T cells (Hanaoka 1984, Nasu et al 1985, Kamihira et al 1992), although Nagatani et al (1990) observed higher expression of CD7 by ATL cells than MF/Sézary cells. In unaffected people approximately 9% of normal T cells have a CD7^ phenotype (Reinhold et al 1993). MF/Sézary cells rarely express CD25 (or other activation markers) whereas CD25, CD30, CD71, Ki67 and MHC-II are usually expressed by ATL cells (Waldmann et al 1984, Maeda and Takahashi 1989, Oishi et al 1994). Transformation of MF to ALCL is also associated with acquisition of CD25, CD30, CD71, Ki67 and MHC-II expression (Gerroni et al 1992). ATL and MF/SS share expression of cell adhesion molecules, some of which are up-regulated by Tax in HTLV-I-infected T cells (Sections 1.6.1.3, 1.8.1.2 and 1.10.1.5). ATL cells exhibit Ts activity (Tendler et al 1994, Yamada et al 1996), whereas MF/Sézary cells exhibit Th or Ts activity, as discussed in Section 1.10.1.3 (Berger et al 1979, Miedema et al 1984, Golstein et al 1986). Immortalised and transformed T cell lines infected with HTLV-I can be readily established from ATL cells in vitro, usually following the addition of IL2 or CM, whereas MF/Sézary cells, with few exceptions, do not proliferate readily under these conditions (Waldmann et al 1984, Abrams et al 1991b, Zucker-Franklin et al 1991) (Chapter 2).

It has been possible to distinguish most cases of MF/SS from ATL using a combination of clinicopathological criteria and the presence or absence of HTLV-I infection (Yamaguchi et al 1984, Levine et al 1994a). Numerous cases initially classified as MF/SS were reclassified as forms of ATL following the discovery of HTLV-I (Poiesz et al 1980a, 1981, Blattner et al 1982, Blayney et al 1983b, D’Incan et al 1995). Most cases of HTLV-I-associated CTCLs in HTLV-I endemic regions such as Japan probably represent cutaneous forms of ATL (Amagasaki et al 1984, Nakamura et al 1993). HTLV-I-infected patients with MF/SS-like diseases in non-endemic regions have subsequently developed ATL-like leukaemia, supporting an aetiopathological classification as ATL (Bunker et al 1990, Whittaker et al 1993, Fujihara et al 1997). Infiltrating neoplastic T cells in the cutaneous form of ATL contain monoclonally integrated HTLV-I provirus and thus may be distinguished from HTLV-I negative MF/SS (Takahashi et al 1988, Yamada et al 1989b, Dosaka et al 1991, Arai et al 1994, Fouchard et al 1996, Setoyama et al 1998). In some cases of smouldering ATL, clonal integration of HTLV-I and rearrangement of the TCR genes may only be found in T cells infiltrating the skin, with no evidence of a neoplastic clone in the peripheral blood (Dosaka et al 1991, Hamada et al 1992). Rare cases of ATL-like leukaemia do not have evidence of HTLV-I infection (Shimoyama et al 1986). Most peripheral T cell and NK cell neoplasms in non-endemic regions are not HTLV-I-associated (Henni et al 1990). However, HTLV-I and HTLV-II have still been implicated in a number of cases that conform to the case definition of classical MF/SS (Kaplanski et al 1986, Zucker-Franklin et al 1991, 1992, Bazarbachi et al 1994, Manca et al 1994).

In several early studies retrovirus-like particles were observed by EM and RT activity was detected in skin lesions and blood of patients with MF/SS, but it is uncertain if these are representative of true retroviruses (Van der Loo 1979, Füllbrandt et al 1983, Slater et al
Positive immunostaining for HTLV-I p19\textsuperscript{Gab} has been detected in involved skin and lymph nodes of patients with CTCL but the specificity of this reactivity is uncertain (Turbitt and MacKie 1985).

1.11.1.3 Serology

There is conflicting serological evidence that HTLV-I, HTLV-II or related viruses are involved in MF/SS in non-endemic regions. Although some studies have reported ELISA seroreactivities as less than 1\% (Posner \textit{et al} 1981, Gallo \textit{et al} 1983\textit{a}), others have reported reactivities of 11\% (Lange Wantzin \textit{et al} 1986) and 24\% (Srivastava \textit{et al} 1990). Reactivities against HTLV-I by WB ELISA have been reported as 15\% (Srivastava \textit{et al} 1990) and 86\% (Ranki \textit{et al} 1990). Interpretation of these results is confounded by the unknown specificity of the serological assays used, including their ability to discriminate HTLV-I and HTLV-II (Section 1.4.2). One group reported antibodies against HTLV-I Tax in 10\% of MF/SS patients who were seronegative for HTLV-I/II structural proteins (Gag and Env) in standard serological assays (Pancake \textit{et al} 1995) (Section 1.11.1.5).

1.11.1.4 Cell culture

Retrovirus-like particles have been detected by EM and RT activity demonstrated in T cell lines propagated from patients with MF/SS (Kaltoft \textit{et al} 1984, 1987, Saal \textit{et al} 1989, Zucker-Franklin \textit{et al} 1991, Bazarbachi \textit{et al} 1994). Reactivity with antiserum against HTLV-I p19\textsuperscript{Gab} was detected in T cell cultures established from involved skin or lymph nodes of three patients with MF using CM and depletion of CD8\(^+\) TILs (Kaltoft \textit{et al} 1984). Extracellular C-type retrovirus-like particles could be induced by withdrawal of serum from a T cell line, Se-Ax, derived from a patient with SS, although no budding particles were observed and little or no RT activity was demonstrable in cell culture supernatants (Kaltoft \textit{et al} 1987). There was no evidence of HTLV-I by SB hybridisation (Kaltoft \textit{et al} 1988). C-type retrovirus-like particles were also observed by EM in cultured PBMCs from a French patient with SS and RT activity was detected in culture supernatants (Saal \textit{et al} 1989). The patient did not have detectable serum antibodies against HTLV-I and DNA from PBMCs was negative for HTLV-I by SB hybridisation.

Retrovirus-like particles were detected by EM in 18 cell lines established from PBMCs of 17 patients with MF and one patient with SS using GMCSF and IL2 as growth stimulants (Zucker-Franklin \textit{et al} 1991). Molecular evidence suggested that incomplete or variant HTLV-I was integrated in four cell lines and HTLV-II was integrated in one (Zucker-Franklin \textit{et al} 1992) (Section 1.11.1.5). However, the viral nature of the observed particles was not confirmed, they were not purified and characterised, their infectivity was not demonstrated and RT activity was not reported. HTLV-I tax sequences were also detected by PCR in 20 (80\%) of 25 T cell lines established from PBMCs of SS patients in the USA, whereas all were negative for HTLV-I gag (Abrams \textit{et al} 1991\textit{b}, Ghosh \textit{et al} 1994) (Section 1.11.1.5).

Retrovirus particles were detected by EM in PBMCs from a SS patient after four months in culture and weak RT activity was detected in culture supernatants, but no HTLV-I-related antigens could be detected (Bazarbachi \textit{et al} 1994). PBMCs from 19 patients with MF/SS
were grown in culture for approximately three months using phytohaemagglutinin (PHA) and IL2 (Capésius et al. 1991). No retrovirus particles or budding forms were detected in cultured cells by EM and only transient RT activity was detected in culture supernatants, mainly from degenerating cultures. No specific HTLV-I products were amplified by PCR from PBMCs using primers and probes for HTLV-I gag and pol.

In comparison with the use of molecular techniques on uncultured clinical samples (skin, PBMCs and lymph nodes), there have been fewer indications of the presence of retroviruses in cultured T cells from patients with MF/SS (Section 1.11.1.5). Only two groups have demonstrated the presence of HTLV-I or HTLV-II sequences in cultured T cells from patients with MF/SS (Zucker-Franklin et al. 1992, Ghosh et al. 1994).

1.11.1.5 Molecular techniques

HTLV-I or HTLV-II sequences have been identified in DNA from PBMCs, skin and lymph nodes of patients with MF/SS from non-endemic regions and low prevalence populations using PCR, SB hybridisation and ISH (Hall et al. 1991, Chan et al. 1993). The proportion of MF/SS cases reported as positive for HTLV-I on the basis of PCR results has been 0% (Capésius et al. 1991, Lisby et al. 1992, Bazarbachi et al. 1993), 1 to 2% (D'Incan et al. 1992, Lapis et al. 1992), 7.5% (Whittaker and Luzatto 1993), 34% (Manca et al. 1994), 45% (Chan et al. 1993) and 92% (Pancake et al. 1995). It is difficult to reconcile these widely differing percentages; selected results are detailed here to illustrate the differences among groups working in this field.

Hall et al. (1991) detected HTLV-I proviral DNA by PCR in cutaneous lesions of five HTLV-I-seronegative Swedish patients with MF using primers for the LTR, gag, pol, env, and pX regions. Sequences from all regions were amplified from both PBMCs and cutaneous lesions in one patient with leukaemic stage MF, whereas incomplete provirus was identified in cutaneous lesions in the other four patients with non-leukaemic MF; one had gag, pol, env and pX, one had LTR and gag, one had gag and pX and one had pX only.

Zucker-Franklin et al. (1991, 1992) provided evidence for the presence of HTLV-I-related viruses in cultured PBMCs from HTLV-II/III seronegative patients with MF in the USA. PCR and SB hybridisation results suggested that incomplete or variant HTLV-I proviruses were integrated in cultured cells of four of 20 patients with MF (HTLV-I pol or env sequences amplified by PCR) and HTLV-II was integrated in cultured cells of one case (HTLV-II pol sequences amplified) (Section 1.11.1.4). The same group detected HTLV-I tax sequences by PCR and RT-PCR in PBMCs from 50 MF patients (Pancake et al. 1995). These patients were seronegative for HTLV-II Gag and Env in standard serological assays but five (10%) had antibodies against HTLV-I Tax (Section 1.11.1.3). Two AIDS patients with MF also had HTLV-I tax and pol sequences amplifiable by PCR from PBMCs and skin (Zucker-Franklin et al. 1994).

HTLV-I tax sequences were amplified by PCR from PBMCs of 18 (72%) of 25 SS patients and three (30%) of 10 skin lesions of MF patients in the USA, whereas no HTLV-I gag or HTLV-II gag or tax sequences were detected (Ghosh et al. 1994).
HTLV-I gag and pX PCR products of identical sequence to a reference strain of HTLV-I were amplified from PBMCs of three of four HTLV-I ELISA positive patients with MF/SS in the USA (Srivastava et al 1992). Manca et al (1994) detected HTLV-I pol and tax sequences by PCR in 10 (34%) of 29 patients with MF from Italy, confirming these results in a double blind trial after a 6 month interval. HTLV-I pol, env and tax sequences were detected by PCR in one (2%) of 51 HTLV-I/II seronegative patients with CTCL from France (D'Incan et al 1992) and HTLV-I tax sequences were amplified by PCR from one (5%) of 20 patients with MF in the USA (Lapis et al 1992).

The wide range of reported frequencies of detection of HTLV-I-related sequences by PCR in patients with MF/SS suggests that there are differences in methods between groups and possible methodological problems such as PCR contamination. There might also be differences in case definitions and selection of cases for inclusion in different studies. If a retrovirus is involved in CTCL, it might be distantly related to HTLV-I or HTLV-II, there might be deletions or mutations, it might be present in the skin in quantities below the detection limit of PCR or it might have been eliminated from the skin before clinical lesions develop (hit-and-run hypothesis) (Lisby et al 1992, Lessin et al 1994).

1.11.1.6 Defective viruses

Incomplete or variant HTLV-I-related viruses have been identified in patients with MF/SS on the basis of PCR, variant restriction endonuclease (RE) patterns on SB hybridisation and sequencing (Hall et al 1991, Zucker-Franklin et al 1991, Srivastava et al 1992, Kiss et al 1993, Whittaker and Luzzato 1993, Bazarbachi et al 1994, Ghosh et al 1994, Pancake et al 1995). The basis for declaration of incomplete or variant HTLV in samples analysed by PCR has been the amplification of HTLV-I or HTLV-II sequences from some regions of the genome, with other regions of the genome being negative (Section 1.11.1.5). The pX region appears to be retained preferentially and tax sequences have been detected more frequently than sequences of other genes (Hall et al 1991, Zucker-Franklin et al 1991, Srivastava et al 1992, Ghosh et al 1994, Pancake et al 1995).

Monoclonally integrated HTLV-I-related proviral sequences were detected by SB hybridisation in DNA from PBMCs of an HTLV-I seronegative patient with SS from France (Kaplanski et al 1988). After digestion of DNA with EcoRI, which does not cleave within the provirus, RE-digested fragments were smaller (7.5 kb) than expected (9.0 kb) using a full-length HTLV-I probe, indicating integration of incomplete or variant HTLV-I. Seven Hungarian patients with MF had evidence of infection with HTLV-I-related viruses by SB hybridisation and serology (Kiss et al 1993). RE digestion suggested differences in the gag region of the integrated provirus compared with a reference strain of HTLV-I. A full-length HTLV-I probe hybridised to SBs of DNA from PBMCs of an HTLV-I seronegative French patient with SS had a RE pattern distinct from that of HTLV-I (Bazarbachi et al 1994). HTLV-I pol and env sequences could not be detected by PCR.

Sequences with homology to HTLV-I were identified in DNA from PBMCs and cutaneous lesions of three of 40 HTLV-I-seronegative West Indian patients with MF in the UK by SB
hybridisation using a full-length HTLV-I probe under conditions of high stringency (Whittaker and Luzzato 1993). After EcoRI digestion the HTLV-I probe hybridised to an 8 kb DNA fragment in one patient, indicating the presence of incomplete provirus. A 119 bp product amplified from the DNA of this patient by PCR using primers specific for the pol region of HTLV-I differed in sequence from the prototype HTLV-I in only one base. HTLV-I could not be detected by PCR in the other two patients using the same HTLV-I pol-specific primers. After HindIII digestion of DNA from both of these patients, a 3 kb fragment was observed in addition to the expected 2 kb fragment when hybridised to the full-length HTLV-I probe. Whittaker and Luzzato (1993) suggested that these three patients had monoclonally integrated defective HTLV-I provirus.

Monoclonally integrated HTLV-I with a 5.5 kb deletion involving the entire pol gene and large portions of gag and env was demonstrated by hybridisation, RE analysis, cloning and sequencing in an EBV-infected B-LCL established from PBMCs of an HTLV-I-seronegative American patient with SS, as well as in uncultured PBMCs (Hall et al 1991). Since the defective virus could not be demonstrated in neoplastic T cells, its pathogenetic role in the patient is uncertain.

Monoclonally integrated HTLV-I-related sequences were identified by SB hybridisation in DNA from a skin lesion of an Italian patient with CTCL (Manzari et al 1984). Although a single fragment of approximately 16 kb was detected by SB hybridisation after EcoRI digestion, BamHI digestion did not reveal an expected internal 1 kb fragment, suggesting that the provirus differed from a reference strain of HTLV-I by the absence of this restriction site. A putative HTLV-I-related virus, designated HTLV-V, was subsequently identified in eight of 150 patients with MF/SS and one patient with acute T cell leukaemia from Italy whose sera had weak reactivity for HTLV-I by ELISA (Manzari et al 1987). A 5 kb probe used in this study was cloned from a continuous B cell line established from the patient with acute T cell leukaemia and may not represent a relevant viral sequence, since no sequence data have been published. Therefore, the authenticity of HTLV-V is questionable.

Evidence from a number of groups thus indicates that defective genomes of HTLV-I and possibly related viruses are sometimes present in patients with CTCL (Hall et al 1991, Whittaker and Luzzato 1993, Pancake et al 1995). Preferential retention of the pX region in these defective HTLV-I proviruses is evidence that pX genes might have a role in the pathogenesis of MF/SS (Hall et al 1991, Pancake et al 1995). As discussed in Section 1.6.1.4, defective HTLV-I proviruses have been detected in a small proportion of patients with ATL (Konishi et al 1984, Korber et al 1991, Ohshima et al 1991, Kubota et al 1995). Infection by HTLV-I or a related virus may be necessary to initiate oncogenesis but there may not be a need to maintain the complete viral genome for maintenance of the transformed phenotype. When defective viruses have been identified in both ATL and HTLV-associated MF/SS there is preferential retention of pX sequences, suggesting that continued expression of genes from this region of the virus may be necessary for oncogenesis. Defective BLV proviruses with preferential retention of the pX region are also found in leukaemic B cells from cattle with EBL (Keltmann et al 1982) (Section 1.9.2.1).
It is possible that recombination and complementation could occur between defective HTLV-I-related retroviral genomes and other exogenous viruses, endogenous retrovirus-like elements or cellular genes. Recombination and complementation leading to enhanced oncogenicity have been demonstrated in avian, feline and murine retroviruses (Weiss et al 1973, Hayward and Hanafusa 1975, Stewart et al 1986, Stoye et al 1991, Sheets et al 1993, Golovkina et al 1997). HERV sequences with similarity to HTLV-I genes and thus potential for recombination have been identified (Mager and Freeman 1987, Perl et al 1989, Fujihara et al 1994). HERV-K sequences, although not homologous to HTLVs, express full-length and spliced mRNA transcripts and form virus particles in human germ cell and trophoblastic neoplasms, indicating that not all endogenous retroviral elements are transcriptionally quiescent (Löwer et al 1993, Herbst et al 1996).

1.11.1.7 Detection of human T lymphotropic viruses in other cutaneous T cell lymphomas

Anagnostopoulos et al (1990) detected HTLV-I by PCR using pol region primers in five patients with cutaneous CD30+ ALCL from Germany and one patient from Iran. SB hybridisation demonstrated monoclonal integration of HTLV-I in cutaneous lesions in five patients and incomplete provirus in four (Anagnostopoulos et al 1990, Detmar et al 1991). The Iranian patient was subsequently reclassified as a case of classical MF (Detmar et al 1991). Conversely, in 24 patients with cutaneous CD30+ ALCL from Europe and Japan there was no evidence of HTLV-I infection by SB hybridisation or PCR using pX primers (MacGrogan et al 1996, Wood et al 1997). Serum antibodies against HTLV-I have been reported in three patients with angiocentric TCL and HTLV-I gag and pX sequences were amplified by PCR from lesional DNA of one (McNutt et al 1990, Shimokawa et al 1993). Insufficient studies have been done to determine whether HTLV-I or related viruses might be involved in cutaneous CD30+ ALCL or angiocentric TCL.

1.11.2 Human T lymphotropic viruses and large granular lymphocytic leukaemia

There is evidence that HTLV-I and HTLV-II are involved in a small proportion of cases of LGL leukaemia. Seroreactivity against HTLV-I/II has been detected in patients with CD3+ LGL leukaemia (Sohn et al 1986, Pandolfi et al 1987, Starkebaum et al 1987, Levitt et al 1988, Loughran et al 1994a). Familial cases of CD3+ LGL leukaemia have been reported and one case had antibodies against HTLV-I/II Gag but not HTLV-I p21Env (Loughran et al 1994a). HTLV-II infection has been demonstrated by PCR in a small proportion of cases (Loughran et al 1992, 1994b). Some patients with CD3+ LGL leukaemia have serological cross-reactivity with epitopes of HTLV-I p21Env (Loughran et al 1997b, 1998). HTLV-I or HTLV-II have been isolated or detected by PCR in a small proportion of cases of CD3+ LGL leukaemia, although not necessarily in the leukaemic cells (Martin et al 1993, Heneine et al 1994, Loughran et al 1998). A case of CD2+ CD3- CD4+ CD5+ CD7+ CD8+ CD16- CD25+ CD56+ CD57+ leukaemia with LGL morphology ("atypical ATL") and monoclonal integration of HTLV-I has been described (Sakamoto et al 1994). Other studies have not demonstrated any association between LGL leukaemia and HTLV-I or HTLV-II (Imamura et al 1988, Woessner et al 1994, Zambello et al 1995). A model for LGL leukaemia in HTLV-I tax-transgenic mice is discussed in Section 1.9.1.
1.11.3 Other viruses associated with cutaneous T cell lymphomas and large granular lymphocytic leukaemia

1.11.3.1 Herpesviruses

Epstein-Barr virus (EBV) is able to infect and transform T cells in addition to B cells and epithelial cells, since all these cell types can express the EBV receptor CR2 (CD21) (Stevenson et al 1986, Fingeroth et al 1988, Watry et al 1991). EBV appears to be aetiologically involved in most cases of nasopharyngeal/sinonasal angiocentric T cell lymphoma (lethal midline granuloma) (Harabuchi et al 1990, Kanavaros et al 1993). An EBV-related virus, HHV-MNE, has been detected in T cell lines and cutaneous lymphoid infiltrates of a non-human primate, the pig-tailed macaque (Macaca nemestrina), with MF (Rivadeneira et al 1999). Although antibodies against EBV-associated nuclear antigen (EBNA) have been detected at increased frequency in patients with MF/SS, EBV genomes are rarely detected in neoplastic cells and the evidence for an aetiological role for EBV in most primary CTCLs, including MF/SS, is doubtful (Lee et al 1990, Dreno et al 1994, Kanavaros et al 1994, Anagnostopoulos et al 1996, Angel et al 1996, Jumbou et al 1997). Similarly, most primary cutaneous CD30⁺ ALCLs are negative for EBV, although EBV is associated with some cases of primary systemic CD30⁺ ALCL (Anagnostopoulos et al 1989, Hamilton-Dutoit and Pallesen 1992, Kanavaros et al 1992, Peris et al 1995, Anagnostopoulos et al 1996, Herbst et al 1997). There is little indication that EBV has an aetiological role in angioimmunoblastic TCL, Lennert's lymphoma, lymphomatoid papulosis or intestinal TCL (Weiss et al 1992, Kadin et al 1993, Khan et al 1993, Pan et al 1993, Anagnostopoulos et al 1994).

Patients with LGL leukaemia usually have antibodies against EBV (Loughran et al 1993). EBV in clonal episomal form has been demonstrated in leukaemic cells in some cases of CD3⁺ and CD3⁻ LGL leukaemia (Kawa-Ha et al 1989, Chan et al 1992, Hart et al 1992). However, other molecular studies have not confirmed an aetiological relationship between EBV and LGL leukaemia (Loughran et al 1993, Pellenz et al 1996).

Although human herpesvirus (HHV) 7 is a T lymphotropic virus, it has not been definitively associated with neoplasia (Berneman et al 1992, 1998). HHV-6 was detected by PCR in skin biopsies of 30 patients with CTCL, whereas all samples were negative by PCR for EBV, HSV-1 and HSV-2 (Brice et al 1993). Skin biopsies from patients with CTCL and PBMCs from patients with T cell LGL leukaemia were also negative for HHV-8 (KS-associated herpesvirus) by PCR (Dupin et al 1997, Henghold et al 1997, Loughran et al 1997a). Thus, there is insufficient evidence to incriminate herpesviruses other than EBV in TCLs (Nagore et al 2000).

1.11.3.2 Human immunodeficiency virus type 1

T cell neoplasms, including MF, have been observed in HIV-1-infected people with AIDS (Crane et al 1991, Nahass et al 1991, Kerschmann et al 1995, Muncz-Perez et al 1999). Most are CD8⁺, possibly a consequence of depletion of the CD4⁺ T cell population in AIDS. EBV may be involved in the development of cutaneous CD30⁺ ALCLs in AIDS patients (Dreno et al 1993). A T cell lymphoma with monoclonal integration of HIV-1 in neoplastic...
cells has been described in an AIDS patient (Herndier et al 1992), but most AIDS-associated TCLs can be attributed to immunosuppression.

1.11.3.3 Hepatitis viruses

In an Italian study, some patients with CD3+ LGL leukaemia had serum antibodies against hepatitis B (HBV) and C (HCV) viruses (Zambello et al 1995). HCV was not associated with lymphoproliferative disease in humans in France (Hausfater et al 2001). A patient with polyclonal proliferation of CD3+ CD8+ LGLs directed against HBV-infected CD4+ T cells has been reported, supporting the hypothesis that LGLs may be proliferating in response to chronic stimulation by viral antigens (Agostini et al 1989).

1.12 Associations between viruses and neoplasia

1.12.1 Viruses and human neoplasia

It has been difficult to establish aetiological associations between viruses and neoplasia in humans (Evans and Mueller 1990, Zur Hausen 1991). The identification of EBV as the cause of Burkitt’s lymphoma was the first such association to be established (Henle et al 1968). The process of discovery leading to the identification of HTLV-I as the cause of ATL (Section 1.6.1.5) further illustrates the difficulties in confirming causality. Only a small proportion of infected people develop ATL and there is a prolonged phase of induction between infection and the occurrence of disease. The process of oncogenesis in ATL appears to be complex, with multiple stages influenced by viral gene expression (Section 1.8) and host factors such as immune status and point mutations or chromosomal translocations involving oncogenes (Section 1.6). Neoplasms such as CTCLs with similar clinicopathological features to ATL may develop in people that do not appear to be infected with HTLV-I. In other neoplasms of humans, for example carcinomas caused by papillomaviruses, different strains of viruses may have different oncogenic potential (Walboomers et al 1999). Cofactors are often required for oncogenic progression in virus-induced neoplasia, for example aflatoxins and alcohol in hepatocellular carcinoma caused by HBV (Monto and Wright 2001) and nitrosamines in nasopharyngeal carcinoma caused by EBV (Liebowitz 1994).

1.12.2 Criteria for establishing aetiological associations

The Henle-Koch postulates provide a clear but limited set of criteria for determining whether an infectious agent is the cause of a disease (Rivers 1936). The agent should be found in all cases of the disease and its location in tissues should correspond with the distribution of lesions. The agent should be isolated from lesions and cultivated to purity outside the host; when inoculated back into the host, the cultivated agent should produce identical disease. However, these criteria cannot be applied to viruses causing human neoplasia. Since viruses are propagated in cell cultures, it is difficult to ensure that inocula are free of other agents that could be alternative sole or cooperating causes of neoplasia. In particular, contamination of cell cultures with non-human viruses has been a major source of confusion in the search for viruses involved in human neoplasia (Reitz et al 1976, Smith et al 1979, Popovic et al 1982). The problem of ensuring purity of viruses has only been overcome by
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An alternative set of criteria for the association of viruses with neoplasia was proposed by Evans (1976) and are developed here. Evidence of exposure to the virus should be demonstrated by detection of infection using virus isolation, molecular assays, antigen assays or tests for other viral markers including serological or cell-mediated immune responses (Section 1.4). The association of a particular virus with a specific neoplasm should be consistent and repeatable by different researchers in different places at different times. Infection should precede the development of neoplasia. However, initial infections with many oncogenic viruses are subclinical and therefore the time of exposure may be difficult to establish. There may be a dose response, with an increased frequency or earlier onset of neoplasia associated with higher levels of infection. Viral markers should be more prominent (higher antibody titres, higher concentration of circulating virus or higher antigenaemia) in cases with neoplasia than in matched controls. It should be possible to demonstrate infection in the neoplasm by virus isolation or molecular, immunological or ultrastructural techniques. However, not all viruses that initiate neoplasia may remain in the affected tissue (hit-and-run mechanism of viral oncogenesis) (Skinner 1976, Smith and Campo 1988, Shen et al 1997, Nevels et al 2001) or viruses may induce neoplasia indirectly, for example lymphoid neoplasia associated with HIV-1-induced immunosuppression in AIDS patients (Cremer et al 1990, Schulz et al 1996, Demario and Liebowitz 1998, Smith et al 1998).

An apparent association between a virus and a neoplasm would be considered more biologically plausible if the virus is from a taxonomic group that has previously been associated with a similar type of neoplasm in the same or related species (Fredricks and Relman 1996). Oncogenic viruses isolated from humans with neoplasia should be able to transform cells in vitro. They may have the capacity to induce comparable neoplasms when inoculated into susceptible non-human animals. Experimental manipulation of the virus or its host may alter the expression of transforming properties. However, suitable in vitro and non-human animal models are not always available because of the difficulty in reproducing neoplasia in alternative hosts with human viruses (Section 1.9). Prevention of infection with a candidate oncogenic virus by vaccination or other prophylactic interventions should be associated with a decrease in the incidence of the neoplasm. T cell neoplasia in domestic fowl caused by Marek’s disease virus (MDV) can be prevented by vaccination with herpesvirus of turkeys or low virulence strains of MDV (Churchill et al 1969, Okazaki et al 1970). Anti-viral therapy might increase the frequency or time to development of neoplasia in infected people or cause regression of neoplasms that have already developed.

Many human oncogenic viruses, for example HTLV-I, HHV-8, HBV, HCV and the human papillomaviruses (HPVs), proved difficult to isolate until the development of specialised cell culture systems and there are likely to be other human viruses that have not been identified. Increasingly, viruses are being identified and characterised by molecular techniques before they have been cultivated in vitro (Fredricks and Relman 1996, Gao and Moore 1996). A λ bacteriophage cDNA expression library was screened with antiserum from patients with non-A, non-B hepatitis to detect sequences of HCV, an RNA virus associated with hepatocellular carcinoma (Young and Davis 1983, Choo et al 1989). HHV-8 was discovered by
amplification of viral sequences from KS, a skin tumour associated with AIDS, using representational difference analysis (RDA) (Lisitsyn et al 1993, Chang et al 1994). Consensus PCR using group-specific primers has been used to amplify sequences of previously unknown HPV genotypes from patients with cervical neoplasia (Van den Brule et al 1992). Novel herpesviruses have been detected by degenerate and consensus PCR in B cell lymphomas of common marmosets (Callithrix jacchus) (Ramer et al 2000), fibropapillomas of green turtles (Chelonia mydas) (Quackenbush et al 1998) and genital carcinomas of California sea lions (Zalophus californianus) (Lipscomb et al 2000).

Fredricks and Reiman (1996) outlined guidelines for establishing an aetiological association between a microorganism and disease based on molecular evidence in the absence of cultivation of the putative pathogen. These guidelines can be applied to novel virus-like sequences and neoplasia. Specific putative viral nucleic acid sequences should be present in neoplastic cells, although this may not apply to hit-and-run agents or those that act indirectly. There should be a lower frequency of detection, a lower copy number or absence of the specific virus-like sequences in unaffected tissues or in regressing neoplasms. Sequences might be detectable in samples that precede the development of neoplasia, indicating prior infection. The taxonomic group to which the putative virus belongs, inferred by sequence analysis, should be one that has known oncogenic members. The type of neoplasm being associated with specific novel virus-like sequences should be consistent with the known biological properties of members of that group. When assessing the significance of retroviral sequences detected in neoplasia, the potential for amplification of endogenous retrovirus-like sequences should be considered, particularly when using primers from regions such as pol where there is a high degree of similarity between endogenous and exogenous sequences. In neoplasia induced by retroviruses, each neoplastic cell would be expected to carry at least one integrated provirus but there is also the possibility that cells might lose part or all of the provirus following neoplastic transformation.

1.12.3 Epidemiological studies

Epidemiological criteria are also important in determining whether associations between viruses and neoplasia are likely to be causal (Evans 1975). The geographical distribution of infection with a virus should be similar to that of the neoplasm with which it is associated, adjusting for age of infection and presence of cofactors. The correlation between the distribution of HTLV-I infection and the occurrence of ATL was the most important factor in identifying a causal association. Even when a virus and a neoplasm have a similar geographical distribution, there is still a need for well-designed epidemiological studies. Not all infected people may develop the neoplasm; with viruses such as HTLV-I neoplasia may develop in only a small proportion of infected people and there may be a long delay between infection and development of the neoplasm. Viruses may not be responsible for all cases of a particular neoplasm if there is multifactorial causation. In these cases, it is necessary to study the degree of association between disease and hypothesised causal factors by measuring relative risks or odds ratios in cross-sectional, case-control and cohort studies. A significantly higher proportion of people infected with a candidate oncogenic virus or with specific viral markers (Section 1.4) should develop neoplasia than uninfected people or those without such markers.
1.12.4 Involvement of HTLV-I in cutaneous T cell lymphomas and large granular lymphocytic leukaemia

Evidence for the involvement of HTLV-I or related viruses in MF/SS, other CTCLs and LGL leukaemia is presented in Section 1.11. Seropositivity for HTLV-I structural and regulatory proteins has been demonstrated in many cases. A number of studies have reported detection of HTLV-I sequences by PCR and SB hybridisation in PBMCs, neoplastic infiltrates and cell lines derived from affected patients. There is some evidence that defective HTLV-I or HTLV-II or variant proviruses are integrated in neoplastic cells of some cases of MF/SS and other CTCLs, with preferential retention of the pX region. However, the discrepancy in reported frequencies of detection of HTLV-I or HTLV-II markers in patients with these neoplasms must result in serious doubts as to the veracity of many studies. Methodological problems such as PCR contamination have to be excluded before positive results can be accepted. Concerns about previous studies have highlighted the need to establish stringent criteria for determining whether HTLV-I, HTLV-II or related viruses are aetiologically involved in CTCLs and LGL leukaemia.

Clear case definitions for MF/SS and other CTCLs must be established in order to distinguish these T cell neoplasms from ATL and similarly the diagnostic criteria for LGL leukaemia must be well-defined. It is then necessary to obtain unambiguous evidence that patients with non-ATL CTCLs and LGL leukaemia are infected with HTLV-I, HTLV-II or related retroviruses. Isolation and propagation of infectious virus from affected patients would be the most definitive form of evidence, particularly if the virus is shown to be a new species of retrovirus or a distinct strain of HTLV-I or HTLV-II based on sequence information and detailed virus characterisation. Since retroviruses establish persistent infections, patients with neoplasms caused by defective integrated proviruses would be expected to be co-infected with intact infectious virus that can be isolated from PBMCs or other non-neoplastic cells. It is conceivable that cells containing intact proviruses could be eliminated by the host immune response, leaving only cells infected by defective viruses that fail to express antigens. In these cases it would be necessary to rely on molecular techniques that detect the remnant sequences. Stringent precautions would have to be taken to eliminate contamination by PCR products. The sequences of amplified products would have to be determined. An additional level of proof would be to accept results only when sequences of PCR products differ by statistically significant percentages from prototypical strains of HTLV-I and HTLV-II, particularly strains that might be handled by the same group in the same laboratory and used as positive controls. Since the level of sequence variation among HTLV-I isolates is low, criteria for interpretation of sequence results might be different to that of more variable viruses such as HIV-1. Sequences should be reproducible when obtained from different PCR products from the same and possibly different patients at different times. Only the study by Manca et al (1994) has so far met these criteria for MF/SS.

Studies conducted at the LRF Virus Centre from 1992 to 1995 to search for HTLV-I-related viruses in MF/SS, LGL leukaemia and other neoplasms are described in Chapters 2 to 4 of this thesis. The results of this work are discussed in Chapter 5 and compared with the findings of other studies.
Chapter 2

Detection of Retroviruses in Human T Cell Leukaemias and Lymphomas by Cell Culture

2.1 Introduction

2.1.1 Approach to isolation of retroviruses from T cell neoplasms

2.1.1.1 Role of human T lymphotropic retroviruses in T cell neoplasia

Human T lymphotropic virus type I (HTLV-I) is the cause of adult T cell leukaemia/lymphoma (ATL), a neoplasm of CD4^ T cells (Section 1.6.1). HTLV-II has been isolated from cases of CD8^ T cell variant hairy cell leukaemia (HCL) but has not been definitively associated with neoplasia (Section 1.7.1). The related retrovirus bovine leukaemia virus (BLV) causes B cell leukaemia and lymphoma in cattle (Section 1.9.2.1). Retroviral aetiologies have been proposed for a variety of diseases of humans for which the cause is currently unknown, including T and B cell lymphoid neoplasms (Sections 1.10.5 and 1.11) and autoimmune diseases (Section 1.6.4). The hypothesis tested in this study was that HTLV-I, HTLV-II, BLV or related viruses are associated with other T cell leukaemias and lymphomas. Combinations of cell and molecular biology techniques were used to search for retroviruses in patients with these neoplasms.

2.1.1.2 Spectrum of clinical diseases investigated

The emphasis in this study was on cutaneous T cell lymphomas (CTCLs), including mycosis fungoides (MF) and its leukaemic variant Sézary syndrome (SS). These neoplasms have similar clinico-pathological features to some forms of ATL and have been the subject of investigations by many groups in an attempt to confirm a retroviral aetiology (Section 1.11.1). Cases of large granular lymphocytic (LGL) leukaemia were examined for the presence of HTLV-related retroviruses because of reports associating this T or natural killer (NK) cell lymphoproliferative disorder with HTLV-I and HTLV-II (Section 1.11.2). Samples from a cluster of adult acute lymphoblastic leukaemia (ALL) cases in butchers and abattoir workers were also examined for the presence of HTLV-I, HTLV-II and BLV, the latter virus because of occupational exposure to cattle (Whittaker 1991) (Sections 1.9.2.1 and 1.10.5).

2.1.1.3 Outline of cell culture approach

The aim of the cell biology section of this project was to isolate retroviruses from human T cell neoplasms by cultivating T cells from peripheral blood mononuclear cells (PBMCs), skin and lymph nodes of affected patients. Cultivated T cells would act as host cells for the propagation of T lymphotropic retroviruses. Protocols for the long-term cultivation of T cells from patients with T cell neoplasia and patients with retroviral infections were designed.
Techniques for the establishment of cultures included stimulation with concanavalin A (ConA) or phytohaemagglutinin (PHA) then interleukin (IL) 2 (Gazdar et al 1980, Poiesz et al 1980b, Ho et al 1990), IL2 alone, IL2 in combination with IL4 (Uchiyama et al 1988, Kaltoft et al 1992, Mori et al 1993b), IL7 (Dalloul et al 1992) and granulocyte-macrophage colony stimulating factor (GMCSF) (Zucker-Franklin et al 1991). Conditioned medium (CM) was prepared by collecting cell culture supernatants from PHA-stimulated umbilical cord blood mononuclear cells (UCMCs), providing a source of T cell growth factor (TCGF) (Poiesz et al 1980a, Hoshino et al 1983). TCGF is an heterogeneous medium containing IL2 and other soluble growth factors (Morgan et al 1976, Ruscetti et al 1977, Gazdar et al 1979, 1980, Mier and Gallo 1980). In addition, CM was prepared by ConA stimulation of PBMCs from a patient with SS, producing Sézary T cell activating factor (SAF) CM (Abrams et al 1991a, b).

Lymphocytes from patients with T cell neoplasia were also cocultivated with UCMCs that would act as recipients for infection with retroviruses released from primary neoplastic cells or other infected cells from the same patient (Miyoshi et al 1981b, Markham et al 1983, Popovic et al 1983). Cultured cells were examined for evidence of retroviruses by electron microscopy (EM) and cell pellets were collected for analysis by the polymerase chain reaction (PCR) (Chapter 3). Culture supernatants were collected for detection of reverse transcriptase (RT) activity. The immunophenotypes of cells from long-term cultures were determined by flow cytometry.

In this chapter the origins and properties of T cell lines established from patients with ATL (Section 2.1.2) and CTCLs (Section 2.1.3) are reviewed to provide a background to the approaches used at the Leukaemia Research Fund (LRF) Virus Centre to establish similar continuous T cell lines. The sources of clinical samples are described in Section 2.2. Techniques for the cultivation of lymphocytes from clinical samples are described in Section 2.3.5 and 2.3.6. All methods relating to collection of samples for subsequent molecular studies are also included in this chapter. The results of investigations for evidence of retroviruses in cultured T cells are described in Section 2.4.

2.1.2 Establishment of adult T cell leukaemia/lymphoma cell lines

2.1.2.1 ATL cell lines and isolation of HTLV-I and HTLV-II

The discovery of HTLV-I and HTLV-II is described in Section 1.3.2.1. HTLV-I (type isolate HTLVcr) was first detected in two CD4+ T cell lines established from a patient with a form of ATL initially diagnosed as MF (Gazdar et al 1979, 1980, Poiesz et al 1980a, b). These cell lines were HUT102, derived from lymph node cells, and CTCL-3, established from PBMCs. A second isolate (HTLVhm) was obtained from a CD4+ T cell line (CTCL-2) derived from PBMCs of another patient with a form of ATL initially diagnosed as SS (Poiesz et al 1981). HTLV-I (ATLV) was also identified in CD4+ T cell lines established from patients with ATL in Japan by direct cultivation of PBMCs (cell line MT1) (Miyoshi et al 1979a, 1980, Hinuma et al 1981) and by cocultivation of UCMCs with ATL cells (cell line MT2) (Miyoshi et al 1981a, b). Numerous other CD4+ T cell lines have been established subsequently from ATL patients (Gootenberg et al 1981, Hinuma et al 1982a, Yoshida et al 1982, Hoshino et al 1983, Markham et al 1983, Sugamura et al 1984a) (Sections 1.3.2 and 1.4.1).
In most cases the cell lines established from PBMCs of patients with ATL are not derived from the neoplastic clone but represent non-leukaemic cells infected in vitro (Miyoshi et al. 1981a, Nowell et al. 1984, Maeda et al. 1985, 1987). HTLV-II (HTLV-III) was first isolated from a CD8⁺ T cell line derived from a patient with T cell variant of HCL (Saxon et al. 1979a, b, Kalyanaraman et al. 1982b). The key to identifying these viruses was the establishment of CD4⁺ or CD8⁺ T cell lines from patients with neoplasia and such an approach continues to be important in attempts to isolate new human T lymphotropic viruses.

2.1.2.2 Methods for establishing HTLV-I-infected T cell lines from ATL patients

HTLV-I-infected T cell lines have been established from ATL patients by stimulation of PBMCs with ConA or PHA and continued cultivation in the presence of CM (TCGF) or IL2 (Gazdar et al. 1979, 1980, Poiesz et al. 1980a, b, Gootenberg et al. 1981, Poiesz et al. 1981, Hinuma et al. 1982a, Hoshino et al. 1983, Sugamura et al. 1984a, Maeda et al. 1987). Activation of T cells by ConA or PHA increases the efficiency of infection and transformation (Merl et al. 1984). CM and IL2 may be withdrawn as cell lines attain IL2 independence (Markham et al. 1983, Yssel et al. 1989, Höllsberg et al. 1992, Rohwer et al. 1994) (Section 1.3.2.3). Cultured neoplastic T cells containing integrated defective HTLV-I from a patient with CD8⁺ ATL also proliferated in response to IL2; these cells were shown to express the IL2 receptor (IL2R) (Matsushita et al. 1994).

Occasionally, HTLV-I-infected T cell lines have been established from ATL patients without the use of stimulants (Katoh et al. 1986). Cocultivation of PBMCs from ATL patients with UCMMCs is a frequently used method for establishing HTLV-I-infected T cell lines (Miyoshi et al. 1981a, b, Yoshida et al. 1982, Markham et al. 1983, Popovic et al. 1983, Hjelle et al. 1992b).

2.1.2.3 Proliferative responses of ATL cells

ATL-derived neoplastic T cells proliferate in response to IL2, IL4, IL7 and IL15 (Uchiyama et al. 1988, Umadome et al. 1988, Mori et al. 1993b, Persaud et al. 1995, Yamada et al. 1998). The receptors for these cytokines share the common γ (γc) chain and this component may be a mutual element in the signalling pathway for stimulation of HTLV-I-infected T cells by these exogenous cytokines during the process of transformation (Persaud et al. 1995) (Section 1.8.1.1). IL4 and IL7 can substitute for IL2 in immortalising some HTLV-I-infected T cells (Uchiyama et al. 1988, Umadome et al. 1988, Mori et al. 1993b, Persaud et al. 1995, Asadullah et al. 1996b, Mastino et al. 1997).

ATL cells also proliferate in response to GMCSF (Matsushita et al. 1997). The receptors for GMCSF, IL3 and IL5 share a common β (βc) receptor but the responses of ATL cells to IL3 and IL5 have not been studied in detail (Guthridge et al. 1998).
2.1.2.4 Infection of T and B cell lines with HTLV-I and herpesviruses

Epstein-Barr virus (EBV)-Infected B lymphoblastoid cell lines (B-LCLs) coinfected with HTLV-I are occasionally established from patients with ATL (Yamamoto et al 1982a, Hi rose et al 1984, Koyanagi et al 1984). These cell lines express IL2R and proliferate in response to IL2 (Sugamura et al 1984b). Since HTLV-I Tax up-regulates complement receptor 2 (CD21), the receptor for EBV (Section 1.8.1.2), HTLV-I-infected T cell lines established from patients with ATL could also be susceptible to coinfection with EBV (Koizumi et al 1992, Kuraya et al 1995, Ohtsubo et al 1997). However, this appears to be an infrequent event (Ohtsubo et al 1997, 1999).

2.1.2.5 Infection of T cell lines with other T lymphotropic retroviruses


2.1.3 Cultivation of T cells from patients with cutaneous T cell lymphomas

2.1.3.1 T cell lines established from patients with mycosis fungoides/Sézary syndrome

The growth requirements of representative MF/Sézary T cell lines are summarised in Table 2.1. MF/SS-derived CD4+ T cell lines that do not require stimulants for growth include Hut78 (Gazdar et al 1980, Poiesz et al 1980a, b), HH (Starkebaum et al 1991) and L-726A (Crae et al 1991). L-726A was established at the LRF Virus Centre from skin lesions of a patient with SS. TCGF (IL2)-dependent CD4+ MF/Sézary T cell lines include two cell lines from skin and one from a lymph node of patients with MF (Kaltoft et al 1984), the Se-Ax cell line cultivated from PBMCs of a patient with SS (Kaltoft et al 1987), a T cell line from PBMCs of a patient with CTCL established by Kadin et al (1988), nine MF/Sézary cell lines (SZ series) from PBMCs of patients with MF (Abrams et al 1991a, b) and a T cell line from a patient with CTCL established by Boehmcke et al (1993). The CD4+ T cell line My-La, from the skin of a patient with MF (Kaltoft et al 1992), and two T cell lines established from patients with CTCL by Boehmcke et al (1993) required IL2 and IL4. Another T cell line established from a CTCL patient by Boehmcke et al (1993) was dependent on IL4 alone. Three IL7-dependent T cell lines (CHA, BEL and RIC) were established from MF/SS patients by Dalloul et al (1992).

In most cases T cell clones cultured from MF lesions are not derived from neoplastic MF/Sézary cells but arise from tumour infiltrating lymphocytes (TILs) (Ho et al 1990, Harwix et al 2001).
Table 2.1: Growth requirements of representative HTLV-I and HTLV-II-negative CD4\(^+\) T cell lines established from patients with mycosis fungoides/Sézary syndrome.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>T cell neoplasm</th>
<th>Source of cells</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Independent of exogenous growth stimulants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hut78</td>
<td>CTCL</td>
<td>PBMCs</td>
<td>Gazdar et al 1980</td>
</tr>
<tr>
<td>L-726A</td>
<td>SS</td>
<td>Skin</td>
<td>Crae et al 1991</td>
</tr>
<tr>
<td>HH</td>
<td>CTCL</td>
<td>PBMCs</td>
<td>Starkebaum et al 1991</td>
</tr>
<tr>
<td><strong>Interleukin 2-dependent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Se-Ax</td>
<td>SS</td>
<td>PBMCs</td>
<td>Kaltoft et al 1987</td>
</tr>
<tr>
<td>SZ-4</td>
<td>SS</td>
<td>PBMCs</td>
<td>Abrams et al 1991a, b</td>
</tr>
<tr>
<td><strong>Interleukin 2 and interleukin 4-dependent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>My-La</td>
<td>MF</td>
<td>Skin</td>
<td>Kaltoft et al 1992</td>
</tr>
<tr>
<td><strong>Interleukin 7-dependent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHA</td>
<td>SS</td>
<td>PBMCs</td>
<td>Dalloul et al 1992</td>
</tr>
</tbody>
</table>

CTCL: Cutaneous T cell lymphoma  
MF: Mycosis fungoides  
SS: Sézary syndrome  
PBMCs: Peripheral blood mononuclear cells
2.1.3.2 T cell lines established from patients with other T cell lymphomas

Selected CD4⁺ T cell lines established from patients with T cell lymphomas (TCLs) other than MF/SS are listed in Table 2.2. These cell lines are not dependent on growth factors and do not appear to be infected with HTLV-I or HTLV-II. As discussed above, a number of T cell lines established from patients initially diagnosed with MF or SS (for example HUT102, CTCL-2, CTC-16 and Sez 627), were found to be infected with HTLV-I and these patients would be regarded as having ATL (Poiesz et al 1980b, Uchiyama et al 1980, Poiesz et al 1981, Roilt et al 1983, Namiuchi et al 1986, Detmar et al 1991). Zucker-Franklin et al (1991) provided equivocal evidence for HTLV-related viruses in cell lines established from PBMCs of 17 patients with MF and one patient with SS using GMCSF and IL2 as growth stimulants (Section 1.11.1.4). An IL2-dependent CD4⁺ T cell line WHN2 has been established from a patient with HTLV-I-negative leukaemia resembling ATL (Kagami et al 1993).

2.1.3.3 Methods for establishing T cell lines from patients with mycosis fungoides/Sézary syndrome

CD4⁺ T cell lines have been established from patients with MF/SS and other CTCLs by in vitro propagation of lymphocytes extracted from skin, lymph nodes or blood. To establish these cell lines, growth of T cells has been stimulated with mitogens such as ConA or PHA, along with various combinations of IL2, IL4, IL7 and GMCSF (Gazdar et al 1980, Ho et al 1990, Abrams et al 1991a, Starkebaum et al 1991, Zucker-Franklin et al 1991, Dalloul et al 1992, Kaltoft et al 1992, Boehncke et al 1993).

CM prepared from PHA-stimulated PBMCs or UCMCs has been used to provide a source of TCGF (Mier and Gallo 1980, Poiesz et al 1980b, Kaltoft et al 1984, 1987). SAF CM produced by ConA-stimulated PBMCs from a patient with SS was used to establish T cell lines from other patients with MF/SS (Abrams et al 1991a, b). MF/SS patient-derived lymphocytes have also been cocultivated with UCMCs and other cells (Detmar et al 1991). Kaltoft et al (1984) found that depletion of CD8⁺ T cells (TILs) permitted outgrowth of neoplastic CD4⁺ T cell clones from skin-derived lymphocyte cultures. Adaptations of these methods to the cell culture studies at the LRF Virus Centre are described in more detail in Section 2.3.6.

2.1.3.4 Proliferative responses of mycosis fungoides/Sézary cells

The in vitro proliferative responses of PBMCs and skin-derived lymphocytes to mitogens are reduced in most patients with MF/SS (Burg et al 1978, Golstein et al 1986, Ho et al 1990, Wood et al 1990b) (Section 1.10.1.3). Although MF/Sézary cells usually express the T cell receptor (TCR)/CD3 complex, they can more readily be activated through antigen-independent pathways such as those involving CDw60, CD2, and CD28 (Hansen et al 1993). Proliferative responses have been demonstrated to IL2 alone or in combination with IL4, IL7 and GMCSF (Kaltoft et al 1987, Zucker-Franklin et al 1991, Dalloul et al 1992, Kaltoft et al 1992, Foss et al 1994), as well as to CM (TCGF) with IL2 activity (Poiesz et al 1980a, Kaltoft et al 1984, Abrams et al 1991b, 1993). In some studies MF/Sézary cells have proliferated strongly in response to IL7 and IL15 and less strongly to IL2 and IL4 (Kaltoft et al 1987, Dalloul et al 1992, Kaltoft et al 1992, Foss et al 1994, Döbbeling et al 1998).
Table 2.2: HTLV-I and HTLV-II-negative CD4\(^+\) T cell lines established from patients with T cell leukaemias and lymphomas other than mycosis fungoides/Sézary syndrome.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>T cell neoplasm</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>CCRF-CEM</td>
<td>T cell ALL</td>
<td>Foley et al 1965</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>T cell ALL</td>
<td>Minowada et al 1972</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>T cell ALL</td>
<td>Minowada et al 1972</td>
</tr>
<tr>
<td>H-SB2</td>
<td>T cell ALL</td>
<td>Royston et al 1974</td>
</tr>
<tr>
<td>JURKAT (JM)</td>
<td>T cell ALL</td>
<td>Schneider et al 1977</td>
</tr>
<tr>
<td>RPMI 8402</td>
<td>T cell ALL</td>
<td>Srivastava et al 1975</td>
</tr>
<tr>
<td>TALL-1</td>
<td>T cell ALL</td>
<td>Hiraki et al 1978</td>
</tr>
<tr>
<td>HPB-ALL</td>
<td>T cell ALL</td>
<td>Morikawa et al 1978</td>
</tr>
<tr>
<td>SUP-T3</td>
<td>T cell ALL</td>
<td>Smith et al 1984</td>
</tr>
<tr>
<td>Kit 225</td>
<td>T cell CLL</td>
<td>Hori et al 1987</td>
</tr>
<tr>
<td>HPB-MLT</td>
<td>T cell lymphoma</td>
<td>Morikawa et al 1978</td>
</tr>
<tr>
<td>SUP-T1</td>
<td>T cell lymphoma</td>
<td>Smith et al 1984</td>
</tr>
<tr>
<td>IARC 301</td>
<td>T cell lymphoma</td>
<td>Duprez et al 1985</td>
</tr>
<tr>
<td>HPB-MLp-W</td>
<td>T cell lymphoma</td>
<td>Morikawa et al 1991</td>
</tr>
</tbody>
</table>

ALL        Acute lymphoblastic leukaemia
CLL        Chronic lymphoblastic leukaemia
Cytokines produced by MF/Sézary cells and other cells in MF lesions are listed in Section 1.10.1.5. Although many MF/SS-derived T cell lines express IL2Rα (CD25) and require IL2 for growth, most MF/Sézary cells in lesions do not express CD25 (Boehnke et al 1993) (Section 1.10.1.3). Therefore, any response to IL2 in vivo is likely to be mediated by a less strongly responsive receptor complex composed of the βc (CD122) and γc (CD132) subunits of the IL2R.

The use of TCGF and IL2 for cultivation of primary T cells from skin lesions or PBMCs of patients with MF/SS could select for T cells that express the high affinity IL2R but are not derived from the neoplastic clone. Mitogen-activated PBMCs from SS patients produce SAF, a 28 kDa protein that induces IL2Rα on MF/Sézary cells, rendering them responsive to IL2 (Abrams et al 1993). This autocrine loop of stimulation could account for the successful propagation of the SZ series of T cell clones from patients with SS by Abrams et al (1991b).

As discussed above, some MF/SS-derived T cell lines require IL4 alone or in combination with IL2 for growth (Kaltoft et al 1992, Boehncke et al 1993). IL4 expression is low in early MF lesions, although it may increase in later stage MF (Vowels et al 1994, Asadullah et al 1996a).

IL7 and IL7 receptor (IL7R) α (CD127) are expressed by a proportion of MF/Sézary cells in cutaneous lesions (Foss et al 1994, Asadullah et al 1996b, Bagot et al 1996, Dobbeling et al 1998). IL7 produced by keratinocytes or MF/Sézary cells could act in an autocrine or paracrine fashion to induce expression of IL7Rα and IL2Rα on MF/Sézary cells (Dalloul et al 1992, Heufler et al 1993, Foss et al 1994, Bagot et al 1996). The presence of such a stimulatory pathway would be consistent with the establishment of IL7-dependent T cell lines from some MF/SS patients (Dalloul et al 1992, Foss et al 1994, Dobbeling et al 1998). However, it has been difficult to demonstrate a role for IL7 in maintaining proliferation of MF/Sézary cells in vivo (Asadullah et al 1996b).

IL15 prolongs the survival of MF/Sézary cells and is a growth factor for some MF/SS cell lines; IL15 mRNA is expressed by PBMCs, basal cell layer keratinocytes and T cells infiltrating the skin in MF/SS patients (Dobbeling et al 1998). These observations strengthen the premise that the γc chain of the receptors for IL2, IL4, IL7 and IL15 plays an important role in maintaining proliferation of MF/Sézary cells in vivo (Asadullah et al 1996b).

2.1.4 Cell lines derived from patients with LGL leukaemia

Although no fresh PBMCs were submitted for culture from patients with LGL leukaemia in this study, a brief description of established NK or T cell lines derived from affected patients is given here. Five CD3 CD8 , one CD3 CD8 and one CD3 CD8 IL2-dependent LGL cell lines were established using PHA and IL2 as growth stimulants (Pistoia et al 1998). Three CD3 CD8 LGL cell lines were established using IL2 (Loughran et al 1988b). TKS-1, a CD3 CD56 cell line with LGL morphology, was derived from a patient with CD3 CD56 LGL leukaemia using IL2 (Kojima et al 1994). NK-92 is an IL2-dependent CD3 CD7 CD56 NK LGL cell line that expresses the IL2Rα (CD25) (Gong et al 1994).
Neoplastic LGL cells proliferate in response to IL2 \textit{in vitro} (Koizumi \textit{et al} 1986). NK-YS is an EBV-infected NK cell line established from a nasal angiocentric NK cell lymphoma with LGL morphology by cocultivation with a murine stromal cell line in the presence of IL9 (Tsuchiyama \textit{et al} 1998). The requirements for establishment of T cell LGL cell lines thus appear to be similar to those for other T cell lines.

2.1.5 \textit{In vitro} proliferation of normal T cells in culture

2.1.5.1 \textit{In vitro} activation and proliferation of normal T cells

T cells are normally activated through binding of specific antigens to the TCR (Minden and Mak 1986, Davis and Bjorkman 1988). The lectins ConA and PHA are non-specific (polyclonal) activators of T cells that bind to the TCR-CD3 complex (Palacios 1982, Chilson and Kelly-Chilson 1989). Monoclonal antibodies (MAbs) against accessory cell surface molecules such as CD2, CD3, CD28 and CDw60 will also activate T cells (Meuer \textit{et al} 1984, Hansen \textit{et al} 1993). T cells from healthy people will not respond to IL2 unless activated. Resting T cells express the low affinity \( \beta \) and \( \gamma_c \) subunits of the IL2R but have limited expression of the high affinity \( \alpha \) subunit (CD25) (Minami \textit{et al} 1993). In addition, the amount of IL2 secreted by resting T cells is very low. Following activation, T cells transiently produce IL2 and express higher levels of IL2R\( \alpha \), which combines with the \( \beta_c \) and \( \gamma_c \) subunits to form the high affinity IL2R complex, allowing the cells to respond strongly to IL2. Activated T cells will proliferate for 1 to 2 days by this IL2-IL2R autocrine mechanism but will not usually persist in culture, even in the continued presence of exogenous IL2, and rarely become immortalised spontaneously (Section 2.1.5.2). In contrast, T cells from patients with some T cell leukaemias and lymphomas, including HTLV-I infected patients with ATL, often respond directly to IL2 and are more readily immortalised (Section 2.1.2.3). There are differences in the \textit{in vitro} proliferative properties of helper (Th) and suppressor (Ts) T cells (Taylor \textit{et al} 1987). Th cells are able to produce IL2 whereas Ts cells require an exogenous source to maintain viability.

The use of cytokines such as IL2, IL4, IL7 and IL15 to stimulate the growth of MF/Sézary cells in culture has been described in Section 2.1.3.2. These four cytokines are important in preventing the death of activated normal T cells \textit{in vitro}, similar to their effects on MF/Sézary cells, although IL4 and IL15 are more effective than IL2 or IL7 (Kaltoft 1998, Vella \textit{et al} 1998). Many other cytokines, such as IL6, IL9, IL12 and interferon (IFN) \( \gamma \), are involved in growth and differentiation of normal T cells (Lanzavecchia and Sallusto 2000).

2.1.5.2 Longevity of normal T cells \textit{in vitro}

The longevity of normal human T cells in culture is limited but varies according to the type of medium and conditions of cultivation (Perillo \textit{et al} 1989). In standard cell cultures, unstimulated primary T cells derived from PBMCs usually survive for 20 to 30 days in the absence of exogenous growth stimulants before undergoing death by apoptosis. Activated T cells proliferate more strongly and will persist in culture for longer; death of most or all members of the population ("crisis") usually occurs at 30 to 60 days. Under such conditions,
normal human T cells are able to undergo 50 ± 10 population doublings before their proliferative capacity is lost, similar to human fibroblasts (Hayflick 1965, Houck et al 1971, Perillo et al 1989, Goletz et al 1994).

2.1.5.3 Role of the cellular environment in maintaining proliferation of T cells

The compartments of the body in which neoplastic cells proliferate in MF/SS are complex and dynamic microenvironments. Cutaneous lesions in MF contain a diverse range of cells, including keratinocytes, fibroblasts, Langerhan's cells, interdigitating cells, melanocytes and TILs, in addition to MF/Sézary cells. Cell-cell contact and secretion of cytokines by these cells is likely to be important in maintaining survival and growth of neoplastic T cells (Section 1.10.1.5). Antigen presenting cells (APCs) are particularly important in activating T cells in vivo (Johnson and Jenkins 1993). T cell-T cell interactions are also significant and can result in activation through costimulatory pathways such as those mediated by CD28-B7 and CD40-CD40 ligand (Grewal and Flavell 1996, Greenfield et al 1998). However, the limited lifespan of T cells in vitro suggests that these interactions cannot maintain the viability of T cell populations in the absence of other proliferative signals. Maintaining the survival of T cells in vitro in the absence of these supporting elements is one of the challenges in attempts to establish continuous T cell lines.

The presence of mixed cell populations in cultures of lymphocytes from skin lesions, PBMCs and lymph nodes could maintain the survival of MF/Sézary cells and promote the outgrowth of continuous T cell lines derived from neoplastic clones. Cultures of adherent cells derived from the peripheral blood were able to support the growth of normal CD4^+ and CD8^+ T cells in the absence of exogenous cytokines for up to 3 months (Sutkowski et al 1995). Apoptosis that follows withdrawal of IL2 from activated normal T cells can be prevented by cultivation in the presence of fibroblasts (Scott et al 1990) (Section 1.8.7.2). These stromal cells produce growth factors and could have an effect similar to that of exogenous CM.

2.1.6 Detection of retroviral infection in cultured cells

Methods for detecting infection with HTLV-I and HTLV-II in cultured T cells are described in Section 1.4.1. With varying degrees of specificity, these methods are applicable to the detection of other retroviruses, including novel T lymphotropic retroviruses. Virions can be visualised by EM in most productive retroviral infections and RT activity is detectable in culture supernatants, but these assays do not permit specific identification. Budding retroviruses in cultured T cells may be difficult to distinguish from cell membrane blebs. The RTs of HTLV-I, HTLV-II and BLV have stronger activity with magnesium (Mg^{2+}) than with manganese (Mn^{2+}) as cofactors (Gilden et al 1975, Graves et al 1977, Poiesz et al 1980a, Rho et al 1981, Kalyanaraman et al 1982b, Hoffman et al 1985). Similarly, HIV-1 and HIV-2 exhibit Mg^{2+}-dependent RT activity (Barré-Sinoussi et al 1983, Popovic et al 1984). RT activity due to the presence of an exogenous retrovirus has to be distinguished from that produced by endogenous retroviruses and from non-specific cellular RT activity. Density gradient centrifugation can be used to associate RT activity with a particular density of particle.
The use of immunological techniques for detection of virus-specific antigens is usually based on prior knowledge and isolation of the virus of interest or closely related viruses in order to generate specific reagents. Sera from patients with ATL were used to detect immunological reactivity in T cell lines infected with HTLV-I before a retrovirus had been identified in these cultures (Miyoshi et al. 1980, Hinuma et al. 1981). Cross-reactivity between antibodies to virus structural group antigens (Gag) has been a useful technique for detecting novel retroviral infections in cultivated cells. The use of molecular techniques can be highly specific, for example using unique primers for amplification of HTLV-I sequences by PCR. Alternatively, group-specific consensus and degenerate primers can be used to amplify retroviral sequences from a number of related viruses, as well as potentially from novel viruses (Chapter 3). In this study EM, RT assays and PCR were used in attempts to identify retroviruses in T cells cultured from patients with T cell neoplasia. A wide range of serological reactivity against HTLV-I/II has been reported in MF/SS patients by different groups (Section 1.11.1.3). Serological testing conducted previously at the LRF Virus Centre had demonstrated that most patients with MF/SS were negative for HTLV-I/II by ELISA and WB ELISA, with only a few patients exhibiting seroindeterminate reactivities. Therefore, serological testing was not performed routinely on samples from MF/SS patients in this study.

2.2 Sources of clinical samples

Samples for testing in this project were obtained from patients with MF/SS and other cutaneous lymphoid infiltrates, LGL leukaemia and ALL in the United Kingdom (UK).

2.2.1 Cutaneous T cell lymphomas and other cutaneous lymphoid infiltrates

To study the involvement of retroviruses in CTCLs, samples of skin, lymph nodes and blood were collected from patients with cutaneous lymphoid infiltrates in the West of Scotland and other parts of the UK. Most samples were submitted by Dr D Tillman, Western Infirmary and Department of Dermatology, University of Glasgow Medical School. Other samples were submitted by Professor R Mackie and Dr D Bilsland, Western Infirmary and Department of Dermatology, University of Glasgow Medical School, Dr D Ellis, Inverclyde Royal Hospital, Greenock, Dr J Norris, Dumfries and Galloway Royal Infirmary, Dumfries, Dr E Spilg, Victoria Royal Infirmary, Glasgow, Dr F Humphreys, Dr J Hunter and Dr M Tidman, Department of Pathology, University Medical School, Edinburgh, and Dr N Kirkham, Department of Histopathology, Royal Sussex County Hospital, Brighton.

Samples were received from 36 patients with cutaneous lymphoid infiltrates during the period 1987 to 1995. Patients in this study were 20 to 91 years of age (mean 58 years, median 61 years). Histological, haematological and immunohistochemical examinations demonstrated that most cases were MF or SS but there were also cases of cutaneous (peripheral) T cell lymphoma (CTCL), angiocentric T cell lymphoma, small plaque parapsoriasis, Langerhan's cell histiocytosis (LCH) and CBCL (Table 2.3).
The clinicopathological features of these cutaneous lymphoproliferative disorders are described in Section 1.10. Most patients with MF/SS conformed to the case definitions for these diseases and the neoplastic population in all cases consisted of CD4+ T cells. One patient with SS (patient 1198) had a marked CD3+ CD4+ lymphocytosis with aberrant expression of CD25 (Section 1.11.1.2). Samples of whole blood (10 ml) were diluted with an equal volume of transport medium containing heparin without preservative (Appendix 4: A4.2.2) and transported chilled to the LRF Virus Centre. Samples of fresh tissues (skin or lymph nodes) were placed in 10 ml of transport medium also containing heparin without preservative and transported similarly.

2.2.2 Large granular lymphocytic leukaemia

Samples of blood from six patients with LGL leukaemia in England were submitted by Dr M Bhavnani, Department of Haematology, Royal Albert Edward Infirmary, Wigan. Patients were 67 to 81 years of age and had CD3+ CD8+ LGL leukaemia (Table 2.4). T cell receptor (TCR) rearrangements were detected in one of four patients tested by SB hybridisation. Whole blood (10 ml) was diluted with an equal volume of transport medium containing heparin without preservative and transported chilled to the LRF Virus Centre.

2.2.3 Adult acute lymphoblastic leukaemia in butchers from Cardiff

PBMCs and bone marrow from five patients with ALL were kindly provided by Dr J Whittaker, Department of Haematology, University Hospital of Wales, Heath Park, Cardiff (Whittaker 1991). All patients were male butchers from Cardiff, 21 to 31 years of age (age of one patient unknown). They had ALL of common, pre-B cell or T cell type (Section 1.10.5) (Table 2.5). PBMCs had been harvested from heparinised blood samples at the University Hospital of Wales by Ficoll density gradient centrifugation (Section 2.2.2) and pellets of 1 x 10^5 cells stored frozen at -80°C. Bone marrow had also been stored frozen at -80°C. PBMCs and bone marrow were packed with solid CO_2 (dry ice) pellets for transportation to the LRF Virus Centre.

2.2.4 Samples from people without neoplasia

Umbilical cord blood was collected at the Southern General Hospital, Glasgow, and Royal Alexandria Hospital, Paisley. The blood was mixed with an equal volume of transport medium with heparin at the time of collection and transported chilled to the LRF Virus Centre. These samples were not screened for viruses but the population was considered to be at low risk for infection with HTLV-I or HTLV-II, since Scotland is not an endemic region for these viruses. UCMCs were used for cocultivation with cells derived from patients with neoplasia and as a source of CM for cell cultures (Section 2.3.6.10). Samples of human placenta were also obtained from the same sources for extraction of DNA. Peripheral blood was collected by venipuncture from adults working in the Department of Veterinary Pathology, University of Glasgow Veterinary School, and placed in transport medium with heparin. PBMCs from adult peripheral blood were used as negative control samples for PCRs.
Table 2.3: Patients with cutaneous lymphoid infiltrates.

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<th>Molecular analysis</th>
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<td>42</td>
<td>F</td>
<td>MF</td>
<td>8/9/92</td>
<td>PBMCs</td>
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<td>+</td>
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<tr>
<td>2522</td>
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<td>M</td>
<td>MF</td>
<td>18/2/93</td>
<td>Skin</td>
<td>+</td>
<td>+</td>
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<td>2531</td>
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<td>M</td>
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<td>4/3/93</td>
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<td>+</td>
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<td>2547</td>
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<td>M</td>
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<td>28/3/93</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>F</td>
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<td>1/4/93</td>
<td>Skin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2552</td>
<td>54</td>
<td>M</td>
<td>MF</td>
<td>1/4/93</td>
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<td>+</td>
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<td>M</td>
<td>MF</td>
<td>16/6/93</td>
<td>PBMCs</td>
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<td>2669</td>
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<td>M</td>
<td>SS</td>
<td>14/10/93</td>
<td>Skin</td>
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<td>+</td>
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<td>2746</td>
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<td>PBSMs</td>
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<td>2757</td>
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<td>17/12/93</td>
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<td>+</td>
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<td>2862</td>
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<td>M</td>
<td>MF</td>
<td>29/12/93</td>
<td>PBMCs</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- **ATCL**: Angiocentric T cell lymphoma
- **CBCL**: Cutaneous B cell lymphoma
- **CTCL**: Cutaneous T cell lymphoma
- **LCH**: Langerhan's cell histiocytosis
- **MF**: Mycosis fungoides
- **SPP**: Small plaque parapsoriasis
- **PBMCs**: Peripheral blood mononuclear cells
- **SS**: Sézary syndrome
### Table 2.4: Patients with large granular lymphocytic leukaemia.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Immunophenotype</th>
<th>Date samples received</th>
<th>Samples</th>
<th>Cell culture</th>
<th>Molecular analysis</th>
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<tr>
<td>1119</td>
<td>74</td>
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<td>9/5/91</td>
<td>PBMCs</td>
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<td>+</td>
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<td>16/5/91</td>
<td>PBMCs</td>
<td>~</td>
<td>+</td>
</tr>
<tr>
<td>1126</td>
<td>76</td>
<td>M</td>
<td>CD3⁺ CD8⁺</td>
<td>16/5/91</td>
<td>PBMCs</td>
<td>~</td>
<td>+</td>
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<td>1148</td>
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<td>M</td>
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<td>20/6/91</td>
<td>PBMCs</td>
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<td>29/8/91</td>
<td>PBMCs</td>
<td>~</td>
<td>+</td>
</tr>
<tr>
<td>1196</td>
<td>67</td>
<td>M</td>
<td>CD3⁺ CD8⁺</td>
<td>4/9/91</td>
<td>PBMCs</td>
<td>~</td>
<td>+</td>
</tr>
</tbody>
</table>

**LGL leukaemia** is Large granular lymphocytic leukaemia. **PBMCs** is Peripheral blood mononuclear cells.
Table 2.5: Cases of acute lymphoblastic leukaemia in butchers from Cardiff.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age (years)</th>
<th>Sex</th>
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<tbody>
<tr>
<td>1123</td>
<td>21</td>
<td>M</td>
<td>Common ALL</td>
<td>16/5/91</td>
<td>PBMCs</td>
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<td>+</td>
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<tr>
<td></td>
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<td></td>
<td>16/5/91</td>
<td>Bone marrow</td>
<td>-</td>
<td>+</td>
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<tr>
<td>1129</td>
<td>U</td>
<td>M</td>
<td>Pre-B cell ALL</td>
<td>12/5/89</td>
<td>PBMCs</td>
<td>-</td>
<td>+</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td>Bone marrow</td>
<td>-</td>
<td>+</td>
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<tr>
<td>1131</td>
<td>22</td>
<td>M</td>
<td>Common ALL</td>
<td>13/6/91</td>
<td>PBMCs</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>1158</td>
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<td>1167</td>
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<td>5/8/91</td>
<td>PBMCs</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

ALL: Acute lymphoblastic leukaemia
PBMCs: Peripheral blood mononuclear cells
U: Unknown
2.3 Materials and methods

2.3.1 Containment facilities for handling clinical samples

2.3.1.1 Level II containment facility

Containers with clinical samples were opened in class II microbiological safety cabinets (MSCs) in the level II containment facility at the LRF Virus Centre. The containment facility operated under negative pressure. Air extracted from MSCs and from the main containment room passed through independent high efficiency particulate air (HEPA) filters. Clean laboratory coats and surgical gowns were worn. MSCs and the main containment room were disinfected with formaldehyde gas every 6 months. Laboratory personnel were routinely vaccinated against hepatitis B virus (HBV) and tuberculosis. Samples entering the laboratory were assigned unique patient and sample identifying numbers. Accompanying documents were filed in the laboratory and sample details recorded in a computerised database.

2.3.1.2 Microbiological safety cabinets

Handling procedures and the laminar flow of air in the class II MSCs prevented cross-contamination of samples and reduced the risk of infection of personnel. Two latex gloves were worn on each hand when working in MSCs. The outer gloves were discarded and replaced every time hands were withdrawn from the operating environment. Sample containers and the interiors of MSCs were sprayed with 1% Virkon-S or 70% ethanol before and after samples were handled. Disinfection was maintained for at least 15 minutes between uses of MSCs by different operators or for different purposes. Disposable plastic pipettes, sample containers, bottles for solutions and media, centrifuge tubes and tissue culture flasks were used for most work. The use of glass was minimised. Sterile round-nosed scissors and rat-toothed forceps were used for manipulation of tissues, including cutting tissues into pieces for extraction of DNA. Solutions were transferred by pipette using battery-operated devices. Components of pipetting devices potentially in contact with tissue culture fluids were dismantled and soaked in 1% Virkon-S between uses and filters were changed.

The three MSCs in the level II containment facility were used for handling different material. MSC 1 was used for handling known HTLV-I, BLV, EBV, human herpesvirus (HHV) 6 or HHV-7-infected cell lines. MSC 2 was used for handling clinical specimens and tissue cultures derived from those specimens. MSC 3 was used for preparing sterile medium and solutions and for handling cell cultures demonstrated to be free of specified exogenous retrovirus or herpesvirus infections. Viable cells were stored in liquid nitrogen (-195.8°C). Samples of serum and cells and tissue samples for DNA extraction were stored at -80°C in freezers in a room adjacent to the containment laboratory. Handling of potentially infectious material outside MSCs was undertaken with appropriate precautions. Cell culture flasks were sealed when in incubators and during handling in the main containment room. Cells were inactivated before removal from MSCs for quantification by haemocytometer counts (Section 2.3.3.3). During centrifugation tubes were placed in sealed screw-top cannisters (Aerosolve, Beckman).
2.3.1.3 Disposal of waste

Used plasticware and glass heparin containers were soaked in 2% Virkon-S in trays for 1 hour before the trays were placed in plastic autoclave bags and autoclaved at 121°C for 1 hour. Discarded samples, solutions and tissue culture media were diluted with at least an equal volume of 2% Virkon-S in screw-top plastic containers and subjected to the same autoclave protocol. All other laboratory waste was placed in double autoclave bags and autoclaved at 121°C for 1 hour before removal from the laboratory and disposal by incineration.

2.3.2 Materials

The sources of frequently used materials are listed in Appendix 3 and the compositions of routinely used solutions for cell biology are listed in Appendix 4. Additional reagents and buffers are specified in individual sections.

2.3.2.1 Cell culture

Cells were cultivated in 50 cm³ offset screw-top tissue culture flasks containing 5 to 10 ml medium (Costar) or in 250 cm³ flasks containing 25 to 50 ml medium (Appendix 4: A4.1). Some cultures were performed in 96-well microtitre plates containing 50 to 200 µl medium per well or 12-well tissue culture plates containing 1 to 2 ml medium per well.

2.3.2.2 Materials for manipulation of fluids

Disposable pipettes (Costar) suitable for handling 1, 5, 10 and 25 ml volumes were used for transferring fluids (Appendix 4: A4.1). Blood samples, cell culture media and other fluids were centrifuged in 15 and 50 ml screw-top polypropylene centrifuge tubes (Falcon, Becton Dickinson and Sterilin, J Bibby). These tubes, as well as 5 ml screw-top polycarbonate bijous and 20 ml Universal tubes, were used for storing fluids and tissues. Small quantities of media and additives for cell culture were sterilised by filtration through 0.2 and 0.45 µm cellulose acetate filters (Acrodisc, Gelman Sciences) attached to syringes. Larger volumes were sterilised by vacuum filtration using 250 and 500 ml bottles with 0.2 or 0.45 µm cellulose acetate filters (Costar).

2.3.2.3 Centrifuges

The main centrifuge used for cell culture work was the GPR bench centrifuge (Beckman) with a GH 3.7 swinging bucket rotor. This held 15 and 50 ml screw-top polypropylene centrifuge tubes in racks inside screw-top canisters (Aerosolve). Microtitre (96-well) plates were centrifuged using microplate carriers. A bench microcentrifuge (Micro Centaur, MSE) was used for centrifuging 0.6 ml flip-top PCR tubes and 1.5 ml flip-top and screw-top polypropylene microcentrifuge tubes.
2.3.2.4 Cell culture medium

Standard tissue culture medium consisted of RPMI 1640 medium nominally containing 10 or 20% foetal bovine serum heat-inactivated at 56°C (hi-FBS), 400 mg/ml streptomycin, 400 U/ml penicillin and 2 mM L-glutamine (RPMI 1640 + 10 or 20% hi-FBS + 2-ME) (Moore et al. 1967) (Appendix 4: A4.2.1). Medium with 10% hi-FBS was used for most established cell lines and medium with 20% hi-FBS for cultivation of cells derived from clinical samples. Phenol red was included as an indicator of changes in pH. Gentamicin was sometimes added at a final concentration of 50 to 70 μg/ml if bacterial contamination developed. Amphotericin B was added to cultures at risk of fungal contamination at a final concentration of 1 μg/ml. However, cultures that developed fungal growth were discarded without opening the contaminated flasks.

2.3.3 Manipulation of cells

2.3.3.1 Standard procedure for cultivation of eucaryotic cells

Established cell lines were usually split at a ratio of 1:4 to 1:6 twice weekly to densities ranging from 5 x 10^3 to 5 x 10^6 cells/ml. The tissue culture flasks were aerated with filtered 5% CO_2 in air for 30 sec then sealed and incubated at 37°C. Cells in suspension were diluted with an appropriate quantity of standard tissue culture medium and unwanted cells and medium were discarded by dilution with at least an equal volume of 2% Virkon-S. Cells that formed adherent monolayers, including BLV-FLK and fibroblast cultures derived from skin samples of patients, were separated from the plastic of the tissue culture flasks by incubation with trypsin or dispase (Appendix 4: A4.2.5). For trypsinisation, the cell culture medium was removed and replaced with 1.5 to 3 ml 0.25% crude trypsin in 1 M ethylenediaminetetraacetic acid (EDTA) (trypsin-EDTA). The flask was incubated at 37°C for 5 to 15 min with occasional gentle tilting. When most cells appeared separated by light microscopy, the flask was tapped firmly to dislodge remaining adherent cells. Alternatively, the fluid was flushed up and down several times using a pipette. Tissue culture medium containing 20% hi-FBS (10 ml) was added to the flask and mixed to dilute and inactivate the trypsin. The cells were then centrifuged at 270 g (1,000 rpm) for 5 min and the supernatant was discarded. The cell pellet was resuspended in an appropriate volume of tissue culture medium, returned to a tissue culture flask and aerated with 5% CO_2 in air before incubation. Dispase was used at a concentration of 1 U/ml for passaging cultures of skin from clinical samples that had adherent fibroblasts underlying lymphocytes in suspension.

When the quantity of cells extracted from a clinical sample was low, 12-well plates or 96-well microtitre plates were used to maintain the density of cells in culture. Cells in 96-well microtitre plates were usually cultured at 1 x 10^6 to 3 x 10^6 cells/ml in 200 μl medium. N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer, gentamicin and amphotericin B were added to the standard tissue culture medium. Approximately 200 μl of autoclaved ultrapure water were added to unused wells to maintain humidity. The lids were secured with tape.
2.3.3.2 Light microscopy

An inverted light microscope (Labovert, Leitz) was used for examination of cells in culture flasks and for haemocytometer counts at powers of 40x, 100x or 200x.

2.3.3.3 Quantification of cells

The concentrations of PBMCs, UCMCs and cultured cells were determined by counting cells in known volumes using an improved Neubauer haemocytometer chamber (Fisons). Cells in even suspension (50 µL) were stained with 0.1% crystal violet in 1% acetic acid (50 µL) for 3 to 5 min in a 5 ml screw-top polycarbonate bijou. Erythrocytes in preparations of PBMCs were lysed by the acetic acid. The stained cells were pipetted under the coverslip of the haemocytometer chamber and the numbers of viable cells were counted in each of four 1 mm x 1 mm x 0.1 mm (0.1 mm³) chambers. The concentration of cells in the original volume was calculated by multiplying the total number of viable cells by 1 x 10⁴. The haemocytometer chamber and glass cover slips were stored in 1% Virkon-S between uses.

2.3.3.4 Cryopreservation of viable cells and cell cultures

Viable cells for cryopreservation were suspended at a density of 1 x 10⁶ to 1 x 10⁷ cells/ml in tissue culture medium containing 20 to 50% hi-FBS and 10% dimethyl sulphoxide (DMSO). Alternatively, cells were suspended in hi-FBS containing 8% DMSO. Aliquots of 1 ml were transferred to screw-top polypropylene low temperature storage vials (Cryotube, Nunc). These were placed in a freezing container (Nalgene) filled with isopropanol and held at -80°C for 4 hours for a controlled decrease (1°C/min) in temperature. The vials were then transferred to a liquid nitrogen (liqN₂) container for storage at ~195.8°C.

2.3.3.5 Cultivation of cryopreserved cells

Cryopreservation tubes were removed from liquid nitrogen and placed in a screw-top metal cannister to prevent dissemination of contents should the tubes explode as they thawed. The metal cannister was placed in a MSC for 5 min until the contents were thawed. The cryotube was removed from the cannister, opened and the contents were pipetted drop by drop into a 15 ml screw-top polypropylene centrifuge tube containing 5 ml of the appropriate tissue culture medium. The 15 ml tube was centrifuged at 270 g for 5 min and the supernatant was discarded. The cell pellet was resuspended in 2 to 5 ml of standard tissue culture medium and the concentration of viable cells was determined (Section 2.3.3.3). Cells were diluted to 5 x 10⁵ to 5 x 10⁶ cells/ml in appropriate tissue culture medium, aerated with 5% CO₂ in air and incubated at 37°C. Cells were examined daily and subcultured as required. If many cells died in the first week of culture, these were removed by centrifuging through a Ficoll gradient (Section 2.3.5.1). The purified viable cells were washed by centrifuging and discarding the supernatant, then were resuspended in fresh medium, aerated with 5% CO₂ in air and returned to cultivation.
2.3.3.6 Collection of cell culture supernatants

Supernatants were collected from selected cell cultures for use as CM and for assay of RT activity after centrifuging at 270 g for 5 min.

2.3.3.7 Cytospins for Epstein-Barr virus latent membrane protein staining

Cultured cells for cytospin preparations for EBV latent membrane protein (LMP) 1 staining were centrifuged to remove the medium and washed three times in PBS 1x. After counting using an haemocytometer (Section 2.3.3.3), cells were resuspended at a concentration of 1 x 10^6 cells/ml. Volumes of 100 μl containing 1 x 10^5 cells were centrifuged onto 3-aminopropyltriethoxysilane (APES)-coated glass cytospin slides (Fisons) at 400 rpm in a cytospin centrifuge (Shandon Cytospin 2) for 5 min. The cytospin preparations were air dried, fixed in ice-cold acetone or methanol for 30 min and stored at -20°C. Staining for EBV LMP-1 with MAbs CS1-4 was performed by Dr A Armstrong at the LRF Virus Centre using techniques established in the laboratory (Armstrong et al 1992).

2.3.3.8 Testing for mycoplasma contamination

Testing for contamination with mycoplasma was performed by June Freeland and Linda Andrew at the LRF Virus Centre. Sub-confluent normal rat kidney (NRK) cells were grown with 0.5 ml test culture supernatant in slide flasks (Nunc, Nunc) for 3 days at 37°C. The cells were fixed with ice-cold methanol:glacial acetic acid (3:1), washed twice with distilled water, then stained with 50 ng/ml Hoescht 33258 (Sigma) for 10 min at room temperature (Appendix 4: A4.7). Stained cells were washed with distilled water and examined under a wet-mounted cover slip using a fluorescent microscope (Laborlux K, Leitz). Mycoplasma typing was performed using a commercial PCR kit (Boehringer-Mannheim).

2.3.4 Established cell lines

2.3.4.1 C8166

C8166 (C81-66-45) is an HTLV-I-infected human CD4+ T cell line derived from the productive HTLV-I-infected cell line HUT102 (Salahuddin et al 1983). HUT102 (ATCC TIB 162) was the first HTLV-I-infected cell line established (HTLV-I type strain HTLV1R) and was derived from lymph node cells of a patient in the USA with cutaneous ATL resembling MF (Gazdar et al 1979, 1980, Poiesz et al 1980a, b, Hay et al 1988) (Section 1.3.2.1). C8166 contains one full length defective and two incomplete integrated HTLV-I proviruses, the incomplete proviruses having deletions of most of their regions from gag to env (Bhat et al 1993). All three proviruses are transcriptionally active and C8166 expresses Tax and p21^\text{\textsuperscript{S}} but has no expression of Rex, Gag or Env, no detectable RT activity and no production of virions (Salahuddin et al 1983, Bhat et al 1993). It is thus a suitable cell line for handling at containment level II. DNA and cell pellets derived from C8166 were used as positive controls for HTLV-I and HTLV-II/III PCR analyses.
2.3.4.2 J.JHAN

J.JHAN is a derivative of the human CD4⁺ T cell line Jurkat (JM) (ATCC TIB-152), cultured from neoplastic T cells of a child with ALL and not known to be infected with any exogenous retrovirus (Schneider et al 1977, Gillis and Watson 1980, Hay et al 1988). It was provided to the LRF Virus Centre by Dr M Steele, Western General Hospital, Edinburgh. DNA and cell pellets from this cell line were used as negative controls for HTLV-I and HTLV-II/III PCR analyses and culture supernatants were collected for use in the RT assay.

2.3.4.3 L-726A

L-726A is an IL2-independent CD4⁺ T cell line established at the LRF Virus Centre from skin lesions of a patient with SS (Crae et al 1991) (Section 2.1.3.1). No viruses have been demonstrated in this cell line. DNA and cell pellets from L-726A were tested for HTLV-I and HTLV-II/III by PCR and culture supernatants were tested for evidence of RT activity.

2.3.4.4 HH

HH is an IL2-independent CD4⁺ T cell line derived from PBMCs of a patient with CTCL and was kindly provided by Dr G Starkebaum, Department of Medicine, Seattle Veterans Affairs Medical Center, Seattle, Washington, USA (Starkebaum et al 1991) (Section 2.1.3.1). This cell line is not known to be infected with HTLV-I. DNA and cell pellets from HH were tested for HTLV-I and HTLV-II/III by PCR and culture supernatants were tested for evidence of RT activity.

2.3.4.5 My-La and My-La MC

My-La is an IL2 and IL4-dependent CD4⁺ T cell line established from the skin of a patient with MF (Kaitoft et al 1992) (Section 2.1.3.1). My-La marker chromosomes (MC) is a derivative of My-La with a 46XY= karyotype. These cell lines were kindly provided by Dr K Kaitoft, Department of Medicine, Institute of Human Genetics, Aarhus University, Denmark. They were grown in standard tissue culture medium containing 10% hi-FBS, 50 U/ml IL2 and 30 U/ml IL4. DNA and cell pellets were tested for HTLV-I and HTLV-II/III by PCR and culture supernatants were tested for evidence of RT activity.

2.3.4.6 BLV-FLK

BLV-FLK is a BLV-infected foetal lamb kidney (FLK) fibroblast cell line (Astier et al 1978, Onuma et al 1981, Rhim et al 1983, Hay et al 1988). It has one or a few intact copies of the BLV provirus and produces infectious virions (Marbaix et al 1981). Cell culture supernatants from this cell line were used as positive controls in RT assays and DNA and cell pellets were used as positive controls in BLV PCR analyses (Chapter 3).
2.3.4.7 FLK

FLK is an ovine fibroblast cell line not known to be infected with exogenous retroviruses (Astier et al 1978). Infection of this cell line with BLV resulted in the persistently infected cell line BLV-FLK described above. Cells obtained from this cell line were used as negative controls in BLV PCR analyses.

2.3.5 Processing of clinical samples

2.3.5.1 Purification of mononuclear cells from peripheral blood

Blood samples had been diluted with an equal volume of heparinised transport medium at the time of collection (Section 2.2.1) (Appendix 4: A4.2.2). On receipt at the LRF Virus Centre they were diluted with an equal volume of Hanks' balanced salt solution (HBSS + 2% hi-FBS) to one quarter of their original concentration. The diluted blood (20 to 25 ml) was carefully layered with a pipette over 15 ml of metrizoate-Ficoll (Ficol 400 solution, Ficoll-Paque, Pharmacia LKB or J.Prep, J.Bio), an aqueous solution of density 1.077 g/ml containing polymerised sucrose and sodium metrizoate/diatrizoate, in each of two 50 ml screw-top polypropylene centrifuge tubes (Falcon). The gradient was centrifuged at 1,500 g for 20 to 30 min at 18°C with no braking. Erythrocytes passed through the layer of Ficoll to sediment in the bottom. PBMCs remained at the interface with the Ficoll layer. PBMCs were removed with a polypropylene bulb pipette, transferred to a 50 ml centrifuge tube containing 30 ml HBSS + 2% hi-FBS and centrifuged at 270 g for 5 min. The PBMCs, forming a pellet at the bottom of the centrifuge tube, were retained and the supernatant was discarded. If the supernatant was red due to lysed erythrocytes, the washing step was repeated by resuspending the cells in 30 ml HBSS and centrifuging. The pelleted PBMCs were resuspended in 10 ml medium and centrifuged again. The final cell pellet was resuspended in 1 to 3 ml medium and the concentration of cells was determined using an haemocytometer (Section 2.3.3.3). PBMCs were diluted to $1 \times 10^5$ to $2.5 \times 10^6$ cells/ml in appropriate medium with selected stimulants for cultivation of T cells (Section 2.3.6). Volumes containing $1 \times 10^5$ to $1 \times 10^6$ PBMCs in PBS 1x were transferred to 15 ml screw-top polypropylene centrifuge tubes to prepare cell pellets for DNA extraction (Section 3.3.5.1). Cell pellets for PCR were prepared with $1 \times 10^5$ PBMCs in PBS 1x in 0.6 ml flip-top PCR tubes (Section 3.3.5.1).

2.3.5.2 Processing of tissue samples

Tissue samples (skin, lymph nodes and human placenta) were processed on the day of receipt for tissue culture and storage at -80°C for later DNA extraction. Tissues were dissected into pieces approximately 1 mm$^3$ in sterile plastic Petri dishes using sterile round-nosed scissors and rats-toothed forceps. Approximately 1 ml of standard tissue culture medium was added to prevent desiccation. Half of each tissue was stored frozen at -80°C for DNA extraction and half was cultivated in an attempt to establish T cell lines (Section 2.3.6). Dispase II was added to the medium at a concentration of 1 U/ml and incubated at 37°C for 10 to 15 min to assist in the dissociation of cells from samples of skin and lymph nodes.
2.3.6 Cultivation of lymphocytes from clinical samples

PBMCs and cells from skin and lymph nodes of patients with MF/SS and other cutaneous lymphoid infiltrates were cultivated in an attempt to establish T cell lines. Cells and tissues were suspended in standard tissue culture medium (RPMI 1640 + 20% hi-FBS + 2-ME) containing selected exogenous cytokines (Appendix 4: A4.2.6), CM or no stimulants, or were cocultivated with UCMCs. Specific protocols were adapted from those used by other groups to establish CD4⁺ cell lines from patients with MF/SS (Sections 2.1.3.3 and 2.1.3.4) (Table 2.6). Once or twice weekly cells were centrifuged and resuspended in fresh medium according to the selected protocol.

2.3.6.1 Unstimulated cultures

Cells harvested from blood, skin and lymph nodes were cultivated in flasks at a density of 1 x 10⁶ cells/ml. Half of the culture volume was removed and centrifuged at 270 g for 5 min once weekly or as required. The cells were resuspended in an equal volume of fresh medium and returned to the same flask. The remaining supernatant was stored for assay of RT activity, as were supernatants collected from other cultures (Section 2.3.3.6).

2.3.6.2 Phytohaemagglutinin and interleukin 2

Cells were cultivated at a density of 1 x 10⁶ cells/ml with 5 μg/ml PHA-P for 48 to 72 hours. The medium was then replaced with medium containing 50 U/ml IL2.

2.3.6.3 Concanavalin A and interleukin 2

Cells were cultivated at a density of 1 x 10⁶ cells/ml with 5 μg/ml ConA for 48 to 72 hours. This was replaced with medium containing 50 U/ml IL2.

2.3.6.4 Interleukin 2

Cells were cultivated at a density of 1 x 10⁶ cells/ml with 50 U/ml IL2.

2.3.6.5 Interleukin 7

The protocol for use of IL7 for cultivation of T cells from patients with MF/SS was adapted from Dalloul et al (1992) and Foss et al (1994). Cells were cultivated at a density of 1 x 10⁶ cells/ml with 10 ng/ml IL7.

2.3.6.6 Interleukin 2 and interleukin 7

Cells were cultivated at a density of 1 x 10⁶ cells/ml with 50 U/ml IL2 and 10 ng/ml IL7.
Table 2.6: Protocols for cultivation of T cells from cutaneous T cell lymphoma patients.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA → IL2</td>
<td>5 μg/ml phytohaemagglutinin then 50 U/ml interleukin 2</td>
</tr>
<tr>
<td>ConA → IL2</td>
<td>2 μg/ml concanavalin A then 50 U/ml interleukin 2</td>
</tr>
<tr>
<td>IL2</td>
<td>50 U/ml interleukin 2</td>
</tr>
<tr>
<td>IL2 + GMCSF</td>
<td>10 U/ml interleukin 2 + 100 U/ml granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>IL2 + IL4</td>
<td>50 U/ml interleukin 2 + 30 U/ml interleukin 4</td>
</tr>
<tr>
<td>IL2 + IL7</td>
<td>50 U/ml interleukin 2 + 10 ng/ml interleukin 7</td>
</tr>
<tr>
<td>IL7</td>
<td>10 ng/ml interleukin 7</td>
</tr>
<tr>
<td>UCMC CM</td>
<td>25% umbilical cord blood mononuclear cell conditioned medium</td>
</tr>
<tr>
<td>IL2 + SAF CM</td>
<td>16 U/ml interleukin 2 + 0.2% Sézary activating factor conditioned medium</td>
</tr>
<tr>
<td>Cocultivation</td>
<td>Cocultivation with umbilical cord blood mononuclear cells</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>No exogenous growth factors</td>
</tr>
</tbody>
</table>
2.3.6.7 Interleukin 2 and interleukin 4

The protocol for use of IL2 and IL4 to stimulate MF/Sézary cells was adapted from Kaito et al (1992). Cells were cultivated at $1 \times 10^6$ cells/ml with 50 U/ml IL2 and 30 U/ml IL4.

2.3.6.8 Interleukin 2 and granulocyte-macrophage colony stimulating factor

GMCSF was used according to the method described by Zucker-Franklin et al (1991). Cells were cultivated at a density of $2.5 \times 10^6$ cells/ml with 10 U/ml IL2 and 100 U/ml GMCSF.

2.3.6.9 Interleukin 2 and Sézary T cell activating factor conditioned medium

SAF CM was prepared according to the method described by Abrams et al (1991b, 1993). PBMCs from a patient with SS (patient 1198) were cultivated at $4 \times 10^5$ cells/ml with 2 µg/ml ConA for 48 hours. The supernatant was harvested by centrifugation at 270 g for 5 min. Residual ConA was inactivated with 1 mg/ml α-methyl mannoside and the supernatant was filtered into a 20 ml screw-top Universal container through a 0.45 µm cellulose acetate filter (Acrodisc, Gelman Sciences) attached to a syringe. This supernatant, designated SAF CM, was stored at -20°C until required. Cultures were initiated from other patients with MF/SS at densities of $1 \times 10^6$ cells/ml with 0.2% SAF CM and 15 U/ml IL2 for 3 days. The cultures were then maintained with 15 U/ml IL2 (without SAF CM), medium being replaced two to three times per week. After 6 weeks, cultures were re-stimulated with 0.2% SAF CM and 15 U/ml IL2 for 3 days before reverting to 15 U/ml IL2 alone.

2.3.6.10 Umbilical cord blood mononuclear cell conditioned medium

UCMC CM, an unmodified culture supernatant also known as lymphocyte conditioned medium, was prepared from UCMCs stimulated with PHA-P. UCMCs were cultivated at a density of $1 \times 10^5$ to $2.5 \times 10^6$ cells/ml with 5 µg/ml PHA-P for 48 hours. The supernatant was harvested after 3 days by centrifuging the cells at 270 g for 5 min and stored at -20°C. UCMC CM was added at a concentration of 25% to cultures of cells from clinical samples containing $1 \times 10^5$ cells/ml that had been stimulated with 5 µg/ml PHA-P for 48 hours. The supernatant was replaced with a mixture of 75% medium and 25% UCMC CM once per week.

2.3.6.11 Cocultivation with umbilical cord blood mononuclear cells

UCMCs were cocultivated with cells from patients with neoplasia to act as recipient cells for infection with retroviruses released from patient cells and to produce growth stimulants that might promote outgrowth of continuous T cell lines. UCMCs were cultivated at $1 \times 10^6$ cells/ml with 5 µg/ml PHA-P for 48 to 72 hours. These cells were centrifuged then resuspended at $2 \times 10^6$ cells/ml. Fresh or cultured cells (donor cells) from patients with neoplasia were suspended at a density of $2 \times 10^6$ cells/ml. Equal volumes of suspensions of donor and recipient cells were mixed in the same flask and cultivated with 50 U/ml IL2. A control flask containing $2 \times 10^6$ UCMCs/ml in the same medium was maintained in parallel. A
different sex was chosen for recipient UCMCs and donor cells from the patient with neoplasia so that any cell line established from cocultures could be karyotyped to determine the origin of the cells.

2.3.7 Immunophenotyping of cultured lymphocytes by flow cytometry

The immunophenotypes of cultured lymphocytes were determined by flow cytometry using an EPICS Elite II (Coulter). Cells were labelled with MAbs to determine the expression of surface antigens with specificity for different cell types (Table 2.7) (Appendix 4: A4.3.2). Cells removed from cultures were centrifuged in 15 ml screw-top polypropylene tubes at 270 g for 5 min. The supernatant was discarded and the cells were resuspended in 5 ml HBSS + 2% hi-FBS. After counting using an haemocytometer (Section 2.3.3.3), aliquots of 1 x 10⁸ cells were transferred to 5 ml polypropylene cytometer tubes (Sarstedt) and centrifuged again at 270 g for 5 min. The supernatant was removed and the cells were resuspended in PBS 1x containing 0.1% sodium azide and 2% bovine serum albumin (PAB). The tubes were centrifuged and the supernatant was removed. The cells were resuspended in 50 μl PAB containing 20% rabbit serum and held on ice for 30 to 60 min to block non-specific binding sites on cells. The cells were again washed with 1 ml PAB, centrifuged and the supernatant discarded. This solution was replaced with 10 μl of a solution containing 20% rabbit serum in PAB and 5 μl/ml fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE)-conjugated murine MAbs against CD2, CD4, CD8, CD15, CD19, CD30, CD33 or CD45. FITC and RPE-conjugated murine MAbs against Aspergillus niger glucose oxidase were used as negative controls. Cells were double-labelled with anti-CD2-FITC + anti-CD19-RPE and anti-CD8-FITC + anti-CD4-RPE for two-colour EPICS analysis. Binding of MAbs to cells was allowed to proceed for 20 to 30 min on ice. Unbound MAbs were removed by centrifuging three times at 270 g for 5 min, the supernatant being removed and the cell pellets resuspended in 1 ml PAB each time. After the last wash step, the cells were fixed by adding 1 ml 1% paraformaldehyde in PBS 1x and held at 4°C until analysed by flow cytometry. EPICS analysis was performed by Linda Andrew in the LRF Virus Centre.

2.3.8 Electron microscopy

Cells from four long-term cultures of T cells (≥ 6 months) established from three patients with MF, as well as lymphocytes from five other patients with MF or SS after 21 to 50 days in culture, were processed for electron microscopy (EM) in an attempt to observe intracellular and budding retrovirus particles. After determining the density of cells by counting using an haemocytometer (Section 2.3.3.3), aliquots of 5 x 10⁸ cells were placed in 15 ml screw-top polypropylene centrifuge tubes. The tubes were centrifuged at 270 g for 5 min and the supernatant was discarded. Cell pellets were resuspended in 5 ml of 2% glutaraldehyde in 0.05 M sodium cacodylate pH 7.2 to 7.3 and the tubes were centrifuged at 900 g (2,000 rpm). The supernatant was removed and carefully replaced with 1 ml glutaraldehyde solution. Glutaraldehyde-fixed cell pellets were submitted to the EM unit in the Department of Veterinary Pathology for postfixing, epoxy embedding and examination by thin section transmission EM (Zeiss 109). Processing and examination of EM samples were performed by Ross Blackley, with additional interpretation by Dr Helen Laird.
Table 2.7: Specificity of selected haematopoietic cell surface markers.

Cell surface markers are indicated by their cluster of differentiation (CD) nomenclature.

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>Predominant cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Haematopoietic cells</td>
</tr>
<tr>
<td>CD2</td>
<td>T cell</td>
</tr>
<tr>
<td>CD19</td>
<td>B cell</td>
</tr>
<tr>
<td>CD3</td>
<td>Mature T cell</td>
</tr>
<tr>
<td>CD4</td>
<td>CD4⁺ helper/inducer T cell</td>
</tr>
<tr>
<td>CD8</td>
<td>CD8⁺ cytotoxic/suppressor T cell</td>
</tr>
<tr>
<td>CD15</td>
<td>Myeloid lineage cells</td>
</tr>
<tr>
<td>CD33</td>
<td>Myeloid lineage cells</td>
</tr>
<tr>
<td>CD30</td>
<td>Activation marker</td>
</tr>
</tbody>
</table>
2.3.9 Reverse transcriptase assay

2.3.9.1 Principle of reverse transcriptase assay

Reverse transcriptase (RT) is essential for retroviral replication and present in all replication competent retrovirus virions (Baltimore 1970, Temin and Mizutani 1970) (Section 1.2.3). An assay based on detection of RT activity can be used as a general technique for revealing the presence of retroviruses. RT assays are based on incorporation of nucleosides into DNA using RNA as a template by utilising the RNA-dependent DNA polymerase activity of viral RTs. The gold standard is the conventional RT assay based on incorporation of radioactive nucleosides into the cDNA product and measurement of radioactivity (Kacian 1977). The inclusion of Mg$^{2+}$ or Mn$^{2+}$ as cofactors in the assay is used to determine the relative Mg$^{2+}$ or Mn$^{2+}$-dependence of any RT activity present. Use of MAbs and biotinylated or digoxigenin-labelled nucleosides has allowed enzyme-linked immunosorbent assay (ELISA)-based assays for RT activity to be developed, increasing sensitivity (Eberle and Seibl 1992, Suzuki et al 1993). Sensitivity has been further increased with PCR-based RT assays that allow exponential amplification of cDNA produced from an RNA template, with detection of product by Southern blot (SB) hybridisation or ELISA (Silver et al 1993, Pyra et al 1994). RNA viruses are used as templates in these assays because they do not have a DNA phase that could contaminate the RT-PCR reaction and are utilised less efficiently than synthetic templates for reverse transcription by cellular DNA polymerases, reducing non-specific reactivity (Robert et al 1972).

2.3.9.2 Conventional reverse transcriptase assay

The conventional reverse transcriptase assay was performed according to the method of Klement and Nicolson (1977). Supernatants collected from cell cultures and stored at -20°C (Section 2.3.3.6) were thawed and clarified by centrifugation at 10,000 g (13,000 rpm) for 20 min in a J2-21 centrifuge (Beckman) with a JA-20 fixed angle rotor. Clarified supernatants were transferred to 10 ml plastic centrifuge tubes (Ultra-Clear, 14 mm x 89 mm, Beckman) in SW41 Ti buckets. The tubes were filled to within 0.5 cm of the top with Tris-NaCl-EDTA (TNE) 1x and opposing buckets were balanced to within 50 mg. The samples were centrifuged at 210,000 g (35,000 rpm) in an L8-60M ultracentrifuge (Beckman) with an SW41 Ti swinging bucket rotor at 4°C for 1 hour. Alternatively, they were centrifuged at 274,000 g (40,000 rpm) for 30 min. Retrovirus virions would be expected to form part of a pellet including some cellular debris at the bottom of the tubes. The supernatants were removed, the tubes were allowed to drain and the inside surfaces were dried, taking care not to dislodge the pellet. Virus disruption buffer, containing 20 mM dithiothreitol (DTT), 0.1% Nonidet P-40 (NP-40), 40 mM Tris pH 8.1 and 50m M KCl (Appendix 4: A4.4.2), was added with a pipette (170 μl for each 10 ml of original supernatant volume) and the bottom of each tube was scraped with the pipette tip to dislodge adherent debris and virus particles. The tubes were placed on ice for 10 to 15 min to allow virions to be disrupted by the buffer. The solution was assayed for RT activity immediately or stored at -80°C.
Reaction solutions (labelled X, Y and Z) were prepared for testing for Mg$^{2+}$ and Mn$^{2+}$- dependent RT activity (Table 2.8). In the presence of RT responsive to either cofactor, tritiated thymidine triphosphate ($^3$H-TTP) (Appendix 4: A4.4.3) would be incorporated by reverse transcription into radiolabelled poly-thymidine deoxyribonucleic acid (poly-dT) that could be quantified in a scintillation counter. Solution X contained poly-adenosine ribonucleic acid (poly-rA) and oligo-thymidine deoxyribonucleic acid (oligo-dT) (Pharmacia); the poly-rA acted as a template and the oligo-dT acted as a primer for RNA-dependent DNA polymerase (RT) activity in the presence of Mg$^{2+}$ or Mn$^{2+}$. Solution Y contained poly-adenosine deoxyribonucleic acid (poly-dA) and oligo-dT (Pharmacia); the poly-dA acted as a template for DNA-dependent DNA polymerase activity and the oligo-dT similarly acted as a primer. Since DNA polymerases of cellular origin may have a degree of RNA-dependent DNA polymerase activity, the quantification of DNA-dependent DNA polymerase activity in a sample gives an indication as to whether RT activity in a sample is likely to be due to the presence of a retrovirus or to contamination by cellular material (Goodman and Speigelman 1971). Solution Z did not contain any synthetic oligonucleotide reagent and acted as a negative control for $^3$H-TTP retention within the assay system.

In each assay, samples of supernatant were labelled 1, 2, 3 ... n. Assays for both Mn$^{2+}$ and Mg$^{2+}$-dependent RT activities were performed in duplicate. For each cofactor, 25 μl of reaction solution X, Y or Z were added to duplicate 15 ml screw-top polypropylene tubes labelled X₁, Y₁, Z₁; X₂, Y₂, Z₂; X₃, Y₃, Z₃; ... Xₙ, Yₙ, Zₙ (Table 2.9). Disrupted virus (25 μl) was added to each of these volumes and the mixtures were incubated in a water bath at 37°C for 1 hour. The tubes were placed on ice and mixed with approximately 10 ml of an aqueous solution containing 10% trichloroacetic acid (TCA) and 1% w/v sodium pyrophosphate (NaPP₄) that had been cooled to 4°C. This mixture was placed on ice for 10 to 20 min to precipitate DNA, including $^3$H-TTP-labelled products of the RT reaction.

A 1225 sampling manifold (Millipore) was used to collect precipitated DNA onto filters (GF/C glass microfibre filters, 2.5 cm, Whatman) by vacuum filtration (OM109 vacuum/pressure pump, Millipore). The top plate had 12 cups, each holding up to 15 ml. It was screwed onto a unit in which 12 glass microfibre filters were placed over polypropylene support screens on a support plate. Fluid passed through the filters under vacuum and was collected into a reservoir. The glass microfibre filters were moistened with a few ml of 10% TCA, 1% NaPP₄ before the RT assay samples (approximately 10 ml) were poured into the cups and vacuum filtration commenced. The RT assay reaction tubes were rinsed with approximately 10 ml of 10% TCA, 1% NaPP₄; this was poured into the cups on the sampling manifold top plate and the vacuum filtration repeated. The filters were rinsed twice with 10 to 15 ml 5% TCA under vacuum, then with 10 ml 98% ethanol (absolute alcohol) under vacuum. The glass microfibre filters were removed from the apparatus with plastic forceps and allowed to dry on blotting paper at room temperature. The sampling manifold was dismantled and soaked in decontaminating solution (Decon) overnight, then rinsed thoroughly with water and allowed to dry.
Table 2.8: Reaction solutions for reverse transcriptase assay.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>X (µl)</th>
<th>Y (µl)</th>
<th>Z (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mn(^{2+})-dependent reverse transcriptase assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 M Tris pH 8.1</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Tris pH 8.1, 0.15 M NaCl</td>
<td>–</td>
<td>–</td>
<td>160</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>20 mM MnCl(_2)</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>5 U/ml Poly(rA).p(dT(_{12-18}))</td>
<td>160</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5 U/ml Poly(dA).p(dT(_{12-18}))</td>
<td>–</td>
<td>160</td>
<td>–</td>
</tr>
<tr>
<td>(^3)H TTP</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Ultrapure water</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td><strong>Mg(^{2+})-dependent reverse transcriptase assay</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8 M Tris pH 8.1</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Tris pH 8.1, 0.15 M NaCl</td>
<td>–</td>
<td>–</td>
<td>160</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>0.1 M MgCl(_2)</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>5 U/ml Poly(rA).p(dT(_{12-18}))</td>
<td>160</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5 U/ml Poly(dA).p(dT(_{12-18}))</td>
<td>–</td>
<td>160</td>
<td>–</td>
</tr>
<tr>
<td>(^3)H TTP</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Ultrapure water</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
</tbody>
</table>
Table 2.9: Preparation of samples for reverse transcriptase assay.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Sample code</th>
<th>RNA activity (rA)</th>
<th>DNA activity (dA)</th>
<th>Negative activity (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn(^{2+})</td>
<td>1</td>
<td>X(_1)</td>
<td>Y(_1)</td>
<td>Z(_1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>X(_2)</td>
<td>Y(_2)</td>
<td>Z(_2)</td>
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<tr>
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<td>3</td>
<td>X(_3)</td>
<td>Y(_3)</td>
<td>Z(_3)</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>X(_n)</td>
<td>Y(_n)</td>
<td>Z(_n)</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>1</td>
<td>X(_4)</td>
<td>Y(_1)</td>
<td>Z(_1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>X(_5)</td>
<td>Y(_2)</td>
<td>Z(_2)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>X(_3)</td>
<td>Y(_3)</td>
<td>Z(_3)</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>X(_n)</td>
<td>Y(_n)</td>
<td>Z(_n)</td>
</tr>
</tbody>
</table>
The dry glass microfibre filters containing RT assay products were placed in 18 ml Type II polyethylene scintillation vials (Poly-Q vials, Beckman) with 5 ml scintillation liquid (Ecoscint A, National Diagnostics). Vials were loaded onto racks in a scintillation counter (LS1701 Liquid Scintillation System, Beckman) and the \(^{3}H\) counts per minute (cpm) were determined by exposure of the detector for 10 min per vial. After use, scintillation vials, fluid and filters were placed in sealed plastic bags and sent for disposal through the same system as for other radioisotopes in use in the Department of Veterinary Pathology.

The RNA-dependent DNA polymerase (RT) activity (rA) in each sample, determined by quantification of \(^{3}H\)-TTP-labelled product transcribed from the poly-rA template, was compared to the DNA-dependent DNA polymerase activity (dA) in the same sample, determined by quantification of \(^{3}H\)-TTP-labelled product transcribed from the poly-dA template, after correction for background \(^{3}H\)-TTP activity with no template (N). Evidence for RT activity was an elevated value of rA divided by N (rA/N) with either Mn\(^{2+}\) or Mg\(^{2+}\), while the values of dA divided by N (dA/N) in the same sample remained low.

**2.3.9.3 Positive and negative control samples**

Positive control samples for testing in the RT assay were supernatants obtained from known retrovirus-infected cell lines that are productive for retrovirus virions. Supernatants from the BLV-infected fibroblast cell line BLV-FLK grown at the LRF Virus Centre were used as positive controls for Mg\(^{2+}\)-dependent RT activity (Section 2.3.4.6). Supernatants from FL4, an IL2-dependent feline cell line infected with the Petaluma strain of feline immunodeficiency virus (FIV) were used as an additional positive control for Mg\(^{2+}\)-dependent RT activity (Yamamoto et al. 1991). Supernatants from this cell line were provided by Dr M Hosie, Department of Veterinary Pathology. SSN-1 is a fish cell line derived from the striped snakehead (Channa striatus) and persistently infected with snakehead fish retrovirus (SnRV) (Frerichs et al. 1991, Hart et al. 1996). Supernatants from this cell line, used as a positive control for Mn\(^{2+}\)-dependent RT activity, were kindly provided by Dr D Hart, Department of Veterinary Pathology.

**2.3.9.4 Product-enhanced reverse transcriptase (PERT) assay**

The product-enhanced RT (PERT) assay is a PCR-based method for detection of RT activity that uses brome mosaic virus as the RNA template (Pyra et al. 1994). PERT allows detection of 1 \(\times\) \(10^{6}\) U (2.1 \(\times\) \(10^{9}\) molecules) of murine leukaemia virus (MuLV) RT, equivalent to the activity present in 3 to 11 MuLV virions. This is 1 \(\times\) \(10^{5}\) to 1 \(\times\) \(10^{7}\) times more sensitive than conventional RT assays. Samples of supernatants from cell cultures of selected patients with CTCL were submitted to Dr J Schüpbach, Swiss National Center for Retroviruses, University of Zurich, Switzerland, for testing in the PERT assay for RT activity, with detection and quantification by ELISA. Positive and negative controls were as described by Pyra et al. (1994).
2.4 Results

Whole blood, skin and lymph nodes submitted to the LRF Virus Centre from patients with cutaneous lymphoid infiltrates during the period 1992 to 1995 were processed for cultivation of T cells and for molecular analyses (Sections 2.2.1 and 2.4.1). Samples received at the LRF Virus Centre from 1987 to 1992 had been cultivated mainly by Shauna Crae and resulted in the establishment of one continuous CD4⁺ T cell line, L-726A (Section 2.1.3.1). Cells derived from skin and PBMCs during this period had been treated with PHA, IL2, CM, polybrene, 12-o-tetradecanoyl phorbol-13 acetate (TPA, phorbol-12-myristate 13-acetate), anti-CD3 and anti-IFNα. Cells had also been cocultivated with UCMCs and depleted of CD8⁺ T cells. Serum samples had tested negative for HTLV-I by ELISA and WB ELISA, except for a few patients who had seroindeterminate bands by WB ELISA.

Results of investigations prior to 1992 will not be presented. However, samples stored from this period were used in molecular analyses described in Chapter 3 (Table 2.3). Cell pellets for PCR and DNA extraction, as well as some viable cells, had been stored from samples submitted from patients with LGL leukaemia and butchers from Cardiff with ALL in 1991 (Sections 2.2.2 and 2.2.3). These included PHA-stimulated PBMCs that had been cultured from one patient with LGL leukaemia. Samples from these patients were processed for molecular analyses (Chapter 3) (Tables 2.4 and 2.5). The results of cell biology studies are described here.

2.4.1 Cultivation of lymphocytes from patients with cutaneous lymphoid infiltrates

PBMCs and cells derived from skin and lymph nodes were cultivated from 15 patients with CTCL and three patients with CBCL (Table 2.10). Most samples of PBMCs were stimulated with PHA or ConA then IL2, IL2 alone or were unstimulated (Table 2.11). PBMCs were also cultivated with IL2 + SAF CM, IL2 + GMCSF, IL2 + IL4, IL2 + IL7, IL7 alone or UCMC CM. PBMCs from eight patients were cocultivated with UCMCs. Skin samples were cultivated with PHA or ConA then IL2, IL2 alone, IL2 + GMCSF, IL2 + IL7 or IL7 alone or were unstimulated. Lymph node samples from two patients were cultivated with ConA then IL2, IL2 alone, IL2 + SAF CM, IL2 + GMCSF, IL2 + IL4, IL7, UCMC CM or with no stimulants and were also cocultivated with UCMCs.

Within 1 week of stimulation with PHA, ConA, IL2 alone or in combination with other cytokines and UCMC CM, lymphocytes in culture formed dense clumps of proliferating cells, between which were many single cells exhibiting morphological changes suggestive of activation. These proliferating cells were larger than non-proliferating cells and had protrusions from the cell membrane. The clumps of proliferating cells dispersed within a few weeks and most cells died within 1 to 2 months of initiation of cultures. The use of cytokines did not appear to result in extended survival of most cultured lymphocytes.
Four long-term T cell cultures (greater than 6 months duration in culture) were established from skin samples of patients with MF (patients 2531, 2600 and 2862) following stimulation with PHA then IL2, IL2 alone or IL2 + IL4 (Table 2.12). These cultures were maintained for 6 to 8 months and remained dependent on IL2 for the duration of cultivation. No viruses were detected by EM in any of these long-term cultures (Section 2.4.2) and supernatants were negative for RT activity (Section 2.4.3). All four long-term cultures were immunophenotyped as CD8^+ T cell populations by flow cytometry. Since the source neoplasms comprised cutaneous infiltrations of CD4^+ T cells, as is typical for MF, the proliferating CD8^+ T cells in these cultures were thought to represent long-lived IL2-driven cytotoxic T cell clones derived from non-neoplastic infiltrates (TILs) in the skin.

One EBV-infected B-LCL was cultivated for 5 months from a skin sample from patient 2132 who had CBCL (Table 2.12). This culture had been stimulated with PHA then IL2. EBV infection was demonstrated by staining for LMP-1 (Section 2.3.3.7).

In skin cultures from patients 2116, 2132, 2552 and 2578, adherent fibroblasts grew out from pieces of tissue, forming monolayers that could be grown for more than 6 months. In several cultures, lymphocytes and other mononuclear cells appeared to have enhanced survival when cultivated in suspension above the fibroblast monolayers, remaining viable for 2 to 4 months.

Testing for mycoplasma revealed infection with *Mycoplasma arginini* in the CD8^+ T cell line established from patient 2600, as well as in short-term cultures from patients 2116, 2560 and 2669.

**2.4.2 Electron microscopy**

Cells were submitted for EM examination from four CD8^+ T cell cultures that had been grown from three patients with MF for 6 to 8 months, as well as selected cultures from skin and PBMCs of five other patients with MF or SS that had been maintained for 21 to 50 days. EM examinations did not reveal any evidence of retrovirus-like particles (Table 2.12). Representative electron micrographs are shown in Figures 2.1 and 2.2.
Table 2.10: Clinical diagnoses of cutaneous T cell lymphoma cases from which viable cells were submitted for culture 1992 to 1995.

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycosis fungoides</td>
<td>11</td>
</tr>
<tr>
<td>Sézary syndrome</td>
<td>3</td>
</tr>
<tr>
<td>Cutaneous T cell lymphoma</td>
<td>1</td>
</tr>
<tr>
<td>Cutaneous B cell lymphoma</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>18</strong></td>
</tr>
</tbody>
</table>
Table 2.11: Methods used for cultivating T cells from patients with cutaneous T cell lymphoma.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Number of PBMC samples</th>
<th>Number of skin samples</th>
<th>Number of lymph node samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA → IL2</td>
<td>13</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>ConA → IL2</td>
<td>11</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>IL2</td>
<td>18</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>IL2 + SAF CM</td>
<td>8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>IL2 + GMCSF</td>
<td>9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>IL2 + IL4</td>
<td>8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>IL2 + IL7</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IL7</td>
<td>8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>UCMC CM</td>
<td>7</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Cocultivation with UCMCs</td>
<td>8</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>13</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

CM: Conditioned medium
ConA: Concanavalin A
IL2: Interleukin 2
IL4: Interleukin 4
IL7: Interleukin 7
GMCSF: Granulocyte macrophage colony stimulating factor
PHA: Phytohaemagglutinin
SAF: Sézary T cell activating factor
UCMC: Umbilical cord blood mononuclear cell
Table 2.12: Features of long-term cultures of cells from patients with cutaneous lymphoid infiltrates.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Clinical diagnosis</th>
<th>Sample number</th>
<th>Sample type</th>
<th>Protocol</th>
<th>Duration in culture (months)</th>
<th>Type of cell line</th>
<th>Electron microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2132</td>
<td>40</td>
<td>M</td>
<td>CBCL</td>
<td>14671</td>
<td>Skin</td>
<td>PHA → IL2</td>
<td>5</td>
<td>EBV LMP+ B LCL</td>
<td>ND</td>
</tr>
<tr>
<td>2351</td>
<td>47</td>
<td>M</td>
<td>MF</td>
<td>13167</td>
<td>Skin</td>
<td>PHA → IL2</td>
<td>7</td>
<td>CD8+ T cell line</td>
<td>-</td>
</tr>
<tr>
<td>2600</td>
<td>62</td>
<td>M</td>
<td>MF</td>
<td>13795</td>
<td>Skin</td>
<td>PHA → IL2</td>
<td>6</td>
<td>CD8+ T cell line</td>
<td>-</td>
</tr>
<tr>
<td>2662</td>
<td>58</td>
<td>M</td>
<td>MF</td>
<td>14679</td>
<td>Skin</td>
<td>IL2 + IL4</td>
<td>6</td>
<td>CD8+ T cell line</td>
<td>-</td>
</tr>
</tbody>
</table>

B-LCL  B lymphoblastoid cell line
CBCL  B cell lymphoma
EBV  Epstein-Barr virus
IL2  Interleukin 2
IL4  Interleukin 4
LMP+  Latent membrane protein positive
MF  Mycosis fungoides
ND  Not done
PHA  Phytohaemagglutinin
-  No viruses detected
Figure 2.1: Electron micrograph of lymphocytes from a CD8\(^+\) T cell line cultivated from a patient with mycosis fungoides.

Culture derived from the skin of patient 2600 following stimulation with IL2. Photograph taken after 49 days in culture. One large lymphocyte (25 \(\mu\)m x 40 \(\mu\)m) exhibits features of activation, including dispersed chromatin and a prominent nucleolus in a slightly indented nucleus. There is abundant cytoplasm with moderately abundant rough endoplasmic reticulum, numerous mitochondria and multiple processes of up to 5 \(\mu\)m length protruding from the cell membrane. One small lymphocyte (10 \(\mu\)m x 12 \(\mu\)m) appears to be relatively inactive, with a small, condensed, indented nucleus and a narrow rim of cytoplasm containing few mitochondria. There are only a few relatively blunt protrusions from the cell membrane. 3,000x. Bar = 10 \(\mu\)m. Courtesy of Ross Blackley.
Figure 2.2: Electron micrograph of lymphocytes cultivated from peripheral blood mononuclear cells of a patient with Sézary syndrome.

Culture derived from PBMCs of patient 2669 following stimulation with IL2 and IL4. Photograph taken after 28 days in culture. One medium-sized lymphocyte (20 µm x 20 µm) has moderately condensed chromatin in a lobulated nucleus, relatively inconspicuous endoplasmic reticulum and few mitochondria in the cytoplasm. There are multiple processes up to 3 µm in length protruding from the cell membrane. 4,400x. Bar = 5 µm. Courtesy of Ross Blackley.
2.4.3 Reverse transcriptase assay

2.4.3.1 Standard reverse transcriptase assay

Results of RT assays are shown in Tables 2.13 to 2.15. All scintillation counts were taken from the average of two readings. RT assays were performed on 88 cell culture supernatants in 29 pools from 15 patients. Most samples were negative for RNA-dependent DNA polymerase (RT) activity (rA:N ≤ 10) with Mn$^{2+}$ and Mg$^{2+}$ as cofactors and also had negligible DNA-dependent DNA polymerase activity (dA:N ≤ 10) (Table 2.13). A pool of three supernatants collected 23 days after initiation of cultures of PBMCs and skin from patient 436 had an elevated rA:N value of 87.9 with Mg$^{2+}$ as a cofactor. However, cultured cells from this patient did not survive longer than 1 month, the positive supernatants were derived from cultures containing mostly necrotic cells and no RT activity was detected in fresh cultures of PBMCs inoculated with the positive supernatants.

Supernatants from positive control cultures exhibited Mn$^{2+}$ or Mg$^{2+}$-dependent RT activity corresponding to the known properties of the retroviruses with which they were infected (Table 2.14). Consistently high Mn$^{2+}$-dependent RT activity was detected in SSN-1 supernatants (rA/N > 200), whereas this cell line exhibited low Mg$^{2+}$-dependent RT activity (rA/N ≤ 10). A wide variation in Mn$^{2+}$-dependent and Mg$^{2+}$-dependent RT activity was detected in BLV-FLK supernatants among assays, even using supernatants collected on the same date, but the level of Mg$^{2+}$-dependent RT activity was always at least five times the level of Mn$^{2+}$-dependent RT activity. There was an increased level of DNA-dependent DNA polymerase activity in some BLV-FLK supernatants with Mn$^{2+}$ as a cofactor and this correlated with increased rA/N values in the same samples tested on the same date. The FIV-infected cell line FL4 exhibited high Mg$^{2+}$-dependent RT activity (rA/N 1,601.4), consistent with the known properties of lentivirus RTs.

The non-productive HTLV-I-infected CD4$^+$ T cell line C8166, as well as three continuous T cell lines not known to be infected with HTLV-I or other exogenous retroviruses (J.JHAN, L-726A, HH and My-La MC), had low Mn$^{2+}$-dependent and Mg$^{2+}$-dependent RT activity (rA:N ≤ 10) (Table 2.15). Normal tissue culture medium (RPMI 1640 + 20% hi-FBS + 2-ME) did not exhibit RT activity with either Mg$^{2+}$ or Mn$^{2+}$ as cofactors (rA/N ≤ 10).

2.4.3.2 Product-enhanced reverse transcriptase (PERT) assay

All of five samples of supernatants from cell cultures of patients with CTCL had negligible reactivity for Mg$^{2+}$-dependent RT activity and low Mn$^{2+}$-dependent RT activity by ELISA (optical densities at 405 nm and 630 nm, OD$_{405/630}$ < 2.0) in the PERT assay (Böni and Schüpbach 1993, Pyra et al 1994) (Table 2.16).
Table 2.13: Results of reverse transcriptase assay on supernatants from cultured cells of patients with cutaneous lymphoid infiltrates.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Source of supernatants (number pooled)</th>
<th>Date of RT assay</th>
<th>Mn&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>rA (cpm)</td>
<td>dA (cpm)</td>
</tr>
<tr>
<td>436</td>
<td>PBMCs/Skin (3)</td>
<td>13/2/94</td>
<td>966</td>
<td>1469</td>
</tr>
<tr>
<td></td>
<td>PBMCs/Skin (3)</td>
<td>25/2/94</td>
<td>678</td>
<td>481</td>
</tr>
<tr>
<td></td>
<td>PBMCs/Skin (3)</td>
<td>10/3/94</td>
<td>439</td>
<td>891</td>
</tr>
<tr>
<td>1198/2</td>
<td>PBMCs (2)</td>
<td>10/3/94</td>
<td>596</td>
<td>539</td>
</tr>
<tr>
<td>1393/2</td>
<td>PBMCs/Skin (4)</td>
<td>10/3/94</td>
<td>484</td>
<td>973</td>
</tr>
<tr>
<td>2116</td>
<td>PBMCs/Skin (3)</td>
<td>10/2/94</td>
<td>1536</td>
<td>760</td>
</tr>
<tr>
<td>2132</td>
<td>Skin (3)</td>
<td>12/9/94</td>
<td>467</td>
<td>690</td>
</tr>
<tr>
<td>2475</td>
<td>LN (B-LCL) (1)</td>
<td>13/2/94</td>
<td>556</td>
<td>661</td>
</tr>
<tr>
<td>2522</td>
<td>PNMCs (5)</td>
<td>13/2/94</td>
<td>1421</td>
<td>729</td>
</tr>
<tr>
<td>2531</td>
<td>PNMCs (5)</td>
<td>12/9/94</td>
<td>676</td>
<td>668</td>
</tr>
<tr>
<td>2551</td>
<td>PNMCs (1)</td>
<td>14/5/93</td>
<td>1493</td>
<td>2352</td>
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<tr>
<td>2600</td>
<td>Skin (4)</td>
<td>13/2/94</td>
<td>698</td>
<td>576</td>
</tr>
<tr>
<td>2666</td>
<td>LN (1)</td>
<td>10/3/94</td>
<td>743</td>
<td>606</td>
</tr>
<tr>
<td>2669</td>
<td>LN/PNMCs (4)</td>
<td>10/3/94</td>
<td>732</td>
<td>2167</td>
</tr>
<tr>
<td>2746</td>
<td>PBMCs (2)</td>
<td>10/3/94</td>
<td>335</td>
<td>245</td>
</tr>
<tr>
<td>2757</td>
<td>PBMCs (1)</td>
<td>10/3/94</td>
<td>401</td>
<td>807</td>
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Table 2.13: (continued)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>B-LCL</td>
<td>Epstein-Barr virus-infected B lymphoblastoid cell line</td>
</tr>
<tr>
<td>LN</td>
<td>Lymphocyte cultures derived from lymph node cells</td>
</tr>
<tr>
<td>PBMCS</td>
<td>Lymphocyte cultures derived from peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Skin</td>
<td>Lymphocyte cultures derived from skin</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>rA</td>
<td>RNA-dependent DNA polymerase activity (poly rA template)</td>
</tr>
<tr>
<td>dA</td>
<td>DNA-dependent DNA polymerase activity (poly dA template)</td>
</tr>
<tr>
<td>N</td>
<td>No template</td>
</tr>
<tr>
<td>dA/N</td>
<td>dA divided by N</td>
</tr>
<tr>
<td>rA/N</td>
<td>rA divided by N</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Magnesium cofactor</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>Manganese cofactor</td>
</tr>
</tbody>
</table>
Table 2.14: Results of reverse transcriptase assay on supernatants from retrovirus infected cell lines and negative control cell culture medium.

<table>
<thead>
<tr>
<th>Source of test sample</th>
<th>Data of test supernatant collected</th>
<th>Data of RT assay</th>
<th>Mn&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rA (cpm)</td>
<td>dA (cpm)</td>
<td>N (cpm)</td>
</tr>
<tr>
<td>SSN-1</td>
<td>28/5/93</td>
<td>130491</td>
<td>3272</td>
<td>463</td>
</tr>
<tr>
<td></td>
<td>13/2/93</td>
<td>141832</td>
<td>1561</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>28/5/93</td>
<td>129651</td>
<td>3266</td>
<td>550</td>
</tr>
<tr>
<td></td>
<td>10/3/94</td>
<td>81659</td>
<td>463</td>
<td>269</td>
</tr>
<tr>
<td>BLV-FLK</td>
<td>3/1/93</td>
<td>23971</td>
<td>8822</td>
<td>369</td>
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<tr>
<td></td>
<td>3/1/93</td>
<td>1042</td>
<td>410</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>28/5/93</td>
<td>14330</td>
<td>4665</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>28/5/93</td>
<td>3834</td>
<td>2338</td>
<td>534</td>
</tr>
<tr>
<td></td>
<td>10/3/94</td>
<td>19360</td>
<td>1100</td>
<td>161</td>
</tr>
<tr>
<td>FL4</td>
<td>20/5/93</td>
<td>181125</td>
<td>69436</td>
<td>2243</td>
</tr>
<tr>
<td>RPMI</td>
<td>22/1/94</td>
<td>821</td>
<td>1422</td>
<td>613</td>
</tr>
</tbody>
</table>

SSN-1: Striped snakehead fish mixed cell line infected with stripehead fish retrovirus (Mn<sup>2+</sup>-dependent RT activity)
BLV-FLK: Fetal bovine kidney fibroblast cell line infected with bovine leukaemia virus (Mg<sup>2+</sup>-dependent RT activity)
FL4: Feline T cell line infected with Petaluma strain of feline immunodeficiency virus (Mg<sup>2+</sup>-dependent RT activity)
RPMI: RPMI 1640 medium with 20% fetal bovine serum + 2-mercaptoethanol

RT: Reverse transcriptase
cpm: Counts per minute
rA: RNA-dependent DNA polymerase activity (poly-rA template)
dA: DNA-dependent DNA polymerase activity (poly-dA template)
N: No template
dA/N: dA divided by N
rA/N: rA divided by N
Mn<sup>2+</sup>: Magnesium cofactor
Mg<sup>2+</sup>: Manganese cofactor
Table 2.15: Results of reverse transcriptase assay on supernatants from established T cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Date supernant collected</th>
<th>Date of RT assay</th>
<th>Mn$^{2+}$</th>
<th>Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>rA (cpm)</td>
<td>dA (cpm)</td>
</tr>
<tr>
<td>C8166</td>
<td>14/2/94</td>
<td>25/2/94</td>
<td>543</td>
<td>969</td>
</tr>
<tr>
<td>J.JHAN</td>
<td>9/4/93</td>
<td>14/5/94</td>
<td>3042</td>
<td>1627</td>
</tr>
<tr>
<td></td>
<td>14/2/94</td>
<td>25/2/94</td>
<td>1313</td>
<td>37547</td>
</tr>
<tr>
<td>L-726A</td>
<td>15/10/92</td>
<td>2/2/94</td>
<td>569</td>
<td>26946</td>
</tr>
<tr>
<td></td>
<td>15/10/92</td>
<td>2/2/94</td>
<td>384</td>
<td>6496</td>
</tr>
<tr>
<td>HH</td>
<td>14/2/94</td>
<td>25/2/94</td>
<td>981</td>
<td>2572</td>
</tr>
<tr>
<td>My-La MC</td>
<td>14/2/94</td>
<td>25/2/94</td>
<td>1480</td>
<td>1820</td>
</tr>
</tbody>
</table>

C8166: IL2-independent non-productive HTLV-I-infected CD4$^+$ T cell line (Salahuddin et al 1983)
J.JHAN: IL2-independent CD4$^+$ T cell line from patient with T cell acute lymphoblastic leukaemia (Schneider et al 1977)
L-726A: IL2-independent CD4$^+$ T cell line from patient with Sézary syndrome (Crae et al 1991)
HH: IL2-independent CD4$^+$ T cell line from patient with cutaneous T cell lymphoma (Staerkbaum et al 1991)
My-La MC: IL2 and IL4-dependent CD4$^+$ T cell line from patient with mycosis fungoides (Katzoff et al 1992)

RT: Reverse transcriptase
cpm: Counts per minute
rA: RNA-dependent DNA polymerase activity (poly-A template)
dA: DNA-dependent DNA polymerase activity (poly-A template)
N: No template
dA/N: dA divided by N
rA/N: rA divided by N
Mg$^{2+}$: Magnesium cofactor
Mn$^{2+}$: Manganese cofactor
Table 2.16: Results of product-enhanced reverse transcriptase (PERT) assay.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical diagnosis</th>
<th>Source of supernatant</th>
<th>Optical density (OD$_{405/630}$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mn$^{2+}$</td>
</tr>
<tr>
<td>1198</td>
<td>SS</td>
<td>Skin</td>
<td>1.7</td>
</tr>
<tr>
<td>2522</td>
<td>CTCL</td>
<td>PBMCs</td>
<td>1.9</td>
</tr>
<tr>
<td>2531</td>
<td>MF</td>
<td>PBMCs</td>
<td>0.0</td>
</tr>
<tr>
<td>2600</td>
<td>MF</td>
<td>Skin</td>
<td>1.7</td>
</tr>
<tr>
<td>2669</td>
<td>SS</td>
<td>PBMCs</td>
<td>1.7</td>
</tr>
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</table>
2.5 Discussion

In the cell biology studies conducted at the LRF Virus Centre from 1992 to 1995, attempts were made to establish continuous CD4⁺ T cell lines from patients with cutaneous lymphoid infiltrates, particularly MF and SS. The rationale for this approach was that HTLV-I had been isolated from continuous CD4⁺ T cell lines established from patients with ATL and other diseases associated with HTLV-I (Poiesz et al. 1980a, b, Miyoshi et al. 1980, Hinuma et al. 1981, Poiesz et al. 1981, Jacobson et al. 1988). T cell lines from patients with other forms of CD4⁺ T cell neoplasia, particularly MF and SS, might also propagate HTLV-I or related retroviruses, allowing a viral aetiology to be established for these conditions. However, in this study no CD4⁺ T cell lines were established from 158 cultures initiated from PBMCs, skin and lymph nodes of 18 patients (Tables 2.10 and 2.11). Most cells from clinical samples of patients with MF/SS, as well as cells from patients with other cutaneous lymphoid infiltrates, died within 3 months of initiation of cultures. This relatively short duration of survival is consistent with the findings of several other groups studying MF/SS (Capesius et al. 1991, Bazarbachi et al. 1997) (Section 1.11.1.4). The observation that the longevity of some lymphocyte cultures in vitro is increased in the presence of fibroblasts is also consistent with the findings of other groups (Scott et al. 1990).

In contrast to the findings in this study, Abrams et al. (1991b) established 25 T cell lines from patients with SS using SAF CM. GMCSF and IL2 were used as growth stimulants by Zucker-Franklin et al. (1991) to establish 18 cell lines from patients with MF/SS but the immunophenotype of these cell lines was not reported. Few studies that have identified HTLV-related retroviruses in cell cultures from patients with CTCL have confirmed that the propagated cells are of T cell lineage (Kaltoft et al. 1987, 1988); most of the cell lines from CTCL patients in which there is evidence for infection with a retrovirus are B cell lines (Manzari et al. 1987, Hall et al. 1991) or are uncharacterised (Zucker-Franklin et al. 1991, 1992). In an HTLV-II-infected cell line established by Zucker-Franklin et al. (1992) from a patient with MF, 80% of cells were CD2⁺ and only 50% were CD4⁺ after 71 days in culture, so this culture may contain a mixed population of cells.

HTLV-II has been isolated from CD8⁺ T cell lines established from patients with T cell variants of HCL, but its role as an aetiological agent in T cell or other neoplasia has not been confirmed (Kalyanaraman et al. 1982b) (Section 1.7.1). The techniques used in this study were also suitable for cultivating CD8⁺ T cells and four long-term CD8⁺ T cell cultures were established from skin samples of three patients with MF. However, these cultures were not derived from neoplastic cells, which in all MF/SS cases consisted of CD4⁺ T cells. Instead, the CD8⁺ T cells would have expanded from TILs accompanying the neoplastic infiltrate. Other studies have found that most T cell clones cultured from MF lesions are not derived from neoplastic MF/Sézary cells but originate from TILs (Ho et al. 1990, Harwix et al. 2001). Depletion of CD8⁺ T cells (TILs) may permit the outgrowth of neoplastic CD4⁺ T cell clones from MF lesions (Kaltoft et al. 1984). This approach was used frequently at the LRF Virus Centre from 1987 to 1991 but did not result in the establishment of any continuous CD4⁺ T cell lines.
EM, RT assays and PCR were used for detection of retroviruses in cultured cells in this study. No retroviruses were detected by EM in four continuous CD8⁺ T cell lines established from three patients with MF or in short term cell cultures from five other patients with MF or SS. RT activity was detected in one pool of supernatants from degenerating cells of one patient, but this result could not be reproduced because all cultured cells from this patient died within one month. The results of PCR analyses conducted on cultured cells are presented in Chapter 3.

In this study one EBV-infected B-LCL was established from the skin of a patient with CBCL, consistent with the in vitro transforming properties of EBV (Pattengale et al 1973). The B-LCL is more likely to be derived by EBV infection of bystander lymphocytes than from neoplastic B cells, since most primary CBCLs are not associated with EBV, except in immunocompromised patients (Dupin et al 1997, Beylot-Barry et al 1999, Nagore et al 2000). However, detailed molecular genetic, viral and immunophenotypic analyses were not undertaken on the B-LCL or CBCL in this case.

Mycoplasma infection was detected in several cell cultures established from clinical samples and was though to be derived from infected source material rather than from contamination in the laboratory.

The results of these studies and the use of cell culture for isolation of novel retroviruses are discussed further in Chapter 5.
Chapter 3

Use of Molecular Techniques to Detect Human T Lymphotropic Virus-Related Retroviruses in Human T Cell Leukaemias and Lymphomas

3.1 Introduction

3.1.1 Molecular approach to detection of human T lymphotropic viruses in T cell neoplasms

The aim of the molecular biology part of this project was to detect proviral sequences of human T lymphotropic virus type I (HTLV-I), HTLV-II or related retroviruses in human T cell neoplasms using the polymerase chain reaction (PCR) in combination with Southern blot (SB) hybridisation. HTLV-I causes adult T cell leukaemia/lymphoma (ATL) (Section 1.6.1), whereas HTLV-II has not been confirmed as the cause of neoplasia (Section 1.7.1). The hypothesis tested in this study was that HTLV-I, HTLV-II or related retroviruses are associated with other T cell leukaemias and lymphomas, particularly mycosis fungoides (MF) and Sézary syndrome (SS), in the United Kingdom (UK). These neoplasms have similar clinicopathological features to some forms of ATL (Section 1.11.1). However, the prevalence of HTLV-I and HTLV-II infection in the UK is low, except in populations derived from endemic regions (Section 1.5.2.1). Cases of large granular lymphocytic (LGL) leukaemia were also studied because HTLV-I and HTLV-II have been identified in a small proportion of cases of this disease (Section 1.11.2).

Bovine leukaemia virus (BLV) causes B cell leukaemia and lymphoma in cattle and is endemic in the UK (Chasey et al 1978) (Section 1.9.2.1). Although BLV is not known to be a zoonosis, samples from cases of acute lymphoblastic leukaemia (ALL) in butchers from Cardiff were examined for this virus because of their occupational exposure to cattle (Whittaker 1991) (Section 1.10.5). Samples were also tested for HTLV-I and HTLV-II because these viruses are related to BLV.

The molecular techniques used in attempts to identify HTLV-I and related retroviruses in samples from patients with cutaneous T cell lymphoma (CTCL), LGL leukaemia and adult ALL at the Leukaemia Research Fund (LRF) Virus Centre are described in this chapter. PCRs were designed to amplify HTLV-I, HTLV-I/II and BLV proviral DNA sequences from clinical samples and cultured cells from these patients. PCR products were hybridized to HTLV-I or BLV-specific nucleotide probes and products of interest were cloned and sequenced. Results of these studies were used to assess the role of HTLV/BLV group viruses in selected cases of CTCL, LGL leukaemia and adult ALL in the UK.
3.1.2 Detection of retroviral sequences in T cell neoplasms by the polymerase chain reaction

PCR is a method for amplifying specific DNA sequences from target DNA using primer extension and a thermostable DNA polymerase (Saiki et al 1985, Mullis and Faloona 1987, Saiki et al 1988). Successive cycles of template denaturation, primer annealing and extension result in exponential amplification of target sequences depending on the efficiency of reaction conditions. The technique is highly sensitive and can detect low copy numbers of target DNA sequences. Thus, it is the tool of choice for detecting integrated proviruses in samples in which infected cells may be rare. The frequency of infected peripheral blood mononuclear cells (PBMCs) in HTLV-I carriers may be low (Section 1.6.3.3). Skin lesions from patients with MF/SS and other CTCLs often have low densities of neoplastic lymphocytes, which form part of a population including non-neoplastic tumour infiltrating lymphocytes (TILs) and other inflammatory cells, as well as keratinocytes, fibroblasts, endothelial cells, melanocytes and other cutaneous cell populations.

The sensitivity of detection can be increased by nested PCR, in which a second round of reactions is performed using primer sets specific for sequences within the first round PCR product. SB hybridisation using sequence-specific nucleotide probes that bind to products amplified by PCR can also be used to increase the sensitivity of detection. The stringency of hybridisation can be adjusted to allow detection of sequences with high or low similarity to that of the probe. Hybridisation under conditions of high stringency provides a measure of confirmation of the specificity of product amplification. Hybridisation under conditions of low stringency can be used to detect target sequences that are similar but not identical to the sequence of the probe and could allow detection of novel retroviruses. PCR is also a potentially useful tool for detecting novel retroviral sequences, since primers can be selected from conserved regions of known viruses (Section 3.1.2.3).

In this study, careful attention was paid to preparation of clinical samples, design of PCR primers, standardisation and optimisation of PCRs, determining the sensitivity of detection of target sequences and preventing PCR contamination.

3.2 Sources of clinical samples

Details of patients from whom clinical samples were obtained are given in Section 2.2. The methods for processing clinical samples and cultured cells are described in Chapter 2.

3.2.1 Cutaneous T cell lymphomas and other cutaneous lymphoid infiltrates

Samples of cultured and uncultured PBMCs and skin from 36 patients with cutaneous lymphoid infiltrates, predominantly MF and SS, were examined by PCR for HTLV-I and HTLV-I/II sequences (Tables 2.3 and 3.1).
Table 3.1: Clinical diagnoses of patients with cutaneous lymphoid infiltrates from whom samples were analysed by the polymerase chain reaction.

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycosis fungoides</td>
<td>21</td>
</tr>
<tr>
<td>Sézary syndrome</td>
<td>5</td>
</tr>
<tr>
<td>Cutaneous T cell lymphoma</td>
<td>1</td>
</tr>
<tr>
<td>Angiocentric T cell lymphoma</td>
<td>3</td>
</tr>
<tr>
<td>Small plaque parapsoriasis</td>
<td>1</td>
</tr>
<tr>
<td>Langerhan's cell histiocytosis</td>
<td>1</td>
</tr>
<tr>
<td>Cutaneous B cell lymphoma</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
</tr>
</tbody>
</table>
3.2.2 Large granular lymphocytic leukaemia

Cultured and uncultured PBMCs from six patients with CD3⁺ CD8⁺ LGL leukaemia were tested for HTLV-I and HTLV-I/II sequences by PCR (Table 2.4).

3.2.3 Adult acute lymphoblastic leukaemia in butchers from Cardiff

PBMCs and bone marrow from six patients with ALL, all male butchers from Cardiff, were tested for HTLV-I, HTLV-I/II and BLV sequences by PCR (Table 2.5).

3.3 Materials and methods

3.3.1 Molecular biology facilities

The molecular biology laboratory in the LRF Virus Centre was used for gel electrophoresis of PCR products and restriction endonuclease (RE)-digested DNA, Southern blotting and electrophotography, molecular cloning, purification of plasmids and most other molecular work. A separate room was used for bacterial culture work and a dark room was used for ultraviolet (UV) transillumination, photography and exposing autoradiographic film. Radioisotopes were handled in a dedicated radioactivity room in the Department of Veterinary Pathology.

3.3.2 Materials

The sources of frequently used materials are listed in Appendix 3 and the compositions of routinely used stock solutions and buffers for molecular biology are listed in Appendix 5. Additional materials and chemicals are specified elsewhere in the text of individual sections. All chemicals used were of Analytical (Analar) or Molecular Biology grade and, unless otherwise stated, were obtained from Sigma or BDH. Most restriction and DNA modifying enzymes were obtained from Life Technologies. Taq polymerase (Amplitaq) and other PCR reagents were obtained from Perkin-Elmer. Ultrapure deionised water obtained from a reverse osmosis filtration system (Millipore) was used in enzymatic manipulations and to dissolve DNA. PCR grade sterile ultrapure water (Sigma) was used for PCRs. Deionised water (Millipore) was used in other general laboratory solutions.

3.3.3 Extraction of high molecular weight DNA from tissues and cells

3.3.3.1 Enzymatic digestion of tissues and cells

High molecular weight (HMW) DNA was extracted in class II microbiological safety cabinets (MSCs) in the level II containment facility (Section 2.3.1). Cells for DNA extraction were collected from established cell lines (Section 2.3.4), cultured clinical samples (Section 2.3.6) and uncultured PBMCs and lymph node cells. Purification of uncultured PBMCs from blood by metrizoate-Ficoll density gradient centrifugation is described in Section 2.3.5.1. The densities of cells in suspension were determined by counting using a haemocytometer (Section 2.3.3.3). Aliquots containing 1 x 10⁶ to 1 x 10⁸ cells were transferred to 15 ml screw-
top polypropylene tubes and centrifuged at 1,600 g (2,700 rpm) in a GPR bench centrifuge (Beckman) with a GH 3.7 swinging bucket rotor for 5 min at room temperature. The supernatant was discarded, cells were resuspended in phosphate-buffered saline (PBS) 1x and the centrifugation was repeated. Cell pellets were stored at -80°C until processed for PCR. Pellets of fresh PBMCs and cultured cells were resuspended in Tris-NaCl-EDTA (TNE) 1x then centrifuged at 270 g for 5 min. The supernatant from each pellet was discarded and the cells were resuspended in 5 ml TNE 1x containing 100 μg/ml proteinase K, 0.2 M NaCl and 0.5% sodium dodecyl sulphate (SDS). The mixture was incubated at 55°C for 1 hour or at 37°C overnight (16 to 20 hours).

Preparation of tissues for DNA extraction is described in Section 2.3.5.2. Frozen tissue was cut into small pieces with sterile scissors, placed in sterile plastic bags with 5 to 25 ml of TNE 1x and mechanically disrupted using a Stomacher 80 (Colworth). The contents were transferred to 15 or 50 ml screw-top polypropylene centrifuge tubes and incubated with proteinase K, NaCl and SDS as above.

3.3.3.2 Phenol-chloroform extraction

After SDS-proteinase K digestion, protein and lipids were separated from dissolved DNA by phenol-chloroform extraction. An equal volume of phenol equilibrated with Tris pH 8.0 (Rathburn Chemical Company) was added to each tube at room temperature and the tube was gently inverted to mix the aqueous and organic phases. Tubes were centrifuged at 484 g (2,000 rpm) for 10 min in a JS-21 centrifuge (Beckman) using a JA-20 fixed angle rotor to separate the aqueous and organic phases. The upper aqueous phase containing dissolved DNA was transferred to a fresh tube using a wide-bore polypropylene bulb pastette to prevent shearing. Phenol extraction was performed twice then followed by extraction with chloroform or isoamyl alcohol:chloroform (24:1) by the same method.

3.3.3.3 Precipitation and dissolution of DNA

After chloroform extraction, the aqueous phase was transferred by pastette into approximately 2.5 times the volume of 99.7% ethanol (absolute alcohol) to precipitate DNA. HMW DNA, which formed a flocculent suspension, was spooled onto a polypropylene pastette or sealed glass pipette and transferred to a sterile 1.5 ml screw-top polypropylene microcentrifuge tube. If the concentration was too low for the precipitate to be visualised, the ethanol solution was transferred to a 30 ml glass centrifuge tube (Corex, Serstedt) and centrifuged at 12,100 g (10,000 rpm) for 30 min in the JS-21 centrifuge using a JA-20 rotor. DNA pellets were washed in 1 ml 70% ethanol and air dried at room temperature. The DNA was dissolved in an appropriate volume of ultrapure water or Tris-EDTA (TE) 1x by incubating at 37°C overnight on a roller mixer.

3.3.3.4 Quantification of genomic DNA

The concentration of double-stranded genomic DNA was determined by measuring the optical densities at 260 nm (OD\textsubscript{260}) and 280 nm (OD\textsubscript{280}) in a spectrophotometer.
(GeneQuant). Calculations were made on the basis that 50 pg/ml of double-stranded DNA has an OD$_{260}$ of approximately 1.0. The ratio of the readings at 260 nm and 280 nm (OD$_{260}$/OD$_{280}$) was used to estimate the purity of the nucleic acid; pure preparations of DNA have an OD$_{260}$/OD$_{280}$ ratio of 1.6 to 1.8 (Sambrook et al 1989).

3.3.3.5 Restriction endonuclease digestion of genomic DNA

Genomic DNA was digested with restriction endonucleases (REs) for gel electrophoresis and Southern blot hybridisation (Section 3.3.10). RE digests were performed with 10 μg HMW DNA incubated with 30 U of the appropriate enzyme (Life Technologies) in a 50 μl volume containing proprietary buffer at 1x concentration and 3 mM spermidine (GeneBloc, International Laboratory Services). To prevent inhibition by glycerol in RE storage buffers, the volume of RE solutions added was kept below 10% of the total reaction volume. Reactions were incubated at 37°C overnight. DNA from the C8166 cell line was digested with EcoRI, PstI, SstI, SstI/HindIII, SstI/XhoI and SstI/BamHI for assessment of proviral integrations by SB hybridisation (Section 3.3.11.2).

3.3.4 Processing of cells for the polymerase chain reaction

3.3.4.1 Preparation of cells for PCR

Cell pellets for PCR were prepared by diluting cultured and uncultured cells to 1 x 10$^5$ cells/ml in PBS 1x in the level II containment facility (Sections 2.3.3.3 and 2.3.5.1). An appropriate number of 0.5 ml aliquots containing 1 x 10$^5$ cells were transferred to 0.6 ml flip-top PCR tubes and centrifuged at 10,500 g (13,000 rpm) for 5 min in a bench microcentrifuge (MicroCentaur, MSE). Supernatants were discarded, leaving the pelleted cells in the bottom of each tube. These cell pellets were stored frozen at -80°C until used for PCR analysis. Some cryopreserved cells were prepared for PCR by thawing as described in Section 2.3.3.5. After centrifuging and washing cells with PBS 1x, the density of viable cells was determined (Section 2.3.3.3) and pellets of 1 x 10$^5$ cells for PCR were prepared as above. Serial dilutions of positive and negative control cells for use in determining PCR sensitivity are described in Section 3.3.8.2.

3.3.4.2 Non-ionic detergent lysis and proteinase K digestion of cells for PCR

To disrupt cells and inactivate proteins in preparation for PCR, 25 μl of non-ionic detergent buffer (NIB) consisting of 1.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris pH 8.2, 100 μg/ml gelatin, 0.45% Nonidet P-40 (NP-40), 0.45% Tween-20 and 60 ng/μl proteinase K were added to pellets of 1 x 10$^6$ cells in PCR tubes in the LRF Virus Centre level II containment facility (Appendix 5: A5.6). The buffer was overlain with two crops (approximately 25 μl) of mineral oil (Sigma) using a polypropylene pastette. Tubes were sealed with parafilm (Nesco sealing film, Bando) or placed in a locking rack (Treff) and incubated at 55°C for 1 hour in a heated water bath (Techne). The proteinase K was inactivated by heating at 95°C in a thermal block (Techne) or boiling for 10 min. Tubes containing lysed cells were then placed on ice and 25 μl of PCR master mix were added in preparation for thermal cycling.
3.3.5 Design of oligonucleotide primers for the polymerase chain reaction

3.3.5.1 Principles of primer selection

Sets of oligonucleotide primers for PCR were designed for annealing to specific sequences of target DNA to act as primers for incorporation of oligonucleotides by Taq DNA polymerase. Forward (sense) primers allowed extension from 5' to 3' on the negative strand of DNA, while complementary reverse (antisense) primers allowed extension from 5' to 3' on the positive strand. The size of the intervening sequence for efficient amplification by PCR was between 100 and 400 base pairs (bp). Primer sets for PCR were selected with a preference for oligonucleotides of length 17 to 21 bases with similar melting temperatures ($T_m$) within the range 55 to 65°C. Strings of identical nucleotides were avoided, as were excesses of G and C residues. The sequence at the 3' end of each primer was considered most important for specificity of annealing and a G or C was selected for the 3' terminal residue. Primers were checked for the absence of complementary sequences that might permit self-annealing and amplification of non-specific products.

Theoretical values for annealing temperatures of oligonucleotide primers can be determined using formulae for calculating the $T_m$ of double-stranded DNA. The following formula is applicable to oligonucleotides of 11 to 23 bases, which includes most PCR primers used in this study (Suggs et al. 1981):

\[
T_m = 4(G+C) + 2(A+T) \, ^\circ C
\]

Where: $A$, $C$, $G$ and $T$ are the number of bases of each nucleotide in the primer. Primers with degenerate bases such as K, M, R or Y were assumed to have been synthesised with an equal proportion of purine and pyrimidine residues and their number was multiplied by three (Appendix 2).

This formula was used to compare the $T_m$ values of forward and reverse primers in a set, with the aim to select primers with similar annealing temperatures. Formula 3.2 was used to calculate the $T_m$ of the 29 base primer 3 (Section 3.3.12.1):

\[
T_m = 81.5 + 16.6 \log_{10}[M^+] + 0.41(G+C) - 500/L - P - 0.63(\% \text{Formamide}) \, ^\circ C
\]

Where:
- $[M^+]$ = Adjusted molar Monovalent cation concentration: $\frac{1 + 0.7 [Na^+]}{[Na^+]}
- \%G+C = $ Percentage of G and C nucleotides in the DNA
- $L =$ Length of the DNA:DNA duplex in bp
- $P =$ Percentage base mismatch
However, such theoretical values do not reliably predict the compatibility of any pair of primers. Similarly, although an annealing temperature 5°C below the calculated $T_m$ of the primers is often used as a guide to selecting PCR thermal cycling conditions, it is usually necessary to determine the optimal annealing temperature empirically (Section 3.3.7.3).

3.3.5.2 Design of primers for amplification of HTLV-I, HTLV-II and BLV by PCR

Primer sets for amplification of HTLV-I, HTLV-II and BLV sequences by PCR were designed at the LRF Virus Centre based on alignments of viral sequences and on previously published information (Appendices 6 to 8). Incomplete proviruses with preferential retention of the $pX$ region are frequent in neoplastic cells of patients with ATL (Section 1.6.1.4) and have been suspected in patients with MF/SS (Section 1.11.1.6). The possibility that proviruses with deletions are involved in the aetiology of CTCLs was addressed by selecting PCR primers from the LTR, $gag$, $pol$, $env$ and $pX$ regions of the HTLV-I genome (Section 1.3.3). Use of the full panel of primer sets on any infected sample would allow detection of some viral sequences by PCR even if other regions of the proviral genome were absent due to deletions. The use of primer sets with different sensitivities could lead to false deductions that incomplete proviruses are present and therefore it was important to optimise and quantify the sensitivity of PCR reactions.

Published sets of primers for PCR that reliably amplified HTLV-I $pol$ and HTLV-II $pX$ sequences were used as standards for detection of HTLV-I and HTLV-II (Kwok et al 1988). LTR, $gag$ and $pX$ primers that had been used to detect defective HTLV-I proviruses in patients with MF were adopted from Hall et al (1991). Additional HTLV-I $gag$ primers were those used to detect variant (Melanesian strain) HTLV-I in Papua New Guinea (Sherman et al 1992). Primers for $env$ had previously been used in attempts to detect HTLV-I sequences by PCR in patients with multiple sclerosis (MS) (Greenberg et al 1989a) (Section 1.6.4.6). Two additional sets of unpublished HTLV-I $pol$ and $pX$ PCR primers that had been used at the LRF Virus Centre previously were also included in the study (primer sets 3 & 4 and 67 & 68). Primers for amplification of BLV $gag$ sequences were adapted from a published study (Murtaugh et al 1991).

Amplification of a sequence of $\beta$-globin was used as a test for the integrity of DNA (Saiki et al 1988) (Appendix 9). These PCR primer sets are shown in Figure 3.1. Accepted letter codes for nucleotides and amino acids, including nomenclature for incompletely specified bases, are listed in Appendix 2.

3.3.5.3 Design of consensus retroviral PCR primers

There is a high degree of sequence divergence over most of the genome of known retroviruses and thus few regions are available with a high degree of similarity from which consensus PCR primers can be selected. However, regions of nucleotide conservation in the retroviral $pol$ gene and amino acid conservation in the translated product of this gene, reverse transcriptase (RT), offer potential for the design of consensus primers that can be used for detection of novel retroviruses. Consensus PCR primers with generic specificity for the HTLV/BLV (Deltaretrovirus) group were derived by alignment of regions of similarity.
among the HTLV-I, HTLV-II and BLV virus LTR and *gag* nucleotide sequences (Figure 3.2). Alignments of sequences in the GenEMBL database were made using the Genetics Computer Group (GCG) programmes on the University of Glasgow VAX and UNIX mainframe computers. Consensus primers were selected from these regions of homology on the assumption that they are conserved in other, as yet unidentified, HTLV-related viruses. Where regions of homology did not provide for a perfect match of bases among the viruses, degenerate primers were used. This resulted in the identification of one LTR sense primer (174) that could be used in conjunction with either of two antisense primers to yield PCR products of 491 to 522 bp (LTR primer 175) or 1823 to 1858 bp (gag primer 176) from HTLV/BLV group viruses (Figure 3.2).

Consensus primers with potential to detect a wide range of retroviruses and hepadnaviruses were also designed by alignment and reverse translation of Pol amino acid sequences (Mack and Sninsky *et al* 1988, Donehawer *et al* 1990). Two regions of conservation in the retroviral RT protein have been identified that are sufficiently close together for amplification of a product of suitable size by PCR (Appendix 10: A10.1). The number of permutations of nucleotide codons for the invariant amino acids in these regions is relatively small, facilitating design of degenerate primers. The 5' conserved region contains the motif LPQG, which is consistent among exogenous retroviruses except for the substitution of R for G in simian retrovirus 1 (SRV-1). Group-specific 5' primers can be derived from a 9 amino acid segment in this region with consistent LPQ and SP residues. The 3' conserved amino acid sequence contains the motifs YMDD or YVDD. The regions between these primer sequences differ among the retroviruses and can be used for specific identification by hybridisation, nested PCR or sequencing.

A potential problem with retroviral consensus primers is that they will bind to endogenous retroviral sequences, resulting in the generation of PCR products that are difficult to distinguish from those of exogenous retroviruses. This can be overcome by the use of RT-PCR to detect viral genomic RNA in purified virions or mRNA transcripts in infected cells. Donehawer *et al* (1990) used RT-PCR with two degenerate oligonucleotide primers to amplify a 117 bp *pol* fragment from genomic RNA of all retroviruses examined.

Consensus primers adapted from Mack and Sninsky (1988), Donehawer *et al* (1990) and designed by aligning retroviral sequences selected from the GenEMBL database using the GCG programmes are shown in Figure 3.3. These primers (296 & 297) have the potential to amplify a retroviral *pol* sequence of approximately 113 bp. *BglII* restriction sites for cloning were added at the ends of both of these primers, resulting in a potential PCR product of approximately 133 bp.
**Figure 3.1:** Oligonucleotide primers for amplification of HTLV-I, HTLV-II, bovine leukaemia virus (BLV) and β-globin sequences by the polymerase chain reaction.

Letter codes for nucleotides, including nomenclature for incompletely specified bases, are listed in Appendix 2.

<table>
<thead>
<tr>
<th>LRF oligonucleotide number</th>
<th>Primer sequence 5' to 3' with HTLV-I nucleotide position (HTLV-II nucleotide positions in brackets)</th>
<th>Length (bp)</th>
<th>T_m (°C)</th>
<th>Size of PCR product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HTLV-I LTR</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>118 Sense</td>
<td>582 TCGACCCGCTGGTCCTCAGCTTCTA 603</td>
<td>22</td>
<td>66</td>
<td>183</td>
<td>(Hall et al. 1991)</td>
</tr>
<tr>
<td>119 Antisense</td>
<td>764 TCTTTCGCCCGAATCCGATAA 744</td>
<td>21</td>
<td>63</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HTLV-I gag</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>120 Sense</td>
<td>1380 CTGGACCAACCCCTGCTTACGGGC 1411</td>
<td>24</td>
<td>76</td>
<td>273</td>
<td>(Sherman et al. 1992)</td>
</tr>
<tr>
<td>121 Sense</td>
<td>1540 CGCTACACCGGGTAAATCTGG 1561</td>
<td>20</td>
<td>60</td>
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<td></td>
</tr>
<tr>
<td>122 Sense 123 Antisense</td>
<td>1423 CTTCGCCCGGCAATGGATACCC 1444</td>
<td>22</td>
<td>68</td>
<td>134</td>
<td>(Hall et al. 1991)</td>
</tr>
<tr>
<td><strong>HTLV-I pol</strong></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1 Sense</td>
<td>2366 CTGGACCAACCCCTGCTTACGGGC 3305</td>
<td>20</td>
<td>50</td>
<td>119</td>
<td>(Kwok et al. 1988)</td>
</tr>
<tr>
<td>2 Antisense</td>
<td>3466 CGCTACACCGGGTAAATCTGG 3451</td>
<td>19</td>
<td>62</td>
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<td>67 Sense</td>
<td>6958 CTTCGCCCGGCAATGGATACCC 4779</td>
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<td>69</td>
<td>186</td>
<td>Unpublished</td>
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<tr>
<td>68 Antisense</td>
<td>4943 CTTCGCCCGGCAATGGATACCC 4925</td>
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<td>69</td>
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</tr>
<tr>
<td><strong>HTLV-I env</strong></td>
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</tr>
<tr>
<td>124 Sense 125 Antisense</td>
<td>5800 CTGGACCAACCCCTGCTTACGGGC 5619</td>
<td>20</td>
<td>62</td>
<td>127</td>
<td>(Greenberg et al. 1989)</td>
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<tr>
<td><strong>HTLV-II pX</strong></td>
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<td>(7246)</td>
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<td>52</td>
<td>139</td>
<td>(Kwok et al. 1988)</td>
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<tr>
<td>(7406)</td>
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<td>(7386)</td>
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<td>68</td>
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<td>120 Sense 127 Antisense</td>
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<td>(Hall et al. 1991)</td>
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<td>20</td>
<td>58</td>
<td>247</td>
<td>(Murtaugh et al. 1991)</td>
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<td><strong>β-globin</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>18 Sense 52 Antisense</td>
<td>14 ACAAGAACCTGACCTGACG 33</td>
<td>20</td>
<td>58</td>
<td>94</td>
<td>(Saiki et al. 1988)</td>
</tr>
</tbody>
</table>
Figure 3.2: Consensus oligonucleotide primers for amplification of human T cell lymphotropic/bovine leukaemia virus group viral LTR sequences by the polymerase chain reaction.

Letter codes for nucleotides, including nomenclature for incompletely specified bases, are listed in Appendix 2.

<table>
<thead>
<tr>
<th>HTLV/BLV group viruses</th>
<th>Sequence alignment</th>
<th>Length (b)</th>
<th>$T_m$ (°C)</th>
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<td>Sense</td>
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<td>HTLV-I</td>
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<td>HTLV-II</td>
<td>TGGGGGCTCGTCCGGGAT</td>
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<td>BLV</td>
<td>TGGGGGCTCGTCCGGGAT</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>Consensus sense</td>
<td>5’ &gt; TTGGGGGCTCGTCCGGGAT &lt; 3</td>
<td>19</td>
<td>61</td>
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<td>LTR primer 174</td>
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<td></td>
</tr>
<tr>
<td>Antisense</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HTLV-I</td>
<td>AGACCTACAGGCCATTAA</td>
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<td>HTLV-II</td>
<td>AGACCTACAGGCCCATCAA</td>
<td>1286</td>
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<tr>
<td>BLV</td>
<td>AGAATTACAAGATATCAA</td>
<td>1022</td>
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<td>Consensus sequence</td>
<td>5’ &gt; AGDMYTAGGYMTAAYAA &lt; 3’</td>
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<td></td>
</tr>
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<td>Consensus antisense</td>
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<td>LTR primer 175</td>
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<td></td>
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<td>HTLV-I</td>
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<td>2355</td>
<td></td>
</tr>
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<td>Consensus sequence</td>
<td>5’ &gt; CCTTTAAACYWGARCGCCTCCAGGCC &lt; 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consensus antisense</td>
<td>5’ &gt; GGCCTGAGGCGGTWRGTKGATTAAAGG &lt; 3’</td>
<td>26</td>
<td>54</td>
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<tr>
<td>gag primer 176</td>
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<tr>
<td>Expected PCR product sizes</td>
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<tr>
<td>HTLV-I</td>
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<td>BLV</td>
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<td>174 &amp; 176</td>
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<td>BLV</td>
<td>1823</td>
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**Figure 3.3: Consensus reverse transcriptase (pol) primers for amplification of retroviral sequences by the polymerase chain reaction.**

Letter codes for nucleotides, including nomenclature for incompletely specified bases, are listed in Appendix 2.

<table>
<thead>
<tr>
<th>LRF oligonucleotide number</th>
<th>Primer sequence 5' to 3' with HTLV-I nucleotide position</th>
<th>Length (b)</th>
<th>( T_m ) (°C)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus retroviral pol</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LRF II</td>
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<td></td>
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<td>296 Sense</td>
<td>2976 GCCGAGATCTYTNCCCARVG 2985</td>
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<td>51</td>
<td>~133</td>
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<tr>
<td>297 Antisense</td>
<td>3088 GCCGAGATCTTCRTCNAYRTA 3078</td>
<td>21</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>
3.3.6 Preparation of oligonucleotides

3.3.6.1 Oligonucleotide synthesis and purification

Oligonucleotides for use as PCR primers were manufactured on an automated synthesiser and cartridge-purified (Alta Bioscience or Genosys). They were dissolved in ultrapure water and stored external to the Department of Veterinary Pathology to reduce the risk of PCR contamination (Section 3.3.8). An aliquot from each solution was quantified (Section 3.3.6.2) and the remaining volume was diluted to an appropriate concentration (usually 10 μM) after optimisation for PCR (Section 3.3.7.3).

Selected oligonucleotides were produced within the Department of Veterinary Pathology using an automated synthesiser (Model 381A, Applied Biosystems). These were used as primers for PCRs not involving clinical samples, for example for cloning, and for use as probes for hybridisation to PCR products. They were cleaved from the synthesis cartridge by flushing with 2 ml of 30% ammonia over a 2 hour period and then deprotected by incubating at 55°C in the ammonia solution overnight. The solution was divided into aliquots and desiccated by centrifugation under vacuum (Speedvac SC100, Savant) to remove the ammonia before being dissolved in ultrapure water.

3.3.6.2 Quantification of oligonucleotides

Concentrations of oligonucleotides were determined by measuring OD$_{260}$ and OD$_{280}$ in a spectrophotometer (Section 3.3.3.4). Calculations were made on the basis that 30 μg/ml single-stranded DNA has an OD$_{260}$ of approximately 1.0. Pure preparations of single stranded DNA have an OD$_{260}$/OD$_{280}$ ratio of 1.6 to 1.8 (Sambrook et al 1989). The molar concentration was calculated on the basis that 1 pmol of a single-strand of the 4.3 kb plasmid pBR322 contains 1.4 μg DNA.

3.3.7 Polymerase chain reaction

3.3.7.1 Standard PCR master mixes

Standard PCRs were performed in 0.6 ml PCR tubes in 50 μl volumes containing target DNA, 200 μM each deoxynucleoside triphosphates (dATP, dCTP, dGTP and dUTP or dTTP), 50 mM KCl, 10 mM Tris pH 8.2, 0.05% Nonidet P-40 (NP-40), 0.02 U/μl Taq thermostable DNA polymerase (Amplitaq DNA Polymerase, Perkin-Elmer) and PCR grade sterile ultrapure water with optimised concentrations of MgCl₂ and oligonucleotide primers. Most oligonucleotides were used at a final concentration of 0.5 or 1.0 mM after optimisation (Section 3.3.7.3). The range of MgCl₂ concentrations used in PCRs was 1.0 to 3.0 mM, with 1.6 mM being optimal for most primer sets (Section 3.3.7.3). To prevent evaporation during thermal cycling, the reaction was overlaid with two drops (approximately 25 μl) of mineral oil.
PCR master mixes were prepared with concentrations of reagents allowing for the final addition of the DNA template to a total volume of 50 µl. Usually 10 µl of a 100 ng/µl solution of purified DNA (1 µg) were mixed with 40 µl of PCR master mix. In PGRs using cell pellets, 25 µl of PCR master mix with constituents at appropriate concentrations were added to PCR tubes in which 1 x 10^6 cells had been lysed with 25 µl NIB-protease K (Section 3.3.4.2).

3.3.7.2 Standard PCR thermal cycling protocol

PGRs were performed by placing tubes containing reaction mixes in a thermal cycling machine (DNA Thermal Cycler, Perkin-Elmer Cetus) using the following protocol: initial denaturing at 94°C for 7 min; then 40 cycles comprising denaturation at 94°C for 10 sec, cooling at 19.5°C/min over 2 min, annealing at an optimised temperature (usually 55°C) for 10 sec, heating at 17°C/min over 1 min, extension at 72°C for 30 sec, then heating at 22°C/min over 1 min to start the next cycle at 94°C; followed by a final extension at 72°C for 7 min. The reaction was then cooled to 6°C. Samples were stored at 4°C in the molecular biology laboratory unless processed immediately.

3.3.7.3 Optimisation of polymerase chain reaction conditions

Optimal concentrations of oligonucleotide primers and MgCl_2 were determined for each primer set using the standard PCR thermal cycling protocol with annealing at 55°C and a standard quantity (1 µg) of positive control DNA template. C8166 DNA was the positive control for HTLV-I PGRs and BLV-FLK DNA was the positive control for BLV PGRs. Serial two-fold dilutions of primers were prepared to give a range of final concentrations of each primer in PGRs from 4.0 to 0.125 mM. The concentration of MgCl_2 used in standard PGRs was 1.5 mM but some primer sets required different Mg^{2+} concentrations for optimal results. PCR optimisations for Mg^{2+} were performed with 0.5 to 3.0 mM final concentrations of MgCl_2 in grades of 0.5 mM. The composition of other constituents was held constant when varying the primer or MgCl_2 concentrations. Amplified products were separated by electrophoresis on 8% polyacrylamide gels, stained with ethidium bromide and examined by UV transillumination (Section 3.3.9).

Using optimised concentrations of primers and MgCl_2, with all other constituents as standard, PGRs were then performed with a range of annealing temperatures from 50 to 70°C to allow the optimal annealing temperature to be selected. The quantity of template used was 1 µg positive control DNA. Optimal conditions were considered to be those that yielded large amounts of PCR product at the highest possible annealing temperature with a preference for final concentrations of 1.0 to 2.5 mM MgCl_2 and 0.5 to 1.0 µM of each primer in a set. An attempt was also made to select PGR conditions that were relatively robust, for example by not using annealing temperatures or concentrations of MgCl_2 or primers that would lead to a loss of sensitivity if altered only slightly.
The reproducibility of PCR results was confirmed by preparing uniform batches of PCR master mixes containing C8166 DNA with primers for amplification of β-globin and HTLV-I sequences. These PCR master mixes were prepared on the same day and frozen at -20°C. PCR thermal cycling was then performed on different days in the same week using reagents thawed on the day of use. The uniformity of amplification in each well of the thermal cycler was also tested by placing an array of PCR tubes containing aliquots from a common master mix across the thermal cycling block and comparing PCR products by gel electrophoresis. The efficacy of amplification was compared using 200 μM dTTP, 200 μM dUTP or 400 μM dUTP and found to be similar.

3.3.7.4 Sensitivity of polymerase chain reactions

The sensitivity of each PCR primer set was determined by preparing PCR master mixes containing graded concentrations of template. All other reaction constituents, including optimised concentrations of primers and MgCl₂, were held constant for each sensitivity assay (Section 3.3.7.1). A standard PCR programme of 40 thermal cycles was then performed using the optimised annealing temperature for each primer set.

Serial ten-fold dilutions of positive control DNA in negative control DNA were prepared in the molecular biology laboratory to determine the sensitivity of PCRs for detecting template in DNA samples from tissues. C8166 DNA was diluted in DNA extracted from human placenta, reactive lymph node, PBMCs or J.JHAN cells to assess the sensitivity of PCRs for detecting HTLV-I. Serial dilutions of BLV-FLK DNA in FLK DNA were used to determine the sensitivity of BLV PCRs. In a standard serial dilution, 50 μl of 100 ng/μl C8166 DNA were added with a direct displacement pipette to 450 μl of 100 ng/μl J.JHAN DNA in a 1.5 ml tube to give 10% C8166 DNA. This mixture was vortexed thoroughly for 1 min, then 50 μl was transferred to another tube containing 450 μl of 100 ng/μl J.JHAN DNA to give 1% C8166 DNA. The process was repeated to give a series of dilutions containing 10 to 0.001% positive control DNA in negative control DNA. Tubes containing 1 ml 100% C8166 DNA and 1 ml 100% J.JHAN DNA were included in the set. Each PCR tube in the sensitivity assay for a particular set of primers contained 1 μg total DNA in a 50 μl reaction volume, with 1 μg, 100 ng, 1 ng, 0.1 ng, 0.01 ng, 0.001 ng, 0.0001 ng or nil positive control DNA. Sets of tubes prepared in a similar way for each primer set allowed the sensitivities of HTLV-I and BLV PCRs to be determined for DNA templates such as DNA extracted from skin and lymph nodes.

To assess the sensitivity of PCRs for detecting HTLV-I and HTLV-I/II in PBMCs from patients or in cultured cells, tubes containing pellets of 1 x 10⁶ cells were prepared with decreasing proportions of HTLV-I-infected C8166 cells diluted in HTLV-I/II negative PBMCs, UCMCs or J.JHAN cells (Section 2.3.4). Cells were prepared and diluted in the level II containment facility (Section 2.3.1). In a standard serial dilution, 1 ml of C8166 cells at 1 x 10⁶ cells/ml was added to 9 ml of PBMCs at 1 x 10⁵ cells/ml in a 15 ml tube to give 10% C8166 cells. After thorough mixing by inversion, a 1 ml aliquot was removed from the tube and added to a second tube containing 9 ml of PBMCs at 1 x 10⁵ cells/ml to give 1% C8166 cells. This process was repeated to give a panel of seven tubes containing 10, 1, 0.1, 0.01 and 0.001% C8166 cells diluted in PBMCs. Tubes were inverted gently at all stages of
dilution to maintain even suspensions of cells. Two aliquots of 0.5 ml, each containing 0.5 x $10^5$ cells, from each mixture were successively transferred to 0.6 ml PCR tubes, centrifuged at 10,500 g for 10 min in a bench microcentrifuge and the supernatants discarded to produce cell pellets of $1 x 10^5$ cells. In addition, two 0.5 ml aliquots of pure suspensions of $1 x 10^5$ cells/ml C8166 and $1 x 10^5$ cells/ml PBMCs were centrifuged in 0.6 ml PCR tubes to produce pellets of $1 x 10^5$ cells representing 100% C8166 and 100% PBMCs, respectively. These tubes were centrifuged at 10,500 g for 10 min in a bench microcentrifuge and the supernatants were discarded. The cell pellets were stored at -80°C until processed for PCR.

3.3.7.5 Nested polymerase chain reaction

Nested PCRs were performed on some samples to increase sensitivity or to re-amplify PCR products for cloning. Nested primers were designed for annealing to sequences internal to primers 50 & 51 for amplification of first round PCR products of HTLV-I (282 & 283) and HTLV-II (288 & 289) (Figure 3.4). The HTLV-I inner primer set was optimised as a single round PCR on C8166 DNA and amplified the expected product of 136 bp. Even though no HTLV-II positive control was available for the HTLV-II inner primer set, primers 288 & 289 also amplified a product of similar size from C8166, due to sufficient sequence similarity between the two viral sequences. Second round PCRs were performed using the inner sets of primers at 0.5 μM each and 1.5 mM MgCl₂ in a standard PCR reaction with 10 μl of a 1 in 100 dilution of first round product amplified from clinical samples using primers 50 & 51.

3.3.7.6 Consensus retroviral PCR

The consensus HTLV/BLV LTR oligonucleotide primers 174 & 175 and 174 & 176 (Figure 3.2) and consensus retroviral primers 296 & 297 (Figure 3.3) were used in PCRs with standard master mixes and thermal cycling conditions (DNA Thermal Cycler, Perkin-Elmer Cetus) in an attempt to amplify HTLV-I PCR products from pMT2 (Section 3.3.11.2), C8166 and BLV-FLK DNA, with DNA from human placenta and the FLK cell line as negative controls. Serial two-fold dilutions of each primer were made to yield final concentrations of 4.0 to 0.125 μM and this titration was tested with 1 μg C8166 DNA in standard PCR mixes using 1.5 or 2.5 mM MgCl₂. Thermal cycling protocols with annealing temperatures of 55, 60 and 65°C were compared.

Primer sets were also used in a touchdown PCR protocol in which the annealing temperature is changed decrementally with each cycle to increase the likelihood of amplifying sequences with the highest similarity to the oligonucleotide primers (Don et al 1991). The following protocol was used on a 9600 GeneAmp PCR System thermal cycler (Perkin-Elmer): initial denaturing at 94°C (5 min); then 26 cycles with denaturation at 94°C (1 min), annealing initially at 68°C then decreasing by 0.5°C per cycle (2 min) and extension at 72°C (1 min); followed by 14 cycles of denaturation at 94°C (1 min), annealing at 55°C (2 min) and extension at 72°C (1 min); final extension at 72°C for 7 min then the reaction was held at 4°C. Primers 174 & 175 were used at 0.5 μM each with 1.5 and 2.5 mM MgCl₂.
Figure 3.4: Nested oligonucleotide primers for amplification of HTLV-I and HTLV-II pX sequences.

<table>
<thead>
<tr>
<th>LRF oligonucleotide number</th>
<th>Primer sequence 5' to 3' with HTLV-I nucleotide position</th>
<th>Length (b)</th>
<th>T_m (°C)</th>
<th>Size of PCR product (bp)</th>
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</thead>
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<td>289 Antisense</td>
<td>CCATCGATGGGGTCCCAGGT 7391</td>
<td>20</td>
<td>66</td>
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</table>
3.3.8 Prevention of PCR contamination

3.3.8.1 Preparation of PCR reagents

Since PCR is highly sensitive, contamination of reagents with positive control DNA samples, plasmids containing target sequences and previously amplified PCR products can be a source of false positive results. Stringent precautions were taken at the LRF Virus Centre to minimise the possibility of contamination of PCRs during the preparation and analysis of samples. Reagents for PCR were ordered from sources outside the Department of Veterinary Pathology and divided into aliquots of the required volume at an external location by a person who did not work in the Department. These reagents were delivered as required and temporarily stored at -20°C in a dedicated freezer in a room separate from both the level II containment facility and the molecular biology laboratory.

PCR master mixes for use with clinical samples were prepared in a designated room separate from other buildings in the Department of Veterinary Pathology and not used for any other purpose. Pipettes, pipette tips, PCR tubes, gloves and other equipment were dedicated to this room. PCR master mixes were prepared at the start of the day before all other laboratory work. A new disposable surgical gown, face mask, surgical cap and two pairs of gloves were worn while handling reagents and tubes. Positive (direct) displacement pipettes or air displacement pipettes (Gilson) with plugged tips were used during the preparation of solutions. Gloves were changed between each sample. Reagents and tubes were placed on ice in disposable foam buckets during the preparation of PCR master mixes. Master mixes were then transported on ice to the level II containment facility.

3.3.8.2 Preparation of clinical samples for PCR

The level II containment facility was used for handling all clinical samples, including processing of PBMCs and tissues, cultivation of cells, extraction of DNA and preparation of cell pellets for PCR. PCR master mixes were combined with DNA samples or added to tubes containing NIB-proteinase K-disrupted cell pellets in this laboratory (Section 3.3.4.2). Cell cultures infected with HTLV-I (C8166) or BLV (BLV-FLK) for use as positive controls were grown in the level II containment facility but were always handled in a separate class II MSC (Section 2.3.1.2). Aliquots of cells from these cell lines, including serial dilutions of positive control cells, were prepared in the same MSC (MSC 1). The use of bench centrifuges was common to all cell culture work in the laboratory. Extractions of DNA from positive control cells were performed in the molecular biology laboratory.

3.3.8.3 Positive and negative controls

PCR tubes containing water in place of DNA or disrupted cells were processed in parallel with all clinical samples to act as negative controls. Control tubes were opened and closed at the same time as their matching sample tubes but care was taken to ensure that the clinical sample was not itself a source of contamination.
DNA from virus negative cell lines and cell pellets containing PBMCs from people in the Department of Veterinary Pathology were included in each batch of PCRs as additional negative controls.

Sealed PCR sample and control tubes were transferred a room adjacent to the level II containment facility for thermal cycling. Positive control samples were added to PCR master mixes in the molecular biology laboratory and placed in the thermal cycler after all tubes containing clinical samples had been handled. After completion of the PCR thermal cycle, tubes were transferred to the molecular biology laboratory to be opened for gel electrophoresis, electroblotting and hybridisation of PCR products.

3.3.8.4 Substitution of dUTP for dTTP in PCR reactions

Deoxyuridine triphosphate (dUTP) was substituted for deoxythymidine triphosphate (dTTP) in PCR nucleotide mixes as a further precaution against contamination. Uracil (U) was thus incorporated into PCR products instead of thymidine (T) and contamination could be reduced or eliminated by adding the enzyme uracil N-glycosylase (UNG) (Cambio or Life Technologies) to PCR master mixes to eliminate carry-over PCR products (Longo et al. 1990). The protocol for prevention of PCR contamination using UNG was to add 0.25 U of enzyme per 100 μl reaction volume of selected PCR mixes prior to amplification. The mixture was incubated at 37°C for 1 hour to break down any U-containing products. The initial 94°C denaturation step in the standard PCR (Section 3.3.7.2) was increased from 7 to 10 min to inactivate the enzyme. Trials using UNG in the molecular biology laboratory showed that it eliminated most potential contamination unless large quantities of PCR products were used.

3.3.9 Polyacrylamide gel electrophoresis and electroblotting of polymerase chain reaction products

3.3.9.1 Polyacrylamide gel electrophoresis

PCR products less than 500 bp were analysed by polyacrylamide gel electrophoresis (PAGE). After removal from the thermal cycling machine, PCR tubes were opened in the molecular biology laboratory and 8 μl of PCR product from each tube were mixed with 2 μl of gel loading buffer/dye 5x. Glycerol in the buffer acted as a carrier for the DNA. Xylene cyanole and bromophenol blue dyes were indicators of electrophoretic migration. Fine pipette tips were used to load the 10 μl mixture into wells of 8% polyacrylamide gels (Appendix 5: A5.6.1) that had been prepared between 10.0 cm x 7.2 cm x 0.1 cm glass plates with a comb inserted at the top to create the wells. A DNA molecular weight (MW) marker, 400 ng φX174/HaeIII (Life Technologies), was loaded on each gel as a size reference. PCR products were separated by gel electrophoresis using a Mini-Protean II gel electrophoresis apparatus (Bio-Rad) for 1 hour at 120 mV (17 mV/cm) with power supplied by a standard electrophoresis unit (Pharmacia). Double-stranded DNA PCR products, as well as single-stranded DNA such as unused oligonucleotide primers, which carry a net negative charge, migrated towards the anode. TBE 1x was used as the buffer in the gel electrophoresis tank.
After electrophoresis, gels were removed from between the glass plates and stained with 0.5 mg/ml ethidium bromide in deionised water for 6 min. Ethidium bromide is a fluorescent agent that intercalates double-stranded DNA. PCR products were viewed using a UV transilluminator (TC-254A, Spectrolite or 3-3504 Foto/Prep I, Fotodyne) while wearing a face shield and photographed with a Polaroid MP4 Land camera using Polaroid Type 57 high speed (3,000 ASA/36 DIN) film (Genetic Research Instrumentation).

3.3.9.2 Electroblotting of PCR products

PCR products separated by PAGE were transferred to 0.45 μm nylon membrane (Hybond-N, Amersham) by electroblotting in preparation for hybridisation to sequence-specific probes. Polyacrylamide gels were immersed in alkali buffer for 8 min to denature double-stranded DNA, neutralising buffer (pH 8.0) for 8 min, then TAE 1x for 5 min (Appendix 5: A5.7). Each gel was positioned onto a sheet of nylon membrane moistened with TAE 1x, then sandwiched between two sheets of moistened blotting paper (No. 3, Whatman or GB003, Schleicher & Schuell) and placed in a rack between two gauze pads to maintain apposition. The rack was inserted into the electrode unit of a mini-electroblotting apparatus (Transblot cell, Bio-Rad), the tank was part-filled with TAE 1x and a frozen ice container was added to keep the buffer cool. Applying 15 V for 1 hour from an electrophoresis power unit transferred DNA from the gel to the nylon membrane, while a magnetic stirrer was used to circulate the buffer and prevent overheating. After electroblotting, the apparatus was dismantled and the nylon membrane exposed to 120 J/cm² UV light for 48 sec (Stratalinker 1800, Stratagene) to fix the DNA to the substrate by cross-linking.

3.3.9.3 Purification of PCR products from polyacrylamide gels

PCR products to be cloned were purified from polyacrylamide gels by excising ethidium bromide-stained fluorescent bands with a clean scalpel blade while examining gels on a UV transilluminator. Excess polyacrylamide was trimmed and the gel slice was cut into small pieces, then placed in a 1.5 ml tube containing an appropriate volume of elution buffer (usually 50 μl) containing 0.5 M ammonium acetate and 1 mM EDTA (Appendix 5: A5.6.1). The tube was incubated at 37°C for 1 hour then centrifuged at 11,600 g (13,000 rpm) in a microcentrifuge. The supernatant was transferred to a fresh 1.5 ml tube and the eluted DNA was precipitated by adding 2.5 volumes (125 μl) of absolute alcohol and holding at -20°C for 2 hours. A carrier such as glycogen (Boehringer Mannheim) was sometimes added to the solution at 20 ng/ml to enhance precipitation of DNA present at low concentration.

After centrifugation, the precipitated DNA was washed in 70% ethanol and air dried, then dissolved in a small volume (10 to 20 μl) of ultrapure water or TE 1x. The concentration of DNA was estimated by electrophoresis of 2 to 5 μl on a 1 or 2% agarose gel next to a MW marker or another DNA sample of known concentration.
3.3.10 Agarose gel electrophoresis and Southern blotting

3.3.10.1 Agarose gel electrophoresis

Electrophoresis of DNA in 1 or 2% agarose gels was used for visualisation of PCR products of 500 bp or more, visualisation of plasmids and products of RE digestion and purification of DNA bands for cloning. Agarose was dissolved in TBE 1x by boiling, allowed to cool to 50 to 60°C, then poured into a Perspex tray and allowed to set at room temperature with a comb to form wells. Ethidium bromide was added to the gel during preparation at a concentration of 5 to 10 μg/ml. The gel was immersed in TBE 1x in an electrophoresis tank (BRL) and the comb was removed. An appropriate volume of gel loading buffer/dye 5x was added to each DNA sample and the mixture was pipetted into the wells. A voltage of 1 V/cm was applied across the electrodes from an electrophoresis power unit. λHindIII (Life Technologies) or αX174/HaeIII were used as molecular weight markers. The DNA was visualised on a UV transilluminator and gels were photographed as required.

HMW DNA samples that had been digested with REs for SB hybridisation (Section 3.3.3.5) were separated by electrophoresis in a 0.8% agarose gel overnight at 1 V/cm. The gel was immersed in 0.5 mg/ml ethidium bromide on a rocking tray for 30 to 60 min to stain the DNA and then placed in deionised water for 30 to 60 min to destain the background. A photograph of the gel was taken under UV transillumination with a ruler adjacent to the MW marker. This photograph provided a reference for estimating the sizes of positive bands that might be revealed following SB hybridisation (Sections 3.3.12). Test samples included C8166 DNA and DNA from patients with neoplasia. Since negative control tissues were not available from the same patients, placental DNA samples digested with the same REs were usually electrophoresed in parallel with each test sample to provide a reference for hybridisation.

3.3.10.2 Southern blotting

RE-digested DNA for hybridisation was transferred to 0.45 μm nylon membrane (Hybond-N, Amersham) using a modified Southern blotting procedure (Southern 1975). After 0.8% agarose gel electrophoresis, the DNA was denatured by immersing the gel in alkali buffer for 1 hour at room temperature. The gel was then submerged in neutralising buffer for 1 hour and finally placed in sodium saline citrate (SSC 10x) transfer buffer for 30 min (Appendix 5: A5.7). Layers of absorbent paper towels were placed in the bottom of a tray and covered with several layers of thick blotting paper (No. 3, Whatman or GB003, Schleicher & Schuell) followed by thin blotting paper (No. 1, Whatman). A piece of nylon membrane that had been cut to the same size as the gel and rinsed with SSC 10x was placed on the blotting paper. The denatured and neutralised gel was laid on top of this membrane and covered with several layers of thin blotting paper that had been moistened with SSC 10x. Care was taken to exclude bubbles. A sponge soaked with SSC 10x was placed over the top layer of blotting paper. The tray and its contents were sealed inside a plastic bag to reduce evaporation during blotting. This system used gravity-assisted capillary action to transfer the DNA downwards onto the nylon membrane.
After overnight blotting, the nylon membrane was rinsed with SSC 3x and exposed to UV light to cross-link the DNA to the substrate (Section 3.3.9.2).

3.3.10.3 Purification of DNA from agarose gels

PCR products to be cloned and inserts to be excised from plasmids by RE digestion for subcloning were purified from agarose gels using diethylaminoethyl (DEAE) membrane (Schleicher & Schuell) or a liquefactive enzyme (Gelase, Cambio). Migrating fragments were visualised by UV transillumination following gel electrophoresis. Where possible, bands were illuminated using a long wavelength (356 nm) hand-held UV light source (UVGL-58 Mineralight Lamp, UVP) or a UV transilluminator set to preparative mode (3-3504 Foto/Prep I, Fotodyne). Purification with DEAE membrane was performed by placing a piece of membrane into a slit cut in the agarose gel on the anode side of a DNA fragment of interest, then the gel was returned to the electrophoresis tank and 1 V/cm was applied for 10 min. When the DNA had migrated onto the DEAE membrane, the membrane was removed, trimmed, placed in 50 µl of elution buffer in a 1.5 ml tube and incubated at 65°C for 1 hour (Appendix 5: A5.6.1). The tube was centrifuged at 11,600 g for 10 min in a microcentrifuge and the supernatant transferred to a fresh tube. After adding 2.5 volumes (125 µl) of absolute alcohol, the tube was held at -20°C for 2 hours to precipitate the eluted DNA. A carrier such as transfer RNA (tRNA) was sometimes added. After centrifugation, the precipitated DNA was washed in 70% ethanol and air dried, then dissolved in a small volume of ultrapure water or TE 1x. The concentration of DNA was estimated by electrophoresis of an aliquot from this volume in parallel with a DNA sample of known concentration (usually serial dilutions of the 3.0 kb plasmid pUC8).

DNA to be purified from agarose gels using Gelase was separated by electrophoresis on a 1% low melting point agarose gel in TAE 1x. The fragment of interest was excised from the gel with a scalpel blade, placed in a 1.5 ml tube and weighed. The Gelase enzyme solution (1 U/ml) was added to the gel slice at 1 U (1 ml) per 100 g agarose. The tube was incubated at 70°C for 20 min or 45°C for 1 hour until the agarose was liquefied, then the liquid was transferred to a 30 ml glass centrifuge tube (Corex). DNA was precipitated by adding a 0.1 times volume of 5 M ammonium acetate, holding at room temperature for 30 min, then centrifuging at 12,100 g for 30 min at room temperature in a JS-21 centrifuge using a JA-20 rotor. After washing with 70% ethanol and drying, the DNA was dissolved in ultrapure water or TE 1x and the concentration was estimated by electrophoresis.

3.3.11 Preparation of radiolabelled probes

3.3.11.1 Applications of radiolabelled probes

Hybridisation using radiolabelled probes was employed as a method of increasing the sensitivity of detection and confirming the specificity of PCRs for HTLV-I and BLV. The stringency of hybridisation was adjusted according to the requirements for sensitivity and specificity. Radiolabelled probes were hybridised to DNA that had been separated by gel electrophoresis, blotted onto nylon membranes and cross-linked (Sections 3.3.9 and 3.3.10).
Probes were either linear double-stranded plasmid inserts radiolabelled with $\alpha^{32}$P dCTP by random priming (Section 3.3.11.4) or synthetic oligonucleotides that had been 5' end-labelled with $\gamma^{32}$P ATP (Section 3.3.11.5) and purified by Sephadex column filtration (Section 3.3.11.6).

3.3.11.2 Selection and design of probes

The 11.7 kb plasmid pMT2 contains a full-length molecular clone of the HTLV-I provirus from the cell line MT2 cloned into the 2.7 kb vector pUC (Miyoshi et al 1981a, b, Seiki et al 1982) (Section 2.1.2.1). pMT2 was grown in *Escherichia coli* (E coli) DH5α. A 9 kb viral insert, designated pMT2i, was excised from purified plasmid by RE digestion using SstI (Section 3.3.13.4). SstI cuts pMT2 in the HTLV-I LTR and the 9 kb insert is nearly full-length. pMT2i was labelled with $\alpha^{32}$P dCTP by random priming (Section 3.3.11.4) and used as a probe for hybridisation to HTLV-I and HTLV-II PCR products and to SBs of RE-digested genomic DNA from C8166 cells. The same probe was also used for hybridisation to RNA blots (Section 4.2.3.2). HTLV-I and HTLV-II have 60% sequence similarity and pMT2i has been used as a probe for detection of HTLV-II sequences amplified by PCR (Shaw et al 1984) (Section 1.5.3.1); in this study primers were used that amplified HTLV-I and HTLV-II sequences from the pX region, which is more highly conserved (Figure 3.1).

Three plasmids containing 2.4, 1.1 and 1.9 kb PstI fragments from pMT2 ligated into the 4.3 kb vector pBR322 were also used as probes for hybridisation to SBs of RE-digested genomic DNA from C8166 cells. These plasmids were designated pMT2-1 (6.7 kb), pMT2-2 (5.4 kb) and pMT2-3 (6.2 kb) and were specific for the pol-env, pol, and gag-env regions, respectively. Hybridisation studies with these probes were used to verify the HTLV-I provirus pattern in C8166, consistent with the findings of Bhat et al (1993) (Section 2.3.4.1). The probe used for hybridisation to BLV PCR products was a $\gamma^{32}$P ATP 5' end-labelled synthetic oligonucleotide (Section 3.3.11.5) (Figure 3.5).

3.3.11.3 Procedures for handling radioactive substances

Radioisotopes for labelling probes for hybridisation were handled in the radioactivity room in the Department of Veterinary Pathology with precautions to prevent radioactive contamination. A radioactivity badge was worn and all surfaces and equipment were checked with a Geiger counter before, during and after handling radioisotopes. All manipulations were made behind a Perspex shield wearing double gloves. Tubes containing radioactive solutions were stored in containers with appropriate shielding and were held in Perspex blocks during manipulations. Direct displacement or plugged pipettes were used for transfer of radioactive solutions. Absorbent blotting paper with a plastic under-surface (Bench-cote) was used in case of spillage of radioactive material. Reusable equipment that had been in contact with radioactive substances was soaked in decontaminating solution (Decon). Surfaces suspected of being contaminated with radioactivity were also cleaned with this solution. Radioactive waste was placed in designated containers for disposal and solutions were flushed down a dedicated sink for containment in a holding tank. The disposition of all radioisotopes was recorded.
Figure 3.5: Oligonucleotide probe for hybridisation to polymerase chain reaction products generated using bovine leukaemia virus primers.

<table>
<thead>
<tr>
<th>LRF oligonucleotide number</th>
<th>Oligonucleotide sequence 5' to 3' with nucleotide position</th>
<th>Length (bp)</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLV gag</td>
<td>AGGACTACGGARCCCTCTGCRAGTTTC 2765</td>
<td>30</td>
<td>48</td>
</tr>
</tbody>
</table>

| BLV gag                    | AGGACTACGGARCCCTCTGCRAGTTTC 2765                         | 30         | 48         |
3.3.11.4 Radiolabelling of linear double-stranded plasmid inserts by random priming

Linear double-stranded DNA inserts purified from plasmids following RE digestion (Section 3.3.13.4) were radiolabelled with $^{32}$P dCTP by random priming to produce radioactive oligonucleotide probes for hybridisation. Double-stranded inserts were denatured by boiling 25 ng linear DNA in 25 µl water for 5 min in a 1.5 ml tube then cooling the tube on ice to maintain separation of DNA strands. Solutions added to this volume for random priming were 10 µl of buffer containing dATP, dGTP and dTTP, 5 µl of primer solution containing random hexanucleotides, 2 µl of enzyme solution containing 1 U/µl DNA polymerase I Klenow fragment (Multiprime Kit, Amersham) and 8 µl (2.96 MBq) of $^{32}$P dCTP with a specific activity of 30 TBq/mmol (R82075, Amersham or Redivue, Amersham) (Appendix 5: A5.8.1). The random priming reaction was allowed to proceed for 4 hours at room temperature (R82075, Amersham) or 30 min at 37°C (Redivue, Amersham). The radiolabelled probe was purified by Sephadex column filtration (Section 3.3.11.6).

3.3.11.5 Radiolabelling of oligonucleotide probes by 5' end-labelling

The oligonucleotide probe for hybridisation to BLV PCR products was synthesised in the Department of Veterinary Pathology (Section 3.3.6.1). The 5' end-labelling reaction mix contained 70 pmol synthetic oligonucleotide, 20 U T4 polynucleotide kinase, T4 kinase buffer 1x (50 mM Tris pH 7.5, 10 mM MgCl$_2$, 5 mM dithiothreitol (DTT), 100 µM spermidine, 100 µM EDTA pH 8.0), and 70 pmol $^{32}$P ATP (ICN) (specific activity 111 MBq/mmol) in a total volume of 100 µl (Appendix 5: A5.8.2). The reaction mix was incubated at 37°C for 1 hour then the radiolabelled probe was purified by Sephadex column filtration (Section 3.3.11.6).

3.3.11.6 Purification of radiolabelled probes by Sephadex column filtration

Radioactive oligonucleotide probes generated by random priming and 5' end-labelling were purified from unincorporated nucleotides by column filtration. Washed Sephadex beads (Sephadex-G50, Pharmacia) were pipetted into a glass column and the column was equilibrated with TE 1x. Pre-packed disposable Sephadex columns (Sephadex G-50 NICK column, Pharmacia) were also used. The 50 µl solutions containing products of random priming or 100 µl solutions containing products of 5' end-labelling were pipetted into the top of the equilibrated column and flushed into the Sephadex with TE 1x. Orange-G dye was added as a marker for elution of unincorporated nucleotides.

The descent of radioactivity was monitored with a Geiger counter. The first peak of radioactivity eluted from the column contained the $^{32}$P-labelled DNA probe. This was collected into a 1.5 ml tube for addition to hybridisation solutions. Unincorporated nucleotides were eluted as a second peak of radioactivity and discarded by flushing down the liquid radioactive waste sink.
3.3.12 Hybridisation of radiolabelled probes to membrane-bound DNA

3.3.12.1 Principles of hybridisation

Pairing of complementary bases in single-stranded DNA molecules results in the formation of DNA:DNA duplexes with a strength of association that depends on the degree of sequence similarity (percentage base mismatch), nucleotide composition, length of sequence, temperature and chemical properties of the solution in which hybridisation takes place. The temperature of hybridisation, composition of hybridisation solutions and stringency of washing can be adjusted according to the predicted properties of probes used for hybridisation. The melting temperature ($T_m$) is a measure of the stability of nucleotide association and for DNA:DNA duplexes can be calculated using formula 3.2 (McConaughy et al 1969, Wetmur 1991) (Section 3.3.5.1). The $T_m$ for hybridisation of DNA probes in solution to target DNA immobilised on nylon membranes is usually slightly lower than the calculated value (Beltz et al 1983). Hybridisations are performed at 20 to 25°C below the calculated $T_m$ to maximise the rate of DNA:DNA association and for aqueous solutions this is usually 60 to 70°C. Washing of blots in aqueous solutions to remove unbound and weakly bound probes is usually conducted at 5 to 25°C below the calculated $T_m$. The effective $T_m$ can be manipulated by adding formamide to the hybridisation solution; $T_m$ decreases by 0.63% for every 1% increase in formamide (Howley et al 1979).

In this study, hybridisations using pMT2| were carried out in standard hybridisation solution containing 50% formamide with SSC 3x ($[\text{Na}^+]=0.59\,\text{M}, \text{thus } [\text{M}^-]=0.42$) (Appendix 5: A5.9). Probes produced by random priming have an average length of 100 bp and thus a value $L=100$ was used for pMT2i (Section 3.3.11.4). The full-length 9,068 bp HTLV-I sequence has 53.8% G+C (2,093 A, 3,165 C, 1,720 G, 2,090 T) (Appendix 6). Assuming a 100% match with a PCR product with a similar %G+C, the calculated $T_m$ for randomly primed pMT2i is 61°C. Hybridisation using pMT2i was routinely performed at 37°C, which is 24°C below the calculated $T_m$. Hybridisations with oligonucleotide probes are usually performed at 5 to 10°C below the calculated $T_m$, since the empirically determined $T_m$ is often up to 10°C higher. The synthetic oligonucleotide (301) used as a BLV probe in this study had 30 bases and 50% G+C (Figure 3.5). This probe was hybridised in standard hybridisation solution containing SSC 3x with 50% formamide (Appendix 5: A5.9) at 37°C; the calculated $T_m$ was 48°C.

3.3.12.2 Prehybridisation

Prehybridisation reduces non-specific binding of radiolabelled probes to nylon membranes during hybridisation. Hybridisation solution was prepared with 50% formamide and SSC 3x to achieve an effective $T_m$ 5 to 10°C below the calculated $T_m$ of the probe, as discussed above (Section 3.3.12.1). This solution was used for both prehybridisation and, following addition of radiolabelled probe, for hybridisation. Nylon membranes with bound DNA were moistened with SSC 3x, 0.1% SDS and inserted into 50 ml screw-top polypropylene tubes with care taken to prevent the formation of bubbles.
Membranes were prehybridised with 2.5 ml hybridisation solution by continuous rotation at 37°C in a hybridisation oven (Hybridiser HB-1, Techne) for at least 4 hours. Alternatively, nylon membranes were placed in glass hybridisation tubes (Techne) with a larger volume of hybridisation solution (10 to 20 ml) depending on the size of the tube and surface area of membrane.

3.3.12.3 Hybridisation

The randomly primed α-32P dCTP radiolabelled probe pMT2 (Section 3.3.11.4) was boiled for 5 min and cooled on ice to denature any DNA duplexes. Usually 25 µl of radiolabelled probe was added to 2.5 ml of the 50% formamide, SSC 3x hybridisation solution that had been used for prehybridisation to yield approximately 1 x 10^6 counts per ml. Nylon membranes were then hybridised with this buffer by rotating the hybridisation tubes in an oven at 37°C overnight. The oligonucleotide probe for BLV was added to a hybridisation solution of the same composition to approximately 1 x 10^6 counts per ml and hybridised overnight at 37°C.

3.3.12.4 Washing blots to remove unbound probe

After overnight hybridisation, the hybridisation solution was removed and disposed of according to normal radioactivity procedures. The nylon membranes were initially washed twice at low stringency by filling the tubes with SSC 2x, 0.1% SDS and rotating in the hybridisation oven at room temperature for five min per wash. Subsequent washes were carried out under high stringency conditions. Usually, this consisted of immersing membranes in SSC 0.5x, 0.1% SDS in a sealed plastic container and washing twice at 65°C for 30 min per wash on an oscillating tray in a heated water bath (Techne). When a SB hybridisation was to be repeated, the nylon membrane was stripped of probe by agitating in 0.1% SDS at 85°C for 30 min then changing the solution and continuing for a further 10 min.

3.3.12.5 Autoradiography

After the final high stringency wash, each nylon membrane was wrapped in plastic (Saran Wrap, Dow, Genetic Research Instrumentation) and exposed to X-ray film (Hyperfilm-MP, Amersham) in an autoradiograph cassette with an intensifying screen under dark room conditions. Cassettes were held at -80°C during exposure and autoradiographs were developed in an automatic processor in the Department of Veterinary Pathology after exposures of 1 to 21 days.

3.3.13 Manipulation of bacteria

3.3.13.1 Growth and storage of bacteria

Strains of bacteria used for most molecular biology purposes were *E coli* HB101 or DH5α. These were grown at 37°C on Luria-Bertani (LB) agar in Petri dishes in a standard incubator or in flasks containing LB broth in an orbital incubator (Fisons) at 225 rpm (Appendix 5:
A5.10.1. Antibiotics were added according to the resistance genes expressed by plasmids within the bacteria. Ampicillin (50 μg/ml) was used for growth of *E. coli* containing pMT2 and TA cloning vectors (Section 3.3.16.2) (Appendix 5: A5.10.2). Tetracycline (12 μg/ml) was used for growth of *E. coli* containing pMT2-1, pMT2-2 and pMT2-3 (Section 3.3.11.2).

Stocks of viable bacteria were prepared by mixing 1 ml of bacteria in suspension from overnight LB broth cultures with 500 μl sterile 80% glycerol in cryopreservation tubes (Cryotubes, Nunc). Cryotubes were frozen in liquid nitrogen (liqN₂) or a mixture of dry ice and absolute alcohol then transferred to a freezer for long-term storage at -80°C. Unwanted bacterial cultures and supernatants were sterilised by autoclaving or inactivated with disinfectant solutions (sodium dichloroisocyanurate, Presept, Surgikos).

3.3.13.2 Transformation of chemically competent bacteria with plasmid DNA

Plasmids are circular double-stranded DNA molecules that replicate in bacteria and are widely used for manipulation of DNA. Plasmids with antibiotic resistance genes can be used for selection of cloned DNA inserts. Transformation is the method by which plasmid DNA is introduced into bacteria. Competent bacteria (Life Technologies) were stored at -80°C and thawed on ice when required for transformation. Volumes of 50 μl were placed in 15 ml polypropylene tubes (2059, Falcon) on ice and 10 to 100 ng of plasmid DNA was added to each tube. The mixture was held on ice for 10 min, then the tubes were placed in a 42°C water bath for 45 sec to induce passage of plasmid DNA across the bacterial cell wall. After the addition of 900 μl of LB broth or SOC, tubes were incubated in an orbital incubator at 37°C for 1 hour to allow expression of plasmid-encoded antibiotic resistance genes. Aliquots of 50 to 200 μl bacteria were spread onto LB agar plates containing the appropriate antibiotic. These plates were incubated at 37°C overnight to allow the selective growth of transformed bacteria.

3.3.13.3 Transformation of bacteria by electroporation

Bacteria were grown at 37°C in 200 ml LB broth containing antibiotics for approximately 6 hours to an OD₆₀₀ of 0.5 to 0.7 (Hanahan 1983). The cultures were centrifuged at 3,740 g (4,500 rpm) for 10 min in the JS-21 centrifuge using a JS-7.5 rotor at 4°C. After removing the supernatant, bacteria were centrifuged and resuspended four times in decreasing volumes (400, 200, 20 and 1 ml) of sterile 10% glycerol in water. Aliquots of 100 μl were stored at -80°C. Bacteria were thawed at room temperature, placed on ice and 40 μl of chilled bacterial suspension was mixed with 1 to 2 μl (5 to 10 ng) plasmid DNA in a 1.5 ml tube on ice. This volume was transferred to an ice cold sterile 0.2 cm electroporation cuvette (Bio-Rad), placed in a chilled Teflon block and electroporated with a voltage of 2.5 kV, charge transfer of 2.4 C (25 μF), resistance of 200 Ω and time constant between 4 and 5 sec (Gene Pulsger and Pulse Controller, Bio-Rad). Following electroporation, 1 ml of LB broth was added to the cuvette and transferred to a 15 ml tube (2059) for incubation at 37°C for 1 hour at 225 rpm in an orbital incubator. Bacteria were then spread on LB agar containing the appropriate antibiotic and incubated at 37°C overnight.
3.3.13.4 Selection and growth of transformed bacteria

A sterile flamed loop was used to pick single colonies of transformed bacteria that had grown on selective agar plates after overnight incubation. Colonies were inoculated into 15 ml tubes (2059) containing 5 ml LB broth with an appropriate antibiotic and incubated at 37°C in an orbital incubator at 225 rpm overnight. Small scale preparations of plasmids were made from these cultures (Section 3.3.14.1). Large scale plasmid preparations (Sections 3.3.14.2 and 3.3.14.3) were made by transferring 5 ml of overnight broth culture into flasks containing 500 ml of LB broth and an appropriate antibiotic. These flasks were incubated overnight in an orbital incubator at 37°C. Chloramphenicol (25 µg/ml) was added to overnight cultures containing tetracycline (12 µg/ml) to maximise expression of pMT2, pMT2-1, pMT2-2 and pMT2-3 in E. coli DH5α for large scale plasmid purification.

3.3.14 Purification of plasmid DNA

3.3.14.1 Small scale purification of plasmid DNA

Small scale preparations of plasmid DNA for RE digestion and screening for inserts by gel electrophoresis were prepared by modified alkaline lysis or boiling methods or by column purification. One or two 1.5 ml aliquots of overnight cultures of bacteria in LB broth containing antibiotics were centrifuged in 1.5 ml tubes at 11,600 g in a microcentrifuge for 5 min. Supernatants were removed, leaving a pellet of bacteria in the bottom of each tube.

Bacteria to be disrupted by alkaline lysis were resuspended by vortexing with 100 µl of Solution I (Birnboim and Doly 1979) (Appendix 5: A5.11.1). This suspension was mixed with 200 µl Solution II, which contained 0.2 M NaOH and 1% SDS to lyse cells, then was vortexed with 150 µl Solution III, containing 3 M potassium acetate to precipitate bacterial proteins and genomic DNA. The tubes were centrifuged at 11,600 g for 5 min in a microcentrifuge and the supernatants, containing plasmid DNA, were transferred to fresh tubes. Extractions with 1 ml phenol and 1 ml chloroform were each followed by centrifugation and transfer of supernatants to fresh tubes. Plasmid DNA was precipitated by the addition of 2.5 volumes of absolute alcohol followed by centrifugation and washing in 70% ethanol. The DNA pellet was allowed to dry then dissolved in 30 to 50 µl ultrapure water or TE 1x. Plasmid DNA was visualised by electrophoresis of 10 µl on an agarose gel (Section 3.3.10.1) and RE digests were prepared to check for the presence of inserts by gel electrophoresis (Section 3.3.14.4).

Bacteria were harvested from LB broth cultures by centrifugation in 1.5 ml tubes for extraction of plasmid DNA by the boiling method. After removing the supernatant, pelleted bacteria were vortexed with 150 µl sucrose-Triton X-100-EDTA-Tris (STET) solution and 10 µl 10 µg/ml lysozyme (Boehringer Mannheim) to disrupt cell walls (Appendix 5: A5.11.2). Tubes were boiled for 40 sec, placed on ice for 5 min and then centrifuged at 11,600 g for 10 min. The glutinous pellet that formed in each tube was removed by pipetting with a wide-bore micropipette tip and discarded. The plasmid DNA was precipitated by adding 100 µl of isopropanol and placing the tubes at -20°C for 15 to 30 min. Tubes were centrifuged at 11,600 g for 5 min, the supernatant was removed and the precipitated DNA was washed with
1 ml 70% ethanol. The tubes were allowed to dry and the plasmid DNA was dissolved in 30 to 50 µl ultrapure water or TE 1x as above. RNase A was added at a final concentration of 50 µg/ml in preparation for gel electrophoresis.

Plasmids were column purified for RE digestion and for sequencing using resin columns (Wizard minipreps DNA purification system, Promega). LB broth cultures of bacteria were centrifuged (1.5 ml twice) in 1.5 ml microcentrifuge tubes at 11,600 g for 5 min and the bacterial pellet was resuspended in 200 µl cell suspension solution. Bacteria were disrupted with 200 µl cell lysis solution then treated with 200 µl neutralisation solution. After centrifuging at 11,600 g for 10 min the supernatant was transferred to a fresh tube and mixed with 1 ml DNA purification resin. This volume was pipetted into a 5 ml syringe attached to a column inserted into a vacuum manifold. A vacuum was applied to filter the liquid through the column then 2 ml column wash solution was added and drawn through under vacuum. The columns were dried by continued vacuum for 2 min then centrifugation for 1 min. DNA was dissolved by adding 50 µl water at 70°C for 5 to 15 min then eluted from the column by centrifuging at 11,600 g for 1 min.

3.3.14.2 Large scale crude extraction of plasmid DNA

Flasks containing 500 ml overnight bacterial cultures were cooled on ice and the contents transferred to 250 ml screw-top centrifuge tubes (Beckman) on ice. The cultures were centrifuged at 9,060 g (7,000 rpm) in the JS-21 centrifuge using a JS-7.5 rotor (Beckman) for 10 min at 4°C. The supernatant was discarded, the bacteria were resuspended in 100 ml TNE 1x and the centrifugation was repeated. The bacterial pellet was resuspended in 4 ml 0.5% lysozyme solution and allowed to stand at room temperature for 5 min to disrupt bacterial cell walls. The DNA was denatured by adding 16 ml of alkaline lysis solution, vortexing and holding on ice for 10 min. The solution was neutralised with 12 ml of potassium acetate solution pH 4.8 and placed on ice for a further 10 min to disrupt plasmid strands to re-anneal. The lysed bacteria were centrifuged at 43,700 g (19,000 rpm) for 30 min at room temperature in the JS-21 centrifuge using a JA-20 rotor. The supernatant was divided between two 30 ml glass centrifuge tubes (Corex). Plasmid DNA was precipitated by adding 0.6 volumes of isopropanol followed by thorough mixing. The precipitate was centrifuged at 12,100 g (10,000 rpm) for 30 min at room temperature in the JS-21 centrifuge using a JA-20 rotor. The supernatant was removed and 5 ml of 70% ethanol was added to the pellet to remove salt from the precipitated plasmid DNA. This solution was centrifuged at 12,100 g for 15 min at room temperature. The pellet was allowed to dry for a maximum of 10 min and then dissolved in 5 ml of TE 1x buffer.

3.3.14.3 Large scale purification of plasmid DNA by caesium chloride density gradient centrifugation

The crude extract of plasmid DNA in 5 ml TE 1x (Section 3.3.13.2) was prepared for density gradient centrifugation by adding 1 g/ml CsCl and 740 mg/ml ethidium bromide. The mixture was transferred to 5 ml ultracentrifuge tubes (Quick-Seal, Beckman), which were balanced, heat sealed and centrifuged overnight at 49,000 rpm in an L-8 60M ultracentrifuge.
(Beckman) using a Vti 65.2 rotor at 20°C. The lower band of supercoiled plasmid DNA was visualised using a hand-held ultraviolet lamp (UVGL-58, UVP) in the dark room. This band was removed using a needle and syringe and was diluted with TE 1x to a volume of 5 ml. Ethidium bromide was removed from the solution by repeated mixing with water-saturated isobutanol. When the aqueous phase was colourless, the plasmid DNA was precipitated by adding 2.5 volumes of absolute alcohol and holding the solution at -20°C for 2 hours. The tubes were centrifuged at 12,100 \( g \) for 30 min in the JS-21 centrifuge using a JA-20 rotor at 4°C. Salt was removed by washing with 70% ethanol then the DNA pellet was allowed to dry before being dissolved in 50 to 200 \( \mu l \) ultrapure water or TE 1x.

### 3.3.14.4 Restriction endonuclease digestion of plasmid DNA

Plasmids were digested with 5 U of the appropriate RE per mg of plasmid DNA in a solution containing proprietary buffer (Life Technologies) at 1x concentration. The most frequently used REs were BamHI, EcoRI, HindIII, PstI, SstI and XbaI (Appendix 5: A5.3.1). Reactions were incubated at 37°C for 1 to 3 hours. If two REs were required to excise a plasmid insert, both enzymes were added to the same solution if they had similar activities in the same buffer. If buffer requirements were substantially different, the enzymes were used in succession and the plasmid DNA was precipitated with ethanol and washed with 70% ethanol to remove salts between each digest. RNase A was sometimes added to RE digests at a final concentration of 50 \( \mu g/ml \) to remove RNA contamination. Inserts were separated from RE-digested plasmids by agarose gel electrophoresis (Section 3.3.10.1) and bands were purified using DEAE membrane or Gelase (Section 3.3.10.3).

### 3.3.15 Subcloning plasmid inserts

Subcloning was used to transfer a double-stranded segment of insert DNA from one plasmid to another. Plasmid DNA was purified by agarose gel electrophoresis using DEAE membrane (Section 3.3.10.3). Supercoiled plasmid DNA migrated most rapidly, followed by linear and nicked forms. Inserts were excised from plasmids by digestion with one or two REs followed by gel electrophoresis and gel purification (Section 3.3.14.4). A recipient plasmid vector was prepared by digestion with the same REs and gel-purified similarly. The insert and recipient plasmid were mixed at an appropriate molar ratio (usually insert:plasmid 3:1) and ligated in a solution containing T4 DNA ligase and ligation buffer 1x in a total volume of 10 to 40 \( \mu l \). Purified plasmid or other DNA that had been dissolved in solutions with a low concentration of salt was concentrated by mixing an aqueous solution with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of absolute alcohol. The mixture was held at -20°C for 1 to 2 hours, centrifuged at 11,600 \( g \) in a microcentrifuge, then washed in 70% ethanol. The DNA was then dissolved in an appropriate volume of ultrapure water or TE 1x.
**3.3.16 Cloning and sequencing PCR products**

3.3.16.1 Re-amplification of PCR products

Selected PCR products were cloned into plasmid vectors for sequencing. When dUTP was substituted for dTTP in PCRs, products of amplification could not be readily cloned because UNGs present in standard laboratory strains of bacteria degrade plasmids containing inserts with U residues. Therefore, PCR products for cloning were re-amplified by PCR using dTTP in the dNTP mix with the same primers as those used for the original PCR reaction. An alternative approach would have been to use strains of bacteria that were defective for UNG activity (UNG' strains) for transformation.

3.3.16.2 TA cloning of PCR products

Non-proof reading DNA polymerases, such as the Taq polymerase (Amplitaq DNA Polymerase) used in standard PCRs (Section 3.3.7.1), add single dA residues to the 3' ends of duplex DNA molecules generated by PCR. These PCR products can be cloned by ligation into vectors with single 3' dT residues. The vector used for this purpose was pCRII (TA Cloning System, Invitrogen). PCR products were cloned directly from unpurified reaction mixes or were gel-purified and dissolved in ultrapure water before ligation. The reaction mix for TA cloning using PClRII contained 1 μl of PCR product, 4 U T4 DNA ligase, commercial ligation buffer 1x and 50 ng plasmid vector in a total volume of 10 μl. E coli DH5α was transformed with the ligation reaction mix (Sections 3.3.13.2) and plated on LB agar containing 50 μg/ml ampicillin and spread with 1 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Blue-white screening was used for selection of plasmids containing inserts. X-gal is a substrate for β-galactosidase, which turns blue when cleaved by active enzyme produced in plasmids that lack inserts. Bacteria transformed with plasmids containing inserts do not produce active enzyme and colonies remain white. LB agar plates spread with transformed bacteria were incubated at 37°C overnight. White colonies were picked and inoculated into 5 ml LB broth containing 50 mg/ml ampicillin. These cultures were grown overnight at 37°C in an orbital incubator at 225 rpm and small scale preparations of plasmids made using the boiling method (Section 3.3.14.1). The plasmids were digested with REs and separated by agarose gel electrophoresis. Plasmids containing inserts of the expected size were identified and the source broth cultures were regrown. Small scale preparations of plasmids were then made using the alkaline lysis method followed by phenol-chloroform extraction or column purified (Wizard minipreps DNA purification system, Promega) (Section 3.3.14.1). The purified plasmids were quantified by spectrophotometry and submitted for sequencing.
3.3.16.3 Sequencing of plasmid inserts

Some plasmids were sequenced at the University of Birmingham (Alta Bioscience) using standard primer sequences (T3 and T7) present in the cloning vector pCRII (Appendix 5: A5.12.1). Other plasmids were sequenced with the Sequencing Kit (Pharmacia), which uses the dideoxy sequencing method in which primer extension by T7 DNA polymerase is terminated in reactions specific for A, C, G and T bases (Sanger *et al* 1977, Tabor and Richardson 1987) (Appendix 5: A5.12.2).

Plasmids that had been purified for sequencing (2 µg) were denatured in 0.4 M NaOH for 10 min at room temperature in 1.5 ml microcentrifuge tubes. The DNA was precipitated with 0.1 volumes of 3 M sodium acetate and 2.5 volumes absolute alcohol at -20°C for 30 min. The tubes were centrifuged at 11,600 g for 15 min then the precipitated DNA was washed with 70% ethanol, vacuum desiccated and dissolved in 10 µl ultrapure water. This volume was mixed with 2 µl (10 pmol) universal primer (M13) and 2 µl annealing buffer and incubated at 65°C for 10 min then 37°C for 10 min. The sequencing enzyme T7 DNA polymerase (2 µl) was mixed with 8 µl enzyme dilution buffer on ice. Each of four labelling reactions consisted of 14 µl annealed template and primer, 3 µl labelling mix (dATP), 1 µl (9.25 MBq) α-35S dATP (Amersham) (Appendix 5: A5.12.3) and 2 µl of diluted T7 DNA polymerase in a total volume of 20 µl. The labelling reactions were incubated at room temperature for 5 min then 4.5 µl was added to each of four 1.5 ml microcentrifuge tubes containing 2.5 µl of one of four sequencing mixes (A', C', G' or T' mix-short) that had been incubated at 37°C for 2 min. The termination reaction was allowed to proceed for 5 min at 37°C then stopped by the addition of 5 µl of a proprietary solution containing formamide, EDTA, bromophenol blue and xylene cyanole.

Aliquots (3 µl) of each reaction were incubated at 80°C for 3 min and placed on ice then 2.5 µl was loaded into one of four adjacent wells of a 5% polyacrylamide sequencing gel (Long Ranger DNA sequencing gel solution Hydro-Link, AT Biochem) (Appendix 5: A5.12.4). The glass sequencing plates had been coated with silicon (Sigmacote SL-2, Sigma) prior to pouring the polyacrylamide gel. The samples were separated by electrophoresis in a sequencing cell (Sequi-Gen II System, 21 cm x 50 cm, Bio-Rad) at 1,500 V for 90 min with power supplied by an electrophoresis unit (ECPS 3000/150, Pharmacia). After dismantling the apparatus and separating the glass plates, the gel was transferred to blotting paper (Number 3, Whatman) and dried at 80°C for 2 hours in a gel-drier (Bio-Rad). Autoradiograph film was exposed for 4 hours then developed. The A, C, G, T sequence was read manually from the pattern of bands in each lane.
3.4 Results

3.4.1 Sensitivity of detection of HTLV-I and BLV by PCR

The primer sets for amplification of HTLV-I, HTLV-I/II and BLV sequences by PCR are shown in Figure 3.1. The sensitivities of these primer sets for detecting HTLV-I and BLV positive control DNA using optimised PCR conditions are indicated in Table 3.2 and depicted in Figures 3.6 to 3.23. Most primer sets were able to detect 0.1 to 1.0 ng positive control DNA in a total quantity of 1 μg DNA when 8 μl from 50 μl of PCR products were electrophoresed on a polyacrylamide gel and stained with ethidium bromide. The sensitivity was increased to 0.01 to 0.1 ng when these PCR products were electroblotted and hybridised to HTLV-I or BLV-specific probes. Some primer sets were less sensitive. Specific PCR products could be detected in not less than 10 ng C8166 DNA on polyacrylamide gels using primers 120 & 121. Primers 67 & 68 also had relatively low sensitivity (10 ng C8166 DNA); both had degenerate bases, increasing the probability of binding to non-specific DNA sequences. Primers 3 & 4 had different calculated $T_m$ values because primer 3 was substantially larger (29 bases) than primer 4 (18 bases), but this primer set retained good sensitivity (1.0 ng C8166 DNA). The primer set used for amplification of BLV sequences (194 & 195) also had relatively low sensitivity (10 ng BLV-FLK DNA). Another set of primers and oligonucleotide probe used for BLV PCRs was found to lack specificity (Agresti et al. 1993).

The sensitivities of PCRs for detection of HTLV-I and BLV in serial dilutions of positive control cells in PBMCs or other negative control cells are shown in Table 3.3. On ethidium bromide-stained polyacrylamide gels, PCR product could be detected with most primer sets when 100 to 1,000 positive control cells were present in a total quantity of 100,000 cells. Some primer sets could detect 10 positive cells in this quantity. Hybridisation to HTLV-I-specific probes increased the sensitivity to 1 to 100 positive cells.

Serial dilutions of positive control DNA or cells were included with each batch of clinical samples tested by PCR to ensure that sensitivity was maintained. Sensitivities of most primer sets were highly repeatable. If the titration of positive controls in any batch of PCRs with clinical samples did not show acceptable sensitivity, the PCRs were repeated on the same samples. In interpretation of these sensitivity assays, it is important to note that C8166 cells contain three copies of each LTR and of the $\beta$X region of HTLV-I, but only one copy of gag, pol and env sequences (Section 2.3.4.1). BLV-FLK cells contain one copy of complete provirus (2.3.4.6).

The $\beta$-globin primers 18 & 52 were used with concentrations of 0.5 μM each primer and a concentration of 1.5 mM MgCl$_2$ in each PCR. Serial dilutions showed that $\beta$-globin sequences could be detected in 0.01 ng DNA using these primers when PCR products were visualised on polyacrylamide gels (Figure 3.24).
Table 3.2: Optimised conditions and sensitivity of polymerase chain reactions.

The listed concentrations of oligonucleotide primers and MgCl₂ were used in PCRs for amplification of HTLV-I, HTLV-I/II and BLV sequences following optimisation. The sensitivity of each PCR primer set is given for detection of template (C8166 DNA for HTLV-I/II PCRs and BLV-FLK DNA for BLV PCRs) in a total of 1 µg DNA. In most cases, hybridisation of the HTLV-I probe pMT2ₗ to electroblots increased the sensitivity of detection by 10-fold relative to examination of ethidium bromide-stained polyacrylamide gels by UV transillumination.

<table>
<thead>
<tr>
<th>LRF primer number</th>
<th>Optimum concentration of each primer (µM)</th>
<th>Optimum concentration of MgCl₂ (mM)</th>
<th>Quantity of positive control DNA detected in a total quantity of 1 µg DNA (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethidium-bromide-stained polyacrylamide gels</td>
<td>Hybridisation using probe pMT2.</td>
<td></td>
</tr>
</tbody>
</table>

HTLV-I LTR
118 & 119 | 0.5 | 2.5 | 0.1 | 0.01 |
HTLV-I gag
120 & 121 | 1.0 | 1.5 | 10 | 0.1 |
122 & 123 | 1.0 | 1.5 | 1.0 | 0.1 |
HTLV-I pol
1 & 2 | 1.0 | 1.5 | 1.0 | 0.1 |
67 & 68 | 0.5 | 2.0 | 10 | 1.0 |
HTLV-I env
124 & 125 | 1.0 | 1.5 | 1.0 | 0.01 |
HTLV-I/II pX
50 & 51 | 1.0 | 1.5 | 0.1 | 0.01 |
3 & 4 | 0.5 | 2.5 | 1.0 | 0.1 |
126 & 127 | 1.0 | 1.5 | 1.0 | 0.1 |
BLV gag
194 & 195 | 0.5 | 1.5 | 10 | 1.0 |
Table 3.3: Sensitivity of polymerase chain reactions for detection of HTLV-I, HTLV-I/II and BLV sequences in cell pellets.

The sensitivity of each PCR primer set is given for detection of template (C8166 DNA for HTLV-I and HTLV-I/II PCRs and BLV-FLK DNA for BLV PCRs) in pellets of $1 \times 10^5$ cells digested with non-ionic detergent buffer and proteinase K. In most cases, hybridisation of the HTLV-I probe pMT2i to electroblots increased the sensitivity of detection by 10-fold relative to examination of ethidium bromide-stained polyacrylamide gels by UV transillumination.

<table>
<thead>
<tr>
<th>LRF primer number</th>
<th>Ethidium-bromide-stained polyacrylamide gels</th>
<th>Hybridisation using probe pMT2i</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HTLV-I LTR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>118 &amp; 119</td>
<td>$1 \times 10^3$</td>
<td>$1 \times 10^1$</td>
</tr>
<tr>
<td><strong>HTLV-I gag</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120 &amp; 121</td>
<td>$1 \times 10^3$</td>
<td>$1 \times 10^1$</td>
</tr>
<tr>
<td>122 &amp; 123</td>
<td>$1 \times 10^3$</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td><strong>HTLV-I pol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 &amp; 2</td>
<td>$1 \times 10^5$</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td><strong>HTLV-I env</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124 &amp; 125</td>
<td>$1 \times 10^2$</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td><strong>HTLV-I/II pX</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 &amp; 51</td>
<td>$1 \times 10^3$</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>126 &amp; 127</td>
<td>$1 \times 10^3$</td>
<td>$1 \times 10^5$</td>
</tr>
</tbody>
</table>
Figure 3.6: Sensitivity of PCR for detection of HTLV-I LTR sequences using primers 118 & 119.

PCR for HTLV-I LTR sequences using primers 118 & 119 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 183 bp. Additional bands at approximately 300 bp represent migration of single-stranded PCR product. M = φX174/HaeIII molecular weight marker. bp = base pairs.
Figure 3.7: Hybridisation of probe pMT2 to HTLV-I LTR PCR products amplified using primers 118 & 119.

HTLV-I LTR PCR products from Figure 3.6 amplified using primers 118 & 119 were electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 183 bp were positive on hybridisation, as were PCR products that migrated as single-stranded bands at approximately 300 bp. M = Molecular weight marker lane. bp = base pairs.

Quantity of C8166 DNA (ng) in total 1 µg DNA

<table>
<thead>
<tr>
<th>M</th>
<th>1,000</th>
<th>100</th>
<th>10</th>
<th>1</th>
<th>0.1</th>
<th>0.01</th>
<th>0</th>
</tr>
</thead>
</table>

183 bp
Figure 3.8: Sensitivity of PCR for detection of HTLV-I gag sequences using primers 120 & 121.

PCR for HTLV-I gag sequences using primers 120 & 121 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 273 bp. Additional larger bands at approximately 400 and 850 bp may represent migration of single-stranded PCR products. M = φX174/HaeIII molecular weight marker. bp = base pairs.
Figure 3.9: Hybridisation of probe pMT2i to HTLV-I gag PCR products amplified using primers 120 & 121.

HTLV-I gag PCR products from Figure 3.8 amplified using primers 120 & 121 were electrophoretically transferred to nylon membrane and hybridised to probe pMT2i radiolabelled by random priming. PCR products of 273 bp, as well as larger bands including probable single-stranded PCR products, were positive on hybridisation. M = Molecular weight marker lane. bp = base pairs.

| Quantity of C8166 DNA (ng) in total 1 μg DNA |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| M | 1,000 | 100 | 10 | 1 | 0.1 | 0.01 | 0 |

<273 bp
Figure 3.10: Sensitivity of PCR for detection of HTLV-I gag sequences using primers 122 & 123.

The sensitivity of PCR for detecting HTLV-I gag sequences using primers 122 & 123 was determined using serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA). In conjunction with the sensitivity assay, PCRs were performed on 1 μg skin DNA from three patients with cutaneous lymphoid infiltrates, along with water controls. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. The expected PCR product size is 134 bp. M = φX174/HaeIII molecular weight marker. P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sèzary syndrome. bp = base pairs.
Figure 3.11: Hybridisation of probe pMT2, to HTLV-I gag PCR products amplified using primers 122 & 123.

HTLV-I gag PCR products from Figure 3.10 amplified using primers 122 & 123 were electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 134 bp were positive on hybridisation and there was hybridisation to products that migrated as single strands at higher molecular weight. M = Molecular weight marker lane. P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sézary syndrome. bp = base pairs.
Figure 3.12: Sensitivity of PCR for detection of HTLV-I pol sequences using primers 1 & 2.

PCR for HTLV-I pol sequences using primers 1 & 2 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 μg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 119 bp. Additional bands at approximately 150 bp represent migration of single-stranded PCR product. M = φX174/HaeIII molecular weight marker. bp = base pairs.
Figure 3.13: Hybridisation of probe pMT2 to HTLV-I\textit{pol} PCR products amplified using primers 1 & 2.

HTLV-I\textit{pol} PCR products from Figure 3.12 amplified using primers 1 & 2 were electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 119 bp were strongly positive on hybridisation. PCR product that migrated as single stranded bands at approximately 150 bp was also hybridisation positive and there was weak hybridisation to unincorporated PCR primers. A faint band visible by eye in the lane with 0.1 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. bp = base pairs.

| Quantity of C8166 DNA (ng) in total 1 µg DNA |
|----------|----------|----------|----------|----------|----------|
| M        | 1,000    | 100      | 10       | 1        | 0.1      | 0.01     | 0        |

119 bp
Figure 3.14: Sensitivity of PCR for detection of HTLV-I pol sequences using primers 67 & 68.

The sensitivity of PCR for detecting HTLV-I pol sequences using primers 67 & 68 was determined using serial dilutions of C8166 DNA in placental DNA (total quantity 1 ug DNA). In conjunction with the sensitivity assay, PCRs were performed on 1 ug skin DNA from three patients with cutaneous lymphoid infiltrates, along with water controls. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. The expected PCR product size is 186 bp. Several bands migrating at larger sizes represent single-stranded PCR products. M = φX174/HaeIII molecular weight marker. P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sézary syndrome. bp = base pairs.
Figure 3.15: Hybridisation of probe pMT2 to HTLV-I pol PCR products amplified using primers 67 & 68.

HTLV-I pol PCR products from Figure 3.14 amplified using primers 67 & 68 were electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 186 bp were positive on hybridisation and there was hybridisation to products that migrated at higher molecular weight. Faint bands visible by eye in the lanes with 10 and 1 ng C8166 DNA are not reproduced in the photograph. M = Molecular weight marker lane. P = Patient number. CBCL = Cutaneous B cell lymphoma. MF = Mycosis fungoides. SS = Sézary syndrome. bp = base pairs.
PCR for HTLV-I env sequences using primers 124 & 125 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 µg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 327 bp. Specific PCR products from C8166 were detected as a doublet, probably reflecting amplification from different integrated proviruses in the DNA. Additional bands migrating at approximately 1,200 and 900 bp represent migration of corresponding single-stranded PCR product. M = φX174/HaeIII molecular weight marker. bp = base pairs.
Figure 3.17: Hybridisation of probe pMT2, to HTLV-I env PCR products amplified using primers 124 & 125.

HTLV-I env PCR products from Figure 3.16 amplified using primers 124 & 125 were electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 327 bp were strongly positive on hybridisation, as were products of other sizes with decreasing intensity as the quantity of positive control template decreased. A faint band visible by eye in the lane with 0.01 ng C8566 DNA is not reproduced in the photograph. bp = base pairs.
Figure 3.18: Sensitivity of PCR for detection of HTLV-I pX sequences using primers 50 & 51.

PCR for HTLV-I pX sequences using primers 50 & 51 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 µg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 159 bp. Bands at approximately 400 bp represent PCR product that has migrated as single-stranded DNA. Non-specific PCR products increase as the proportion of C8166 DNA decreases. M = φX174/HaeIII molecular weight marker. bp = base pairs.

| Quantity of C8166 DNA (ng) in total 1 µg DNA |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| M               | 1,000           | 100             | 10              | 1               | 0.1             | 0.01            | 0               |
| bp              | 1,353           | 872             | 603             | 281             | 234             | 194             | 118             | 72              |

159 bp
Figure 3.19: Hybridisation of probe pMT2| to HTLV-I pX PCR products amplified using primers 50 & 51.

HTLV-I pX PCR products from Figure 3.18 amplified using primers 50 & 51 were electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 159 bp were positive on hybridisation and there was hybridisation to bands that migrated at approximately 400 bp and represent single-stranded PCR product. Hybridisation to several non-specific bands is visible in the lane containing product from the PCR with no C8166 DNA template (1 μg placental DNA only). A faint band visible by eye in the lane with 0.01 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. bp = base pairs.
Figure 3.20: Sensitivity of PCR for detection of HTLV-I pX sequences using primers 126 & 127.

PCR for HTLV-I pX sequences using primers 126 & 127 was performed on serial dilutions of C8166 DNA in placental DNA (total quantity 1 µg DNA) and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 127 bp. A slightly larger band at approximately 150 bp in some lanes probably represents an alternative product from one of three proviruses in C8166 DNA. Bands at approximately 275 bp are due to migration of single-stranded PCR products. M = φX174/HaeIII molecular weight marker. bp = base pairs.
Figure 3.21: Hybridisation of probe pMT2, to HTLV-I pX PCR products amplified using primers 126 & 127.

HTLV-I pX PCR products from Figure 3.20 amplified using primers 126 & 127 were electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 127 bp, as well as larger single-stranded PCR products migrating at approximately 275 bp, were positive on hybridisation. A faint band visible by eye in the lane with 0.1 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. bp = base pairs.

<table>
<thead>
<tr>
<th>Quantity of C8166 DNA (ng) in total 1 µg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
</tr>
</tbody>
</table>

![Image of hybridisation result with 127 bp marker]
The sensitivity of PCR for detecting bovine leukaemia virus *gag* sequences with primers 194 & 195 was determined using serial dilutions of BLV-FLK DNA in FLK DNA (total quantity 1 µg DNA). In conjunction with the sensitivity assay, PCRs were performed on three samples of 1 x 10^5 peripheral blood mononuclear cells (PBMCs) from two patients with T cell acute lymphoblastic leukaemia (ALL) (patient 1130) and common ALL (patient 1131), along with water controls. PCR products were separated by polyacrylamide gel electrophoresis and stained with ethidium bromide. The expected PCR product size is 247 bp. M = φX174/HaeIII molecular weight marker. P = Patient number. T-ALL = T cell acute lymphoblastic leukaemia. C-ALL = Common acute lymphoblastic leukaemia. BLV-FLK = Bovine leukaemia virus-infected foetal lamb kidney cells. bp = base pairs.
Figure 3.23: Hybridisation of a bovine leukaemia virus probe to gag PCR products amplified from BLV-FLK DNA using primers 194 & 195.

Bovine leukaemia virus (BLV) gag PCR products from Figure 3.22 amplified using primers 194 & 195 were electroblotted onto nylon membrane and hybridised to the BLV oligonucleotide probe 301 radiolabelled by 5' end-labelling. PCR products of 247 bp were positive on hybridisation and there was hybridisation to single-stranded PCR products that migrated at higher molecular weight. A faint band visible by eye in the lane with 1 ng C8166 DNA is not reproduced in the photograph. M = Molecular weight marker lane. P = Patient number. T-ALL = T cell acute lymphoblastic leukaemia. C-ALL = Common acute lymphoblastic leukaemia. BLV-FLK = Bovine leukaemia virus-infected foetal lamb kidney cells. bp = base pairs.
Figure 3.24: Sensitivity of PCR for detection of β-globin sequences in genomic DNA using primers 18 & 52.

PCR for β-globin sequences using primers 18 & 52 was performed on serial dilutions of C8166 DNA in water and products were examined on an ethidium bromide-stained polyacrylamide gel following electrophoresis. The expected PCR product size is 91 bp. M = φX174/HaeIII molecular weight marker. bp = base pairs.
3.4.2 Cutaneous T cell lymphomas

Samples from 36 patients with cutaneous lymphoid infiltrates were examined by PCR for HTLV-I sequences. Clinical diagnoses in these patients were 21 cases of MF, five cases of SS, four cases of CBCL, three cases of angiocentric T cell lymphoma (ATCL) and one case each of CTCL (peripheral T cell lymphoma), small plaque parapsoriasis (SPP) and Langerhan's cell histiocytosis (LCH) (Table 3.1). Samples from all patients were positive with primers 18 & 52 for β globin by PCR, indicating the presence of amplifiable DNA (Tables 3.4, 3.5 and 3.6).

Three samples of PBMCs from two patients, one with SS (patient 1198) and one with CBCL (patient 1359), had faintly positive bands of approximately 159 bp on hybridisation for HTLV-I/II tax sequences amplified using primers 50 & 51 (Table 3.7) (Figures 3.25 and 3.26). Attempts were made to clone and sequence the 159 bp first round PCR products after gel purification, but the quantity of amplified material was too small to yield clonable DNA. The PCR products were re-amplified using nested primers that annealed to sequences internal to primers 50 & 51 (Section 3.3.7.5). Bands of 136 bp were obtained using the HTLV-I-specific primer set 282 & 283, whereas multiple non-specific bands, as well as bands of the approximate expected size, were obtained using the HTLV-II-specific primers 288 & 289. This suggested that HTLV-I-specific product was present.

The second round (nested) PCR products amplified using primers 282 & 283 from two submissions of PBMCs from patient 1198 (1198-1 and 1198-3) were successfully TA cloned and sequenced (Section 3.3.16.2), along with PCR products cloned from C8166 DNA following a single round amplification using the outer set of primers (50 & 51). The sequences amplified from these two samples from patient 1198 were identical and matched the sequence from C8166, but differed at four bases from the sequence of the same region of prototypical HTLV-I (Seiki et al. 1983) (Figure 3.27). The first round PCR product from patient 1198 had been handled in the potentially contaminated molecular biology laboratory before the nested PCR was performed.

One sample of skin from a patient with MF (patient 436) had a faintly positive band of approximately 127 bp on hybridisation for HTLV-I tax sequences amplified using primers 126 & 127 (Table 3.7) (Figures 3.28 and 3.29). The first round PCR product from this sample was cloned after gel purification of the 127 bp band. The sequence of this PCR product was identical to that expected from the prototypical HTLV-I sequence, as well as the sequence amplified from positive control C8166 DNA using the same primers (Figure 3.30).

All other samples were negative by PCR using primers specific for HTLV-I LTR, gag, pol, env and pX regions.
3.4.3 Large granular lymphocytic leukaemia

Fresh and cultured PBMCs were tested from six patients with CD3⁺ CD8⁺ LGL leukaemia (Table 2.4). All samples were positive for β globin, indicating the presence of amplifiable DNA, although the signal from one patient was weak (Table 3.8).

Of six samples tested for HTLV-I/II tax using primers 50 & 51 and four samples tested for HTLV-I pol using primers 1 & 2, all were negative by PCR. Faint bands with sizes of approximately 119 and 180 bp were observed on ethidium bromide-stained gels in one patient following amplification with primers 1 & 2 but neither of these bands hybridised to the specific HTLV-I probe. No BLV gag PCR products were detected in two PBMC samples from two patients tested using primers 194 & 195.

3.4.4 Acute lymphoblastic leukaemia cases

PBMCs and bone marrow were tested from six patients with ALL of common, pre-B cell or T cell type (Table 2.5). All patients were male butchers from Cardiff. Seven samples of PBMCs and three of four bone marrow samples were positive for β globin, indicating the presence of amplifiable DNA (Table 3.8).

PBMCs and bone marrow samples from four patients tested for HTLV-I/II tax using primers 50 & 51 and HTLV-I pol using primers 1 & 2 were negative by PCR. Following amplification with primers 50 & 51, a faint band with a size of approximately 118 bp was detected in one sample, but this was negative on hybridisation. Using primers 1 & 2, faint bands with a size of approximately 120 bp were observed on ethidium bromide-stained gels in four samples from three patients. Additional bands also observed on ethidium bromide-stained gels were 90 and 230 bp in one sample, 350 bp in a second sample from the same patient and 170 bp in a sample from another patient. Faint bands of approximately 119 bp were detected in samples from two of these patients when amplification with primers 1 & 2 was repeated, whereas no PCR product was detected in another sample from one of these patients. These additional bands were negative on hybridisation to the specific HTLV-I probe.

No BLV gag PCR products were detected in six PBMC and four bone marrow samples from five patients tested using primers 194 & 195 (Table 3.9).
3.4.5 Polymerase chain reaction for detection of HTLV-I and HTLV-II in cultured cells

PCR was used to test for HTLV-I and HTLV-I/II sequences in four long-term T cell cultures established from skin samples of three patients with MF (patients 2531, 2600 and 2862) and one Epstein-Barr virus (EBV)-infected B-LCL established from a skin sample from a patient with CBCL (patient 2132) (Section 2.4.1) (Table 2.12). Established T cell lines grown in the laboratory were also tested for HTLV-I, HTLV-I/II and BLV sequences by PCR. These cell lines were J.JHAN, L-726A, HH, My-La and My-La MC, none of which is known to be infected with HTLV-I or HTLV-II (Sections 2.1.3.1 and 2.3.4). C8166 cells were used as positive controls. Pellets of $1 \times 10^5$ cells and 1 µg DNA extracted from cultured cells were tested by PCR with the complete panel of HTLV-I, HTLV-I/II and BLV primers using optimised conditions for each primer set. Parallel samples were also tested for β-globin sequences using primers 18 & 52. C8166 was positive with all HTLV-I and HTLV-I/II primer sets but was negative with the BLV primer sets. All other cultured cells tested by PCR were negative for HTLV-I, HTLV-I/II and BLV sequences.
Table 3.4: Polymerase chain reaction results from skin samples of patients with cutaneous lymphoid infiltrates.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>PCR product</th>
<th>Number tested</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 &amp; 52</td>
<td>β globin</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>118 &amp; 119</td>
<td>HTLV-I LTR</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>120 &amp; 121</td>
<td>HTLV-I gag</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>122 &amp; 123</td>
<td>HTLV-I gag</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>1 &amp; 2</td>
<td>HTLV-I pol</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>67 &amp; 68</td>
<td>HTLV-I pol</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>124 &amp; 125</td>
<td>HTLV-I env</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>3 &amp; 4</td>
<td>HTLV-I tax</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>50 &amp; 51</td>
<td>HTLV-I tax</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>126 &amp; 127</td>
<td>HTLV-I tax</td>
<td>28</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3.5: Polymerase chain reaction results from peripheral blood mononuclear cells of patients with cutaneous lymphoid infiltrates.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>PCR product</th>
<th>Number tested</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 &amp; 52</td>
<td>β globin</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>118 &amp; 119</td>
<td>HTLV-I LTR</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>120 &amp; 121</td>
<td>HTLV-I gag</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>1 &amp; 2</td>
<td>HTLV-I pol</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>124 &amp; 125</td>
<td>HTLV-I env</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>50 &amp; 51</td>
<td>HTLV-I tax</td>
<td>15</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3.6: Polymerase chain reaction results from lymph node samples of cutaneous T cell lymphoma patients.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>PCR product</th>
<th>Number tested</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 &amp; 52</td>
<td>β globin</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>118 &amp; 119</td>
<td>HTLV-I LTR</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>120 &amp; 121</td>
<td>HTLV-I gag</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>1 &amp; 2</td>
<td>HTLV-I pol</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>124 &amp; 125</td>
<td>HTLV-I env</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>50 &amp; 51</td>
<td>HTLV-I tax</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>126 &amp; 127</td>
<td>HTLV-I tax</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3.7: Details of polymerase chain reaction positive cutaneous T cell lymphoma cases.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Clinical diagnosis</th>
<th>Sample number</th>
<th>Sample type</th>
<th>Primer set</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>436</td>
<td>64</td>
<td>M</td>
<td>MF</td>
<td>15757</td>
<td>Skin</td>
<td>126 &amp; 127</td>
<td>HTLV-I tax</td>
</tr>
<tr>
<td>1198</td>
<td>81</td>
<td>M</td>
<td>SS</td>
<td>13702, 13944</td>
<td>PBMCs, PBMCs</td>
<td>50 &amp; 51</td>
<td>HTLV-I tax</td>
</tr>
<tr>
<td>1359</td>
<td>NA</td>
<td>M</td>
<td>DCL</td>
<td>15644</td>
<td>PBMCs</td>
<td>50 &amp; 51</td>
<td>HTLV-I tax</td>
</tr>
</tbody>
</table>
Figure 3.25: Amplification of HTLV-I \( pX \) sequences from clinical samples of patients 1198 and 1359 using primers 50 & 51.

Peripheral blood mononuclear cells (PBMCs) from a patient with Sézary syndrome (patient 1198) and a patient with cutaneous B cell lymphoma (patient 1359) were positive for HTLV-I \( pX \) sequences when tested by PCR using primers 50 & 51. Samples are shown on a polyacrylamide gel stained with ethidium bromide. Water controls were run next to each sample. C8166 cells (1 x 10^5) were used as a positive control. Sensitivity was determined by electrophoresis of PCR products amplified from serial dilutions of C8166 cells in PBMCs on a different polyacrylamide gel (sensitivity 10 C8166 cells). Bands of the expected size (159 bp) could not be distinguished from non-specific bands on the polyacrylamide gel, but bands representing single-stranded DNA migrated at approximately 400 bp in lanes containing two samples from patient 1198 and one sample from patient 1359. These bands hybridised to a probe for HTLV-I (Figure 3.26). M = \( \Phi X 174/HaeIII \) molecular weight marker. P = Patient number. SS = Sézary syndrome. MF = Mycosis fungoides. CBCL = Cutaneous B cell lymphoma. bp = base pairs.
Figure 3.26: Hybridisation of probe pMT2, to HTLV-I pX PCR products amplified from clinical samples of patients 1198 and 1359 using primers 50 & 51.

HTLV-I pX PCR products from Figure 3.25 amplified using primers 50 & 51 were electrophoretically separated and electroblotted onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 159 bp from two samples of PBMCs from patient 1198 (Sézary syndrome) and one sample of PBMCs from patient 1359 (cutaneous B cell lymphoma) were faintly positive on hybridisation. Positive control cells from the C8166 cell line were strongly positive on hybridisation. Bands that migrated at approximately 400 bp, representing single-stranded PCR products, were also positive on hybridisation. M = Molecular weight marker lane. P = Patient number. SS = Sézary syndrome. MF = Mycosis fungoides. CBCL = Cutaneous B cell lymphoma. bp = base pairs.
**Figure 3.27: Sequences of nested HTLV-I pX PCR products amplified using primers 282 & 283 from patient 1198.**

First round PCR products were amplified from peripheral blood mononuclear cells of patient 1198, who had SS, using primers 50 & 51. Second round (nested) PCR products were amplified using primers 282 & 283, then TA cloned and sequenced. The 136 bp sequence is aligned with prototypical HTLV-I (Seiki et al 1983) and sequences amplified from positive control DNA (C8166) using primers 50 & 51 in the same laboratory. Primers are underlined. The sequences from patient 1198 and C8166 are identical but differ at four bases from the prototypical HTLV-I sequence.

<table>
<thead>
<tr>
<th></th>
<th>Prototypical HTLV-I sequence</th>
<th>Positive control HTLV-I-infected T cell line</th>
<th>Second round PCR product from PBMCs of patient 1198, sample number 13702</th>
<th>Second round PCR product from PBMCs of patient 1198, sample number 13944</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTLV-I</td>
<td>AGTCTACGTGTTTGGAGACTGTGTAAGGCGACTGGTGCCCCATCTGCGGGGACTATGTTCGGCCC</td>
<td>C8166</td>
<td>1198-1</td>
<td>1198-3</td>
</tr>
<tr>
<td>C8166</td>
<td>AGTCTACGTGTTTGGAGACTGTGTAAGGCGACTGGTGCCCCATCTGCGGGGACTATGTTCGGCCC</td>
<td>1198-1</td>
<td>1198-3</td>
<td></td>
</tr>
<tr>
<td>1198-1</td>
<td>AGTCTACGTGTTTGGAGACTGTGTAAGGCGACTGGTGCCCCATCTGCGGGGACTATGTTCGGCCC</td>
<td>1198-3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DNA samples (1 μg) from involved skin of five patients with mycosis fungoides (MF) and one patient with angiocentric T cell lymphoma were tested for HTLV-I pX sequences by PCR using primers 126 & 127. Water control PCRs were performed along with each sample. C8166 DNA (1 μg) was used as a positive control. Sensitivity was determined by electrophoresis of PCR products amplified from serial dilutions of C8166 DNA in placental DNA on a different polyacrylamide gel (sensitivity 1 ng positive control DNA). Bands of the expected size (127 bp) were detected in PCR products from lesional skin of patient 436, who had MF, as well as in positive control DNA. These bands hybridised to a probe for HTLV-I (Figure 3.29). Multiple non-specific bands were also amplified from clinical samples but did not hybridise to the HTLV-I probe. Polyacrylamide gel electrophoresis and ethidium bromide staining. M = φX174/HaeIII molecular weight marker. P = Patient number. MF = Mycosis fungoides. ATCL = Angiocentric T cell lymphoma. bp = base pairs.
Figure 3.29: Hybridisation of probe pMT2 to HTLV-I pX PCR products amplified from skin DNA of patient 436 using primers 126 & 127.

HTLV-I pX PCR products from Figure 3.28 amplified using primers 126 & 127 were electrophoretically transferred onto nylon membrane and hybridised to probe pMT2, radiolabelled by random priming. PCR products of 127 bp from skin DNA of patient 436 and positive control DNA (C8166) were positive on hybridisation, whereas there was no hybridisation to non-specific PCR products. M = Molecular weight marker lane. P = Patient number. MF = Mycosis fungoides. ATCL = Angiocentric T cell lymphoma. bp = base pairs.
Figure 3.30: Sequences of polymerase chain reaction products amplified using primers 126 & 127 from patient 436.

PCR products were amplified by PCR using primers 126 & 127 from a skin sample of patient 436, who had mycosis fungoides, then cloned and sequenced. The 127 bp sequence is aligned with prototypical HTLV-I (Seiki et al 1983) and sequences amplified from positive control DNA (C8166) in the same laboratory. Primers are underlined.

<table>
<thead>
<tr>
<th>127 bp sequence</th>
<th>Prototypical HTLV-I sequence</th>
<th>Positive control HTLV-I-infected T cell line</th>
<th>First round amplification product</th>
<th>Second round amplification product</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC</td>
<td>CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC</td>
<td>CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC</td>
<td>CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC</td>
<td>CCAATCACTCATACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATGCGCAAATACTCCC</td>
</tr>
</tbody>
</table>

Primer 126

Primer 127
Table 3.8: Polymerase chain reaction results from peripheral blood mononuclear cells of cases of large granular lymphocytic leukaemia.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>PCR product</th>
<th>Number tested</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 &amp; 52</td>
<td>β globin</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1 &amp; 2</td>
<td>HTLV-I pol</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>50 &amp; 51</td>
<td>HTLV-I tax</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>194 &amp; 195</td>
<td>BLV gag</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3.9: Polymerase chain reaction results from peripheral blood mononuclear cells of acute lymphoblastic leukaemia cases in butchers from Cardiff.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>PCR product</th>
<th>Number tested</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 &amp; 52</td>
<td>β globin</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>1 &amp; 2</td>
<td>HTLV-I pol</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>50 &amp; 51</td>
<td>HTLV-I tax</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>194 &amp; 195</td>
<td>BLV gag</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>
Consensus retroviral polymerase chain reaction

The expected size of HTLV-I PCR products amplified using the consensus HTLV/BLV LTR primers 174 & 175 was 506 bp (Figure 3.2). Products of approximately 550 bp that hybridised to the HTLV-I probe pMT2 were amplified from pMT2 when these primers were used in a standard PCR (annealing temperature 55°C) at concentrations of 1.0 μM with 1.5 mM MgCl₂ (Section 3.3.7.6). Bands of approximately 600 bp were amplified from C8166 DNA and human placental DNA under the same PCR conditions but did not hybridise to the HTLV-I probe. PCR products of a variety of different sizes were amplified from pMT2, C8166 and placental DNA at different annealing temperatures, but only the products from pMT2 hybridised to pMT2. Specific PCR products could be amplified from pMT2 using 1.0 to 2.5 mM MgCl₂. Touchdown PCR resulted in PCR products of approximately 400 bp from pMT2 with 1.5 and 2.5 mM MgCl₂ and 450 bp from C8166 with 2.5 mM MgCl₂ only. Although smaller than the expected product size, both bands hybridised to pMT2, suggesting that non-specific priming from plasmid and proviral sequences may have occurred.

PCR using 1.0 μM each of primers 174 & 176 and 1.5 mM MgCl₂ resulted in variable, weak amplification of a specific 1,858 bp LTR-gag product from pMT2, as well as smaller bands that hybridised to pMT2. Several bands of 100 to 250 bp amplified from C8166 also hybridised to pMT2, whereas similar bands amplified from placental DNA were negative. In a MgCl₂ titration using pMT2 as template, the number of non-specific PCR bands increased as the MgCl₂ concentration increased from 1.0 to 3.0 mM. The specific 1,858 bp product was amplified from pMT2 only using touchdown PCR. This band, as well as several smaller bands amplified from pMT2 and C8166, hybridised to pMT2.

The consensus retroviral primers 296 & 297 were used in an attempt to amplify HTLV-I pol sequences from pMT2, C8166 and BLV-FLK DNA using human placental DNA and FLK DNA as controls. Titrations of C8166 DNA in human placental DNA from 1 μg to 0.01 ng in a total of 1 μg DNA were tested, as were titrations of C8166 DNA in water. The primers were used at 1.0 to 4.0 μM each with MgCl₂ concentrations of 1.5, 2.5 and 3.5 mM in standard PCR reactions with annealing temperatures of 55, 60 and 65°C. A touchdown PCR protocol with the same compositions of PCR reaction mixes was also applied. A band of approximately 130 bp consistent with the expected product size of 133 bp was amplified from pMT2 using 1.0 μM of each primer and 3.5 mM MgCl₂. A band of moderately high intensity that migrated at approximately 160 bp was frequently amplified from both C8166 and human placental DNA using 3.5 or 2.5 and sometimes 1.5 mM MgCl₂. However, at 2.5 and 1.5 mM MgCl₂ there were many additional bands in all samples. Only PCR products from pMT2 hybridised to pMT2. The 180 bp PCR product from C8166 was gel-purified, TA cloned and sequenced. Excluding the primers, the sequence had 98% identity over 137 bases to a sequence on human chromosome 4 using the BLAST programme but had no significant matches with retroviral sequences. The specific PCR product amplified from pMT2 was also sequenced and shown to contain the expected 133 bp HTLV-I retroviral pol sequence. Bands of approximately 160 and 130 bp were amplified from both BLV-FLK and FLK DNA using 3.5, 2.5 and 1.5 mM MgCl₂.
3.5 Discussion


The amplification of HTLV-I/II tax sequences from two patients with CTCL (one SS, one MF) in this study would have been of significance if these results were verified as genuine evidence of infection with HTLV-I. To eliminate the possibility of contamination with PCR products, probes or positive control DNA, attempts were made to clone and sequence PCR products from the hybridisation-positive samples. The PCR products amplified from two different samples from a patient with SS were identical and matched the sequence from the positive control DNA used in the laboratory, but differed at four bases from the sequence of prototypical HTLV-I. It was concluded that these PCR products were the result of contamination from positive control (C8166) DNA. The first round PCR products from these samples had been handled in the molecular biology laboratory before the nested PCR was performed and therefore contamination could have resulted during either the first or second round PCRs.

The first round PCR product amplified from a patient with MF using a different set of primers also had an identical sequence to that amplified from positive control DNA, as well as prototypical HTLV-I. Therefore, it cannot be determined whether this PCR product was the result of contamination. It would be necessary to amplify HTLV-I sequences from these same clinical samples using other sets of tax primers and identify unique sequence signatures to confirm that the patients were infected with HTLV-I or a related virus. An alternative approach would be to have another sample of the same type collected from the same patient at the same time tested by PCR for HTLV-I tax sequences in a different laboratory. Sequence errors introduced by Taq polymerase could also be resolved by replicating the amplification, cloning and sequencing of PCR products from samples. However, in the cases in which false positive results were suspected in this study there was insufficient DNA available to retest all samples.

HTLV-I/II tax PCR products were also detected in one patient with CBCL, but it was not possible to re-amplify, clone and sequence these products. Therefore, this result cannot be verified. The detection of HTLV-I/II sequences in patients with cutaneous BCL has not previously been reported, but HTLV-I has been associated with B cell CLL (Mann et al 1987).
HTLV-II sequences have also been detected by PCR in patients with CTCL (Zucker-Franklin et al. 1992) and LGL leukaemia (Loughran et al. 1992, Martin et al. 1993, Heneine et al. 1994, Loughran et al. 1994). In this study, no HTLV-I, HTLV-II or related sequences were detected in six patients with LGL leukaemia. Similarly, no HTLV-I, HTLV-II or BLV sequences were detected in six butchers from Cardiff with ALL.

Differences in sensitivities of primer sets used for amplification of HTLV-I and HTLV-II sequences were noted in this study, despite careful optimisation (Section 3.4.1) (Table 3.3). This means that it might be possible to detect sequences from one portion of a provirus by PCR using a highly sensitive set of primers, whereas other proviral regions are negative by PCR because the specific sets of primers for those regions are less sensitive. In addition, highly sensitive primer sets are more likely to yield false positive results if there is contamination from PCR products, plasmids or positive control DNA. The implications of these results are discussed further in Chapter 5.

The use of two sets of consensus oligonucleotide primers based on alignment of HTLV/BLV group virus LTR and gag sequences showed limited potential for a group-specific PCR assay. Specific PCR products of the appropriate sizes could be amplified from purified plasmid (pMT2) containing the full-length HTLV-I sequence, but not from the HTLV-I-infected cell line C8166. Similar non-specific bands were frequently amplified from C8166 and placental DNA.

While consensus primers can be designed for amplification of pol sequences of most known retroviruses, they lack specificity when used on complex (genomic) DNA, resulting in amplification of many endogenous sequences. Preferential amplification of endogenous viruses, retrotransposons or non-specific cellular sequences reduces the sensitivity of PCR for detection of accompanying proviral DNA using consensus primers. This can be overcome by reverse transcribing purified viral RNA to cDNA before amplification by PCR but for this approach it is necessary to isolate the uncharacterised virus to ensure adequate quantities of template (Donehawer et al. 1990).

In this study most samples from patients with CTCL, LGL leukaemia and ALL were negative for HTLV-I, HTLV-II and BLV sequences where tested by PCR in combination with Southern blot hybridisation. Positive results could be accounted for by contamination. Therefore, no unequivocal evidence for infection with HTLV/BLV group retroviruses was demonstrated. Molecular approaches to the detection of HTLV-I-related retroviruses in patients with CTCL are discussed further in Chapter 5.
Chapter 4
The Role of Human T Lymphotropic Virus Type I
pX Gene Products in Apoptosis

4.1 Introduction

4.1.1 Role of apoptosis in HTLV-I leukaemogenesis

4.1.1.1 Oncogenic properties of HTLV-I pX proteins

The Tax protein of human T lymphotropic virus type I (HTLV-I) is the main regulator of viral transcription and has transforming properties in a number of experimental systems (Section 1.8.3). Established rodent fibroblast cell lines can be transformed by Tax (Tanaka et al. 1990, Smith and Greene 1991). Primary rat embryo fibroblasts (REFs) can be immortalised by Tax alone or transformed by Tax in cooperation with oncogenes such as Ras (Pozzatti et al. 1990, Matsumoto et al. 1994). Oncogenic features of the HTLV-I p12\textsuperscript{I} protein have also been demonstrated (Section 1.8.4). p12\textsuperscript{I} enhances transformation of C127 fibroblasts by the bovine papillomavirus type 1 (BPV-1) E5 protein (Schlegel et al. 1986, Franchini et al. 1993). The contribution of other HTLV-I pX gene products to leukaemogenesis is unknown. Rex acts as a post-transcriptional regulator of HTLV-I gene expression (Inoue et al. 1987, Hidaka et al. 1988, Nosaka et al. 1989) (Section 1.3.5.2). The presence of a nuclear export signal (NES) in p21\textsuperscript{II} and a nucleolar localisation signal (NLS) in p30\textsuperscript{II} (Tof) suggest that these proteins could have a regulatory function. However, there is insufficient information available to attribute roles to p21\textsuperscript{II}, Tof, p13\textsuperscript{II} and p12\textsuperscript{III}.

4.1.1.2 Induction of apoptosis by Tax

Rodent fibroblasts expressing Tax, including transformed Rat1 fibroblasts and non-transformed REFs and 3Y1 rat fibroblasts, undergo apoptosis when deprived of serum and this effect is inhibited by Bcl-2 (Sakurai et al. 1992b, Yamada et al. 1994, Fujita and Shiku 1995) (Section 1.8.7.4). Tax appears to promote cell cycle progression in the absence of external growth factors (Yamada et al. 1994) and thus the response of cells expressing Tax to withdrawal of growth factors is similar to that of cells expressing c-Myc (Evan et al. 1992) (Sections 1.8.6 and 1.8.7). This has led to the hypothesis that Tax activates pathways leading either to proliferation or cell death, depending on the availability of survival factors. Tax might exert this effect directly or indirectly through up-regulation of c-myc or fos (Fujita and Shiku 1993, Smeyne et al. 1993). Since several oncogenic viruses produce proteins that induce proliferation and inhibit apoptosis (Section 4.1.3), it is possible that HTLV-I pX region gene products with currently unknown function could oppose the pro-apoptotic properties of Tax and thus cooperate with Tax in oncogenesis.
4.1.2 c-Myc-model of proliferation and apoptosis

4.1.2.1 Differential responses of rodent fibroblasts to c-Myc expression

The oncogene c-Myc appears to prime cells for either proliferation or apoptosis, depending on whether they are permissive for survival (Evan et al. 1992) (Section 1.8.6). Rat1 cells constitutively expressing c-Myc undergo transformation and have a high rate of proliferation when grown in medium containing normal concentrations of serum (Small et al. 1987). Eilers et al. (1989) transfected Rat1 cells with a gene encoding a chimaeric c-Myc-murine oestrogen receptor (c-Myc-ER) protein, allowing translocation of functional c-Myc to the nucleus when the cells were exposed to oestrogen. Transient expression of c-Myc in the presence of oestrogen resulted in reversible proliferation (conditional transformation) in the absence of other mitogenic stimuli (Eilers et al. 1989). Evan et al. (1992) demonstrated that these Rat1 cells undergo apoptosis when c-Myc expression induced by oestrogen occurs under conditions of serum deprivation. Cells with deregulated expression of c-Myc are unable to withdraw from the cell cycle and arrest growth, but when grown in medium with a low concentration of serum the rate of population increase is slowed due to an increase in the rate of apoptosis. Induction of apoptosis by c-Myc has also been demonstrated in REFs, rat vascular smooth muscle cells and Swiss 3T3 cells (Harrington et al. 1994).

4.1.2.2 Chimaeric c-Myc-modified oestrogen receptor system

The system of c-Myc induction by oestrogen in Rat1-c-myc-ER cells (Section 4.1.2.1) has been improved by substitution of the oestrogen receptor (ER) with a modified receptor (ER™) that responds to 4-hydroxytamoxifen (4-HT) (Littlewood et al. 1995). This removes the need for charcoal-dextran stripping of natural steroid hormones from foetal bovine serum (FBS), previously necessary to prevent constitutive activation of the ER. Cells transfected with chimaeric ER proteins could not be grown in phenol red, whereas this indicator can be included in the medium used to grow cells with the ER™. Therefore, Rat1 cells stably transfected with a chimaeric c-myc-ER™ gene (Rat1-c-myc-ER™ cells) were used in this study (Littlewood et al. 1995).

4.1.3 Inhibition of apoptosis by oncogenic DNA viruses

Several oncogenic viruses express proteins that inhibit apoptosis. These may cooperate with growth-promoting oncoproteins produced by the same viruses. This strategy allows cell proliferation to be switched on without activating pathways that lead to apoptosis. The E1B proteins of adenovirus type 5 block apoptosis induced by the viral oncoprotein E1A; p55\textsuperscript{E1B} binds to and inactivates p53 by promoting degradation through the ubiquitin system, whereas p19\textsuperscript{E1B} inhibits apoptosis by a mechanism similar to that of Bcl-2 (Scheffner et al. 1990, Werness et al. 1990, Rao et al. 1992, White et al. 1992, Lowe and Ruley 1993, Lowe et al. 1994). The simian virus 40 (SV40) large tumour (large T) antigen and the E6 proteins of highly oncogenic human papillomaviruses (HPVs) also bind to and inactivate or promote the degradation of p53 (Lance and Crawford 1979, Sarnow et al. 1982, Metz et al. 1992).
The Epstein-Barr virus (EBV) gene product BHRF1 is a Bcl-2 homologue that inhibits apoptosis and may cooperate with other EBV transforming genes (Henderson et al. 1993, Marshall et al. 1999). Other oncogenic herpesviruses, including human herpesvirus type 8 (HHV-8, Kaposi’s sarcoma-associated virus) and Herpesvirus saimiri, also encode Bcl-2 homologues (Cheng et al. 1997, Nava et al. 1997). SV40 large T and EBV BHRF1 inhibit c-Myc-induced apoptosis (Hermeking et al. 1994, Fanidi et al. 1998). Expression of anti-apoptotic proteins thus appears to be a common strategy in infection by oncogenic DNA viruses. HTLV-I could use a similar mechanism to maintain the survival of infected T cells in vivo.

4.1.4 Investigation of the role of HTLV-I pX genes in proliferation and apoptosis

In this study the hypothesis was tested that the HTLV-I pX gene products Tax, Rex, p21\textsuperscript{Tat}, Tof, p13\textsuperscript{Tat} and p12\textsuperscript{Tat} contribute to leukaemogenesis by modulating apoptosis. One model used to test this hypothesis was the ability of pX gene products, in particular Tax, to induce apoptosis in Rat1 cells under conditions of serum deprivation, similar to c-Myc (Evan et al. 1992). The other model was the ability of Tax or other pX gene products to prevent apoptosis induced by c-Myc in Rat1 cells under conditions of serum deprivation. At the start of the study it was not known that Tax induces apoptosis in rat fibroblasts deprived of serum. However, as this property became apparent, it was realised that Tax provided a parallel model in which to test for anti-apoptotic properties of other pX proteins. The pX genes tax, rex, p21\textsuperscript{Tat}, p30\textsuperscript{Tat}, p13\textsuperscript{Tat} and p12\textsuperscript{Tat} were cloned into the eucaryotic expression vector pcDNAI/Neo and introduced into Rat1-c-myc-ER\textsuperscript{TM} cells (Section 4.1.2.2). When these cells are exposed to 4-HT, the chimaeric protein translocates to the nucleus, allowing expression of c-Myc transcriptional activity. c-Myc activates the cell cycle, inducing proliferation, but at the same time activates the apoptosis pathway, leading to programmed death when cells are deprived of survival factors present in serum (Evan et al. 1992). HTLV-I pX region gene products were tested for their ability to maintain the survival of cells deprived of serum that would otherwise die by apoptosis induced by c-Myc or Tax.

4.2 Methods

4.2.1 Cloning HTLV-I pX genes

4.2.1.1 Source of HTLV-I tax and rex plasmids

Plasmids containing p40\textsuperscript{Tat} (tax) and p27\textsuperscript{Tat} (rex) were kindly provided by Dr Moyra Campbell, Leukaemia Research Fund (LRF) Virus Centre. Competent bacteria (E. coli DH5\textalpha) were transformed with these plasmids and grown on LB agar containing 50 \(\mu\)g/ml ampicillin (Section 3.3.13.2). Large scale preparations of plasmids were purified by CsCl gradient centrifugation (Sections 3.3.14.2 and 3.3.14.3). The 6.6 kb plasmid LRF-MC-p27 contained a 1.6 kb tax insert cloned into pUC18 (2.7 kb) at BamH1 and XbaI sites (Appendix 1). An SphI site within the insert had been deleted to remove the rex initiation codon. The tax insert was flanked by the 255 bp CD3\epsilon promoter and 2.0 kb CD3\epsilon enhancer (Hall et al. 1998).
The 4.8 kb plasmid LRF-MC-p33 contained a 2.1 kb rex insert cloned into pC20R (2.7 kb) at BamHI and HindIII restriction sites (Appendix 1). The rex insert had been subcloned from HindIII sites in pKCR27x (Siomi et al. 1988, Nosaka et al. 1989). The insert expressed Rex but mutations prevented expression of p24 and allowed expression of an inactive, truncated protein comprising the N terminal 60 amino acids of Tax.

4.2.1.2 Amplification and cloning of p24

The HTLV-I p24 gene was amplified by PCR from pMT2, a full-length HTLV-I molecular clone in the vector pUC (Seiki et al. 1982) (Section 3.3.11.2). Primers were designed with BamHI and XbaI restriction sites (Figure 4.1) (Appendix 1) and the PCR was performed under standard conditions with an annealing temperature of 55°C (Section 3.3.7.1). The reaction mix contained dTTP, 1.0 μM each primer and 1.5 mM MgCl₂. The PCR product (356 bp) was ligated into the TA cloning vector pCRII (Invitrogen) (Section 3.3.16.2), grown in E. coli DH5α and the plasmid construct was purified by CsCl gradient centrifugation.

4.2.1.3 Source of HTLV-I p30, p13 and p12 plasmid constructs

Plasmids containing the HTLV-I p30, p13 and p12 genes were kindly provided by Dr. Genoveffa Franchini, Laboratory of Tumor Cell Biology, National Institutes of Health, Bethesda, Maryland, USA. These consisted of tagged pX inserts cloned into the 3.2 kb vector HCMV-HSpA: p30-HA1, p13-HA1 and p12-AU1. HCMV-HSpA contains the pUC19 polylinker cloned into HindIII/SmaI sites of HCMV-SEAP (Schwartz et al. 1990) (Appendix 1). Competent E. coli DH5α were transformed with the p30-HA1, p13-HA1 and p12-AU1-HCMV-HSpA constructs. Transformants were grown on LB agar containing 50 μg/ml ampicillin and the plasmids were purified by CsCl gradient centrifugation then quantified by spectrophotometry.

4.2.1.4 Subcloning HTLV-I pX genes into pcDNA1/Neo

The 7.0 kb (6,969 bp) vector pcDNA1/Neo (Invitrogen) was used for expression of HTLV-I pX gene products in eukaryotic cells. Genes cloned into this vector are constitutively expressed from the human cytomegalovirus (HCMV) promoter. pcDNA1/Neo has a neomycin resistance gene, permitting selection in eukaryotic cells with Geneticin (G418, Life Technologies).

The HTLV-I pX inserts tax, rex, p21, p30, p13 and p12 were subcloned from their source vectors into pcDNA1/Neo using RE sites that permitted directional cloning (Table 4.1). The tax, p24, p30, p13 and p12 inserts were excised from their respective vectors using BamHI and XbaI and the rex insert was excised from LRF-MC-p27 by digestion with BamHI and EcoRV (Section 3.3.15). Inserts were separated from the vector backbone by agarose gel electrophoresis and gel purified (Gelase, Cambio) (Section 3.3.10.3). BamHI/XbaI and BamHI/EcoRV restriction endonuclease (RE) digests of pcDNA1/Neo were prepared and gel-purified at the same time. Inserts were ligated into pcDNA1/Neo using T4 ligase (Section 3.3.14).
**Figure 4.1: Specific oligonucleotide primers for amplification of the HTLV-I p21<sup>III</sup> sequence by the polymerase chain reaction.**

<table>
<thead>
<tr>
<th>LRF oligonucleotide number</th>
<th>Primer sequence 5' to 3' with HTLV-I nucleotide position</th>
<th>Length (b)</th>
<th>$T_m$ (°C)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HTLV-I p21&lt;sup&gt;III&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190 Sense</td>
<td>7499 GCCGAGTCCAMGGACGGTTATCGGTCCA 7518</td>
<td>30</td>
<td>38</td>
<td>356</td>
</tr>
<tr>
<td>191 Antisense</td>
<td>7834 GCCCTCTAGATCGGGGCGGAGGGGC 7815</td>
<td>30</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>XbaI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2.1.5 Transformation of MC1061/P3 with pcDNAI/Neo constructs

Competent bacteria of E coll\i\ strain MC1061/P3 (Ultracomp E coli, Invitrogen) were transformed with pcDNAI/Neo alone or with pcDNAI/Neo containing ligated inserts according to a modification of the protocol outlined in Section 3.3.13.2. Bacteria that had been stored at -80°C were thawed on ice and 100 μl transferred to a 5 ml polypropylene tube (2097, Falcon). A fresh solution of 2-mercaptoethanol (2-ME) was added to the bacteria to a final concentration of 25 mM. Ligation reaction products (5 μl) were mixed with the bacteria and held on ice for 30 min. The tubes were treated at 42°C in a water bath for 75 sec and then placed on ice for 2 min. SOC medium (900 μl) (Appendix 5: A5.10.1) was added and the transformed bacteria were incubated at 37°C for 1 hour on an orbital incubator (225 rpm). After centrifuging at 400 g (1500 rpm) in a bench centrifuge (BR401, Denley), the supernatant was discarded and the bacteria were resuspended in 200 μl SOC medium. Equal volumes were spread on LB agar plates containing 25 μg/ml ampicillin or 10 μg/ml tetracycline (Appendix 5: A5.10). Growth on LB agar containing ampicillin, with no growth on medium containing tetracycline, was an indication of successful transformation without reversion of MC1061/P3 from type.

4.2.1.6 Selection and purification of pcDNAI/Neo pX constructs

Small scale preparations of plasmid DNA (Section 3.3.14.1) followed by RE digestion were used to select MC1061/P3 transformants that contained the desired pcDNAI/Neo constructs. A large scale preparation of each plasmid was made by alkaline lysis and CsCl density gradient centrifugation, then quantified by spectrophotometry (Sections 3.3.14.2 and 3.3.14.3). Inserts were sequenced in both directions at the University of Birmingham (Alta Bioscience) using T7 and SP6 sequencing primers (Appendix 5: A5.12.1). Each purified plasmid construct was diluted to 0.4 μg/μl in preparation for transfection of Rat1 cells.

4.2.2 Transfection of Rat1 cells

4.2.2.1 Culture of Rat1 and Rat1-c-myc-ER™ cells

Rat1 and Rat1-c-myc-ER™ cells were obtained from Dr Gerard Evan, Imperial Cancer Research Fund (ICRF), Lincoln's Inn Fields, London (Littlewood et al 1995). Rat1 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies) containing 10% heat-inactivated foetal bovine serum (hi-FBS), 400 mg/ml streptomycin, 400 U/ml penicillin, and 2 mM L-glutamine (Appendix 4: A4.2.1.3). Rat1-c-myc-ER™ cells were grown in the same medium containing 5 μg/ml puromycin to maintain selection for the transfected genotype. These cell lines formed adherent monolayers and were subcultured by trypsinisation with 1.5 to 3 ml 0.25% crude trypsin in 1 M EDTA (trypsin-EDTA) (Section 2.3.3.1). Cells cultured in sealed flasks were aerated with 5% CO₂ and cells grown in tissue culture dishes (Nunclon, Nunc) were grown in a 5% CO₂ incubator at 37°C.
Table 4.1 Plasmids used in studying the role of HTLV-I pX genes in apoptosis.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Size of vector (kb)</th>
<th>Insert</th>
<th>Size of insert (bp)</th>
<th>Insertion site</th>
<th>Tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tax-pcDNA1/Neo</td>
<td>pcDNA1/Neo</td>
<td>7.0</td>
<td>HTLV-I p40&lt;sub&gt;IV&lt;/sub&gt;</td>
<td>2600</td>
<td>BamHI/XbaI</td>
<td>–</td>
</tr>
<tr>
<td>Rex-pcDNA1/Neo</td>
<td>pcDNA1/Neo</td>
<td>7.0</td>
<td>HTLV-I p27&lt;sub&gt;III&lt;/sub&gt;</td>
<td>2100</td>
<td>BamHI/EcoRV</td>
<td>–</td>
</tr>
<tr>
<td>p21&lt;sup&gt;III&lt;/sup&gt;-pcDNA1/Neo</td>
<td>pcDNA1/Neo</td>
<td>7.0</td>
<td>HTLV-I p21&lt;sub&gt;III&lt;/sub&gt;</td>
<td>2100</td>
<td>BamHI/XbaI</td>
<td>–</td>
</tr>
<tr>
<td>p30&lt;sup&gt;II&lt;/sup&gt;-TSP-pcDNA1/Neo</td>
<td>pcDNA1/Neo</td>
<td>3.2</td>
<td>HTLV-I p30&lt;sup&gt;II&lt;/sup&gt;</td>
<td>770</td>
<td>BamHI/XbaI</td>
<td>TSP</td>
</tr>
<tr>
<td>p13&lt;sup&gt;III&lt;/sup&gt;-HA1-pcDNA1/Neo</td>
<td>pcDNA1/Neo</td>
<td>3.2</td>
<td>HTLV-I p13&lt;sup&gt;III&lt;/sup&gt;</td>
<td>380</td>
<td>BamHI/XbaI</td>
<td>HA1</td>
</tr>
<tr>
<td>p12&lt;sup&gt;II&lt;/sup&gt;-AU1-pcDNA1/Neo</td>
<td>pcDNA1/Neo</td>
<td>3.2</td>
<td>HTLV-I p12&lt;sup&gt;II&lt;/sup&gt;</td>
<td>430</td>
<td>BamHI/XbaI</td>
<td>AU1</td>
</tr>
<tr>
<td>pcDNA1/Neo</td>
<td>pcDNA1/Neo</td>
<td>7.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Tag:**
- AU1: Bovine papillomavirus type 1 AU1 epitope (DTYRYI) (~30 bp)
- HA1: Influenza virus haemagglutinin epitope (YPYDVPDYASL) (~50 bp)
- TSP: Thrombospondin epitope (CSVTGC) (~30 bp)
- –: Not present
4.2.2.2 Transfection of Rat1 cells with plasmid constructs

Rat1-c-myc-ER™ cells were transfected with 1 μg of each pcDNA1/Neo plasmid construct (Section 4.4.1). Rat1 cells without the c-myc-ER™ gene were also transfected with selected plasmids. Some cultures were transfected with two active plasmids, for example p12^1-AU1-pcDNA1/Neo and Tax-pcDNA1/Neo, in which case 1 μg of each plasmid was co-transfected.

Cells were grown to 50% confluence in 10 cm diameter tissue culture dishes, then the medium was withdrawn and replaced with 10 ml serum-free DMEM. Plasmid DNA (1 μg) was diluted in 50 μl sterile ultrapure water in a 1.5 ml sterile polypropylene microcentrifuge tube. In a separate 1.5 ml tube 20 μl liposomal transfectant reagent (LipofectAMINE, Life Technologies) was mixed with 30 μl sterile ultrapure water. The DNA and liposomes were combined in a 15 ml screw-top polypropylene centrifuge tube and held at room temperature for 30 min to allow complexes to form. The 100 μl volume containing DNA-liposome complexes was then poured gently into the tissue culture dish containing cells in serum-free medium, mixed by gentle swirling and incubated at 37°C for 5 hours in a CO₂ incubator.

The serum-free medium was replaced with DMEM + 20% hi-FBS and the cells were grown in this medium with daily replacement for two days. The cells were trypsinised, serial two-fold dilutions prepared in DMEM + 10% hi-FBS and re-seeded into a series of six tissue culture dishes at 1 x 10^1 to 1 x 10^3 cells/ml. After one day in culture, the medium was replaced with medium containing 5 μg/ml puromycin and 1 mg/ml Geneticin to select for transfectants while maintaining the c-myc-ER™ phenotype. This medium was replaced after 3 days.

4.2.2.3 Selection of transfected Rat1 clones

Clones were selected 7 days after cells had been reseeded at different densities on tissue culture dishes. Colonies derived from a single cell were identified, the medium was removed and plates were rinsed briefly with 1.5 ml trypsin-EDTA. Marked colonies were isolated using metal or plastic cloning rings affixed to the bottom of the tissue culture dishes with sterile Vaseline.

Trypsin-EDTA (200 μl) was pipetted into the well formed by each ring and the tissue culture dishes were incubated at 37°C for 10 min in 5% CO₂ to dissociate the cells. The solution in each well was pipetted up and down to dislodge remaining adherent cells and the trypsinised cells were transferred to a flask containing 5 ml DMEM + 10% hi-FBS with puromycin and Geneticin at standard concentrations. Each transfectant was subjected to a second round of clonal selection. These cell lines were maintained in medium containing puromycin and Geneticin at 37°C in 5% CO₂ and trypsinised for subculturing or to prepare cells for seeding of tissue culture dishes.
4.2.3 Detection of pX mRNA expression

4.2.3.1 Isolation of RNA from Rat1 cells

RNA was extracted using the acid guanidinium thiocyanate method with phenol-chloroform extraction (Chomczynski and Sacchi 1987). Rat1 cells and Rat1-c-myc-ER™ cells transfected with HTLV-I pX plasmid constructs were grown to near-confluency in 75 cm² tissue culture flasks. The medium was removed and 4 ml RNAzol B (Life Technologies) was added to lyse the cells (Appendix 5: A5.13.1). The lysate was pipetted up and down several times to dislodge adherent cells, then transferred to a 15 ml polypropylene centrifuge tube. The mixture was shaken vigorously with 200 µl chloroform for 15 sec, placed on ice for 5 min and centrifuged at 1,500 g (2,700 rpm) in a GPR bench centrifuge (Beckman) with a GH 3.7 swinging bucket rotor for 15 min at 4°C.

The upper aqueous phase containing dissolved RNA was transferred to one or more 1.5 ml microcentrifuge tubes and the RNA was precipitated by mixing with an equal volume of isopropanol and holding at 4°C for 15 min. Tubes were centrifuged at 10,600 g (13,000 rpm) for 5 min in a bench microcentrifuge (MicroCentaur) at 4°C, then the RNA pellet was washed with 70% ethanol in ultrapure water containing 0.05% diethyl pyrocarbonate (DEPC) and re-centrifuged.

The tubes were desiccated under vacuum for 10 min then dissolved in ultrapure water containing 1 mM EDTA pH 7.0, 0.5% SDS and 0.05% DEPC. The concentration was determined by spectrophotometry with calculations made on the basis that 40 µg/ml of single-stranded RNA has an optical density at 260 nm (OD₂₆₀) of approximately 1.0.

4.2.3.2 Northern blot hybridisation

Northern blot hybridisation to detect expression of HTLV-I pX mRNA in transfected cells was performed by Dr Moyra Campbell in the LRF Virus Centre. A 10 µl volume containing 10 µg RNA was mixed with 20 µl formamide buffer (Appendix 5: A5.13.2), heated at 85°C for 15 min to denature the RNA, then placed on ice for 5 min. RNA loading buffer/dye (2 µl) was added and the samples were separated by gel electrophoresis (5 V/cm) on a 1% agarose gel in 3-(N-morpholino)-2-hydroxypropane sulphonic acid (MOPS) buffer 1x with 6% formaldehyde prepared using ultrapure water with 0.05% DEPC (Appendix 5: A5.13.2). λHindIII and φX174/HaeIII (Life Technologies) were used as size markers in combination with visualisation of 28S and 18S ribosomal RNA bands after staining with ethidium bromide.

The RNA was transferred to nylon membrane (Hybond-N, Amersham) by Northern blotting using SSC 20x as the transfer buffer, rinsed in SSC 3x, then cross-linked with ultraviolet (UV) light (Section 3.3.10) (Appendix 5: A5.7). Blots were hybridised to the HTLV-I probe pMT2, labelled with α³²P dCTP by random priming (Sections 3.3.11.2 and 3.3.11.4). The following formula was used for calculation of the T_m for RNA:RNA hybridisations (Section 3.3.12.1):
Formula 4.1: $T_m = 87 + 16.6 \log_{10}(M') + 0.8(\%G+C) - \frac{500}{L} - P - 0.5(\%Formamide) \degree C$

Where: $[M'] = \frac{\text{Adjusted molar Monovalent cation concentration}}{1 + 0.7 [Na^+]}$

$\%G+C = \text{Percentage of G and C nucleotides in the DNA probe}$

$L = \text{Length of the DNA:RNA duplex in bp}$

$P = \text{Percentage base mismatch}$

The calculated $T_m$ for pMT2j labelled with $\alpha^{32}$P dCTP by random priming was 74°C. Hybridisation was performed overnight at 37°C in standard hybridisation solution containing SSC 3x with 50% formamide, as for Southern blot (SB) hybridisation (Section 3.3.12) (Appendix 5: A5.9). The high stringency washes were performed with SSC 0.5x, 0.1% SDS. Autoradiographic film was exposed for 3 days.

4.2.4 Induction of apoptosis

4.2.4.1 Serum starvation of Rat1 and Rat1-c-myc-ER™ cells

Rat1 cells, Rat1-c-myc-ER™ cells (selected with puromycin) and cells transfected with HTLV-1 pX pcDNA1/Neo constructs (selected with puromycin and Geneticin) were trypsinised and resuspended in DMEM + 10% hi-FBS. Serial two-fold dilutions were prepared and 500 µl aliquots were pipetted into duplicate wells of an eight well chamber slide (ChamberSlide, Lab-Tek, Nunc). This resulted in a series of approximately $2 \times 10^5$, $1 \times 10^5$, $5 \times 10^4$ and $2.5 \times 10^4$ cells in each of two duplicate rows.

The chamber slides were incubated at 37°C in 5% CO$_2$ for 24 hours to allow cells to adhere to the substrate. The medium was aspirated from each well and replaced with 500 µl DMEM without serum. Cells were maintained in this medium for 48 hours to undergo cell cycle arrest.

4.2.4.2 Induction of c-Myc-ER™ expression

Nuclear expression of the c-Myc-ER™ protein was activated in Rat1-c-myc-ER™ cells on day 2 of serum starvation by the addition of 4-HT to a final concentration of 0.1 µM. Cells in 500 µl medium in each well of one row of a chamber slide were induced by adding 5 µl of 10 µM 4-HT. Cells in the duplicate row remained uninduced as negative controls. Experiments were performed twice with duplicate cultures in each experiment.
4.2.4.3. Microscopic examination

Cells were examined microscopically using an inverted light microscope (Labovert, Leitz) 6, 24 and 48 hours following withdrawal of serum and 6 and 24 hours following induction of c-Myc-ER™ expression with 4-HT.

Morphological changes occurring in cells undergoing apoptosis include loss of cell-cell contact, cell shrinkage, condensation of chromatin in the nucleus and disintegration of the nucleolus and nuclear membrane (Kerr et al 1972) (Section 1.8.7.1). As the dying cell disintegrates, the cell contents are fragmented to produce membrane-bound apoptotic bodies.

4.2.4.4 Staining with propidium iodide

Cells were stained with propidium iodide (PI) 24 or 48 hours following withdrawal of serum and 6 and 24 hours following induction with 4-HT. PI is a fluorescent vital dye that does not cross the plasma membrane of viable cells. In dying cells in the late stages of apoptosis, PI enters the cell and intercalates DNA. It also stains DNA within apoptotic bodies. The dye fluoresces in the orange range of the spectrum.

Medium was removed from the wells of chamber slides to be stained with PI and the cells were fixed with 200 µl acetic acid-alcohol fixative for 30 min at room temperature (Appendix 4; A4.8). The fixative was aspirated and replaced with 200 µl 4 M HCl for 30 min at room temperature to denature the DNA. This solution was replaced with 0.1 M sodium borate for 5 min to neutralise the cells. After aspiration, the chamber slides were air dried for 5 min. The cells were then incubated at 37°C for 30 min in 200 µl of a solution containing 5 µg/ml PI and 100 µg/ml RNase A in PBS 1x. This solution was removed and the walls of the chambers were removed from the glass slide along with the gasket.

Approximately 100 µl of mounting solution was pipetted onto each slide, overlaid with a glass coverslip and sealed at the edges with clear nail varnish. The mounting solution (Vectashield, Vector Laboratories) contained glycerol and PBS 1x at a ratio of 9:1 with 1,4-diazobicyclo(2,2,2)-octane (DABCO) as an antiquenching agent (Johnson et al 1982). The slide was wrapped in aluminium foil to protect the cells from light and held at 4°C for 5 to 10 min for the mounting solution to set. The slides were examined under oil immersion using a fluorescent microscope (Laborlux K, Leitz) with a 562 to 588 nm band pass (green) filter.
4.3 Results

4.3.1 Detection of HTLV-I pX expression in Rat1 cells

The HTLV-I pX genes tax, rex, p21\textsuperscript{\textprime}, p30\textsuperscript{\textprime}, p13\textsuperscript{\textprime} and p12\textsuperscript{\textprime} were cloned into pcDNAI/Neo and transfected into Rat1 and Rat1-c-myc-ER\textsuperscript{TM} cells. Each plasmid construct was transfected alone and the Tax-pcDNAI/Neo construct was also co-transfected with each of the other pX-pcDNAI/Neo plasmids. Cells transfected with pcDNAI/Neo without an insert were used as negative controls. Transfectants were selected by cultivation in medium containing Geneticin and puromycin. RNA extracted from transfected cell cultures was hybridised to the HTLV-I probe pMT2\textsuperscript{\textprime}, labelled with \textsuperscript{32}P dCTP by random priming. Positive hybridisation to bands of the appropriate size was detected in all transfected cell lines (Table 4.1), whereas non-transfected Rat1 cells were negative. Cells co-transfected with pX-pcDNAI/Neo and one other pX plasmid had two bands corresponding to the respective inserts. The assay for pX mRNA expression was not quantitative but the strongest bands were detected with tax, rex and p21\textsuperscript{\textprime}.

4.3.2 Induction of apoptosis by expression of c-Myc

Rat1 cells and Rat1-c-myc-ER\textsuperscript{TM} cells grew to confluence when cultivated in DMEM + 10% hi-FBS, forming adherent monolayers in tissue culture flasks and dishes. When serum was withdrawn, there was cessation of proliferation and spreading in non-confluent cultures. Confluent cultures, which had stopped proliferating due to contact inhibition in the presence of serum, exhibited no obvious morphological changes by light microscopy when serum was withdrawn (Figure 4.2). Addition of 4-HT to Rat1-c-myc-ER\textsuperscript{TM} cultures grown in the absence of serum resulted in cell death, with rounding up and detachment of fibroblasts, while cells that remained adherent had attenuated cytoplasmic extensions (Figure 4.3). Cell death was visible initially at 6 hours and more than 50% of cells had died and detached by 24 hours. These changes were also observed consistently in Rat1-c-myc-ER\textsuperscript{TM} cells transfected with the vector pcDNAI/Neo that did not contain an insert and acted as a negative control. Addition of 4-HT to confluent Rat1-c-myc-ER\textsuperscript{TM} cells in the presence of serum had no visible effect within the time frame of observations. Addition of 4-HT had no effect on Rat1 cells cultured with or without serum.

4.3.3 Induction of apoptosis by expression of Tax

Transfection of cells with Tax-pcDNAI/Neo would have resulted in constitutive expression of Tax from the HCMV promoter. Rat1 and Rat1-c-myc-ER\textsuperscript{TM} cells expressing Tax exhibited mild piling up (focus formation), suggestive of transformation, with occasional large multinucleate cells scattered through cultures (Figure 4.4). When serum was withdrawn from these cultures, cell death was visible initially at 24 hours and by 48 hours 50 to 90% of cells had died (Table 4.2) (Figure 4.5). It was not possible to perform experiments with induction of c-Myc expression by 4-HT in these cells according to the selected protocol. However, the addition of 4-HT at 24 or 48 hours following withdrawal of serum did not alter the fate of Rat1 and Rat1-c-myc-ER\textsuperscript{TM} cells transfected with Tax-pcDNAI/Neo (Table 4.2) (Figure 4.6).
4.3.4 Effects of Rex, p21\textsuperscript{II}, p30\textsuperscript{II}, p13\textsuperscript{II} and p12\textsuperscript{I} on c-Myc and Tax-induced apoptosis

Constitutive expression of Rex, p21\textsuperscript{II}, Tof, p13\textsuperscript{II} and p12\textsuperscript{I} from the pcDNAI/Neo HCMV promoter had no effect on Rat1 or Rat1-c-myc-ER\textsuperscript{TM} cells transfected with single plasmids containing any one of these genes (Table 4.2). In the presence of serum Rat1-c-myc-ER\textsuperscript{TM} cells transfected with these genes continued to proliferate and spread until confluent, whereas withdrawal of serum resulted in cessation of growth, similar to non-transfected cells. When Rat1-c-myc-ER\textsuperscript{TM} cells expressing these genes were exposed to 4-HT under conditions of serum deprivation, the cells died in a temporal pattern that was similar to non-transfected cells (Figure 4.7). Thus, there was no inhibition of c-Myc-induced apoptosis by any HTLV-I pX genes.

Rat1-c-myc-ER\textsuperscript{TM} cells were co-transfected with Tax-pcDNAI/Neo and pcDNAI/Neo expressing Rex, p21\textsuperscript{II}, p30\textsuperscript{II} (Tof) p13\textsuperscript{II} or p12\textsuperscript{I}. No morphological changes were evident in these cultures that were different from cells transfected with Tax-pcDNAI/Neo alone. When serum was withdrawn, the cells died with a time course and morphological pattern similar to that of Rat1 and Rat1-c-myc-ER\textsuperscript{TM} cells expressing Tax alone (Figure 4.8). Thus, Rex, p21\textsuperscript{II}, Tof, p13\textsuperscript{II} and p12\textsuperscript{I} did not oppose the apoptotic effects of Tax (Table 4.2).

4.3.5 Staining with propidium iodide

Staining of nuclei with propidium iodide (PI) in detaching and degenerating cells was visible by fluorescence microscopy in only small numbers of Rat1-c-myc-ER\textsuperscript{TM} cells from confluent monolayers grown in the presence of serum or 24 and 48 hours following withdrawal of serum (Figure 4.9). There was increased intranuclear staining in serum-deprived Rat1-c-myc-ER\textsuperscript{TM} cells stained 6 hours after treatment with 4-HT, as well as staining of DNA in fragments of degenerating cells (apoptotic bodies) (Figure 4.10). Serum-deprived Rat1-c-myc-ER\textsuperscript{TM} cells stained with PI 48 hours after treatment with 4-HT exhibited extensive degeneration and detachment. PI-stained DNA was visible in adherent debris and in many cells that remained attached. However, most degenerating cells were washed away during the staining procedure.

Rat1-c-myc-ER\textsuperscript{TM} cells transfected with Tax-pcDNAI/Neo had mild piling up and occasional multinucleate cells when grown in the presence of serum (Figure 4.11). Only a few degenerating cells with condensed nuclei or apoptotic bodies were visible. Rat1-c-myc-ER\textsuperscript{TM} cells transfected with Tax-pcDNAI/Neo exhibited extensive degeneration and detachment of cells when PI staining was performed 24 and 48 hours after withdrawal of serum. PI staining of DNA was visible in fragmented cells, as well as attenuated cells that remained adherent (Figure 4.12). Induced expression of c-Myc in serum-deprived cells resulted in extensive degeneration and detachment, with numerous apoptotic bodies visible in PI-stained slides. c-Myc-induced apoptosis was not inhibited by Rex, p21\textsuperscript{II}, p30\textsuperscript{II} (Tof) p13\textsuperscript{II} or p12\textsuperscript{I} (Figure 4.13). Similarly, apoptosis induced by Tax in serum-deprived Rat1-c-myc-ER\textsuperscript{TM} cells was not inhibited by Rex, p21\textsuperscript{II}, p30\textsuperscript{II} (Tof) p13\textsuperscript{II} or p12\textsuperscript{I} (Figure 4.14).
Table 4.2 Effect of induced expression of c-Myc following withdrawal of serum on survival of Rat1-c-myc-ER\textsuperscript{TM} cells constitutively expressing HTLV-I pX region genes.

<table>
<thead>
<tr>
<th>HTLV-I pX gene expression</th>
<th>Normal serum</th>
<th>Low serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c-Myc−</td>
<td>c-Myc−</td>
</tr>
<tr>
<td>pcDNA1/Neo</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p12\textsuperscript{1}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p13\textsuperscript{II}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p30\textsuperscript{II}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p21\textsuperscript{III}</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rex</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tax</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tax + p12\textsuperscript{1}</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tax + p13\textsuperscript{II}</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tax + p30\textsuperscript{II}</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tax + p21\textsuperscript{III}</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Tax + Rex</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

- c-Myc−: No c-Myc expression
- c-Myc+: c-Myc expression induced by 4-HT
- +: Survival
- −: Death by apoptosis
- *: Many cells already dead at time of addition of 4-HT
- ND: Not done
Figure 4.2: Rat1-c-myc-ER™ cells following withdrawal of serum.

Rat1-c-myc-ER™ cells grown in the presence of serum had multiplied and spread across the bottom of the tissue culture flask to form a confluent monolayer that had ceased proliferating due to contact inhibition. These cells remained stable following withdrawal of serum. A few rounded, detached cells are visible (arrow). Original 200x magnification.
Figure 4.3: Apoptosis in serum-starved Rat1-c-myc-ER\textsuperscript{TM} cells following induction of c-Myc expression.

c-Myc expression was induced in Rat1-c-myc-ER\textsuperscript{TM} cells by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 6 hours following c-Myc induction there is attenuation (arrows), rounding up (arrow heads) and detachment of fibroblasts dying by apoptosis. Original 200x magnification.
Figure 4.4: Rat1-c-myc-ER™ cells expressing Tax grown in serum.

Rat1-c-myc-ER™ cells transfected with Tax-pcDNAI/Neo were grown in the presence of serum. These cells proliferated to form a confluent monolayers with mild piling up (focus formation) (arrow) and occasional multinucleate cells (arrow head). A few rounded, detached cells are visible. Original 200x magnification.
Figure 4.5: Effect of withdrawal of serum on Rat1-c-myc-ER™ cells expressing Tax.

Rat1-c-myc-ER™ cells transfected with Tax-pcDNAI/Neo formed confluent monolayers when grown in the presence of serum. At 24 hours following withdrawal of serum there is extensive cell death characterised by attenuation (arrows), rounding up (arrow heads) and detachment. Original 200x magnification.
Figure 4.6: Effect of induction of c-Myc expression in serum-starved Rat1-c-myc-ER™ cells expressing Tax.

Rat1-c-myc-ER™ cells transfected with Tax-pcDNAI/Neo experienced apoptosis following withdrawal of serum (Figure 4.5). When c-Myc was induced in these cells by addition of 4-hydroxytamoxifen 24 hours following withdrawal of serum, there was no alteration in the pattern of cell death. Cells exhibit attenuation (arrow), rounding up (arrow heads) and detachment. Original 200x magnification.
Figure 4.7: Effect of induction of c-Myc expression on serum-starved Rat1-c-myc-ER™ cells expressing p12†.

c-Myc expression was induced in Rat1-c-myc-ER™ cells transfected with p12†-pcDNAI/Neo by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 24 hours after induction of c-Myc dying cells have rounded up and detached from the substrate. Original 200x magnification.
Figure 4.8: Effect of serum starvation on Rat1-c-myc-ER™ cells expressing HTLV-I Tax and p12'.

Rat1-c-myc-ER™ cells co-transfected with Tax-pcDNAI/Neo and p12'-pcDNAI/Neo were allowed to grow to near-confluence in the presence of serum. At 24 hours following withdrawal of serum, a substantial proportion of cells are dying, characterised by rounding up and detachment. Expression of p12' has not inhibited cell death induced by Tax in serum-starved cells. Original 200x magnification.
Figure 4.9: Propidium iodide staining of Rat1-c-myc-ER$^{TM}$ cells following withdrawal of serum.

Near-confluent monolayer of Rat1-c-myc-ER$^{TM}$ cells grown in the presence of serum shows survival of most cells 48 hours after withdrawal of serum. Cells detached from two sites in the field have been washed away during the staining procedure (arrows). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.
c-Myc expression was induced in Rat1-c-myc-ER\textsuperscript{TM} cells by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 6 hours following c-Myc induction there are fragmented nuclear bodies containing condensed DNA that have arisen from fibroblasts dying by apoptosis (arrows). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.
Rat1-c-myc-ER\textsuperscript{TM} cells transfected with Tax-pcDNA1/Neo and grown in the presence of serum form a near-confluent monolayer with mild piling up and some multinucleate cells. A single rounded, condensed nucleus from a dead cell can be seen (arrow). A mitotic figure is visible in a dividing cell (arrow head). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.
Figure 4.12: Propidium iodide staining of Rat1-c-myc-ER™ cells expressing Tax following withdrawal of serum.

Rat1-c-myc-ER™ cells transfected with Tax-pcDNA1/Neo photographed 24 hours following withdrawal of serum exhibit attenuation (arrows) and formation of apoptotic bodies in degenerating nuclei of dying cells (arrow heads). Detached cells have been washed away during the staining procedure. Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.
c-Myc expression was induced in Rat1-c-myc-ER™ cells transfected with p12'-pcDNA1/Neo by addition of 4-hydroxytamoxifen 48 hours following withdrawal of serum. At 24 hours after induction of c-Myc there are multiple foci of cell loss with numerous degenerating cells and apoptotic bodies (arrows). Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.
Figure 4.14: Propidium iodide staining of Rat1-c-myc-ER™ cells expressing HTLV-I Tax and p12\textsuperscript{i} following serum starvation.

Serum was withdrawn from near-confluent Rat1-c-myc-ER™ cells co-transfected with Tax-pcDNAI/Neo and p12\textsuperscript{i}-pcDNAI/Neo. At 24 hours following serum deprivation there are multiple foci of cell loss and formation of apoptotic bodies in degenerating cells (arrows). Expression of p12\textsuperscript{i} has not inhibited cell death induced by Tax in serum-starved cells. Propidium iodide staining. Fluorescence microscopy. Original 400x magnification.
4.4 Discussion

In this study combinations of HTLV-I pX genes were transfected into Rat1-c-myc-ER™ cells and tested for their ability to protect these cells from apoptosis following activation of c-Myc by 4-HT under conditions of serum deprivation. Rat1 and Rat1-c-myc-ER™ cells survived but ceased growing when serum was withdrawn. Activation of c-Myc in these cells under conditions of serum deprivation resulted in cell death due to apoptosis (Evan et al., 1992). Constitutive expression of Tax induced cell death in Rat1 and Rat1-c-myc-ER™ cells following withdrawal of serum, consistent with the observations of other groups using rodent fibroblasts in different experimental systems (Sakurai et al., 1992b, Yamada et al., 1994, Fujita and Shiku, 1995). Within the constraints of the temporal pattern of cell death in serum-starved Rat1-c-myc-ER™ cells expressing Tax, the fate of these cells was unaffected by induced expression of c-Myc.

Constitutive expression of Rex, p21Ⅲ, Tof, p13Ⅱ and p12Ⅰ in Rat1-c-myc-ER™ cells did not have any detectable effect on proliferation or survival when cells were cultivated in normal or reduced concentrations of serum. Co-expression of these gene products with Tax did not prevent Tax-induced apoptosis of serum-starved Rat1-c-myc-ER™ cells. Similarly, constitutive expression of Rex, p21Ⅲ, Tof, p13Ⅱ or p12Ⅰ did not prevent apoptosis induced in serum-starved Rat1-c-myc-ER™ cells by activation of c-Myc. Therefore, no role in induction or inhibition of apoptosis by Rex, p21Ⅲ, Tof, p13Ⅱ or p12Ⅰ was demonstrated in this experimental system and these gene products did not appear to cooperate with Tax or c-Myc. It is uncertain whether prevention of apoptosis is one of the oncogenic mechanisms used by HTLV-I.

In this study cell death due to apoptosis was demonstrated by visible changes in cells by standard light microscopy and by examination of PI-stained cells by fluorescence light microscopy. These changes are largely qualitative. Quantification of apoptosis could be achieved by a variety of techniques, including the use of video time-lapse photography to follow the fate of individual cells (Evan et al., 1992), flow cytometry analysis of DNA strand breaks labelled with bromo-dUTP (Li and Darzynkiewicz, 1995), detection of phosphatidylserine expression on apoptotic cells by flow cytometry using annexin V (Koopman et al., 1994) and staining of DNA strand breaks in cells on slides stained by the terminal deoxynucleotidyl transfer-mediated dUTP-biotin nick end labelling (TUNEL) (Kasagi et al., 1994). Use of these techniques could allow more subtle effects resulting from interactions between HTLV-I pX gene products to be detected.

HTLV-I pX mRNA expression was detected in transfected Rat1-c-myc-ER™ cells in this study by Northern blot hybridisation. Expression of mRNA does not necessarily imply expression of a functional protein. The observed effects in Tax-pcDNAI/Neo-transfected cells deprived of serum indicate that functional Tax was expressed in these cells. However, it would be desirable to demonstrate expression of Rex, p21Ⅲ, Tof, p13Ⅱ and p12Ⅰ proteins by functional or immunological assays to be confident that the apparent absence of effects due to these gene products was not due to lack of expression of active proteins. Monoclonal antibodies (Mabs) could be used to stain specific HTLV-I pX proteins or Mabs against amino acid tags such as AU1, HA1 and TSP in Tof, p13Ⅱ and p12Ⅰ (Table 4.1) could be used as
markers for protein expression. In 2003 an attempt was made to revive 35 transfected cell lines that had been stored in liquid nitrogen to test for expression of HTLV-I pX proteins but none of the cell lines were viable.

This study demonstrated that Tax induces apoptosis in Rat1 cells deprived of serum and that other HTLV-I pX gene products did not prevent apoptosis induced by either Tax or c-Myc under these conditions. The implications of these results and potential future studies are discussed further in Chapter 5.
5.1 Investigation of the role of retroviruses in human T cell leukaemias and lymphomas

The main aim of this project was to determine if human T lymphotropic virus type I (HTLV-I) or related retroviruses are associated with cutaneous T cell lymphomas (CTCLs), particularly mycosis fungoides (MF) and Sézary syndrome (SS). Additional aims were to determine if HTLV-I-related viruses are involved in large granular lymphocytic (LGL) leukaemia and if infection with bovine leukaemia virus (BLV) or other members of the HTLV/BLV group of viruses is present in selected cases of adult acute lymphoblastic leukaemia (ALL). The possibility that undiscovered retroviruses might be involved in human T cell leukaemias and lymphomas was also considered. Identifying an association between a virus and a neoplasm is a first step in confirming the aetiology of virus-induced neoplasia. A final aim of the project was to determine if HTLV-I pX region gene products are able to prevent apoptosis. Identifying anti-apoptotic properties in pX proteins with currently unknown function would contribute to an understanding of the mechanisms of HTLV-I leukaemogenesis.

MF is an epidermotropic CD4+ CTCL and SS is considered to be the leukaemic form of the same disease (Edelson 1980). These neoplasms were targeted for investigation because they have clinical and pathological similarities to some forms of adult T cell leukaemia/lymphoma (ATL), an aggressive CD4+ T cell neoplasm that is caused by HTLV-I (Blattner et al 1982, Hinuma et al 1982b, Nagatani et al 1990). Infections with HTLV-I and HTLV-II have been demonstrated in some patients with LGL leukaemia and therefore samples from cases of this lymphoproliferative disorder were included in the study (Loughran et al 1992, Martin et al 1993). Cases of acute ALL in butchers from the Cardiff area were examined for evidence of infection with BLV because of their occupational exposure to cattle (Whittaker 1991). A combination of cell culture and molecular biology techniques was used in an attempt to identify HTLV-I and related viruses in these neoplasms.

Many other groups have attempted to establish associations between HTLV-I, HTLV-II or related retroviruses and CTCLs (Hall 1994, Lessin et al 1994). Most cases of MF/SS can be distinguished from ATL on the basis of clinicopathological features and the presence or absence of HTLV-I infection (Yamaguchi et al 1984, Nagatani et al 1990, Levine et al 1994a). Many cases initially diagnosed as MF/SS were reclassified as forms of ATL following the discovery of HTLV-I (Poiesz et al 1980a, 1981, Blattner et al 1982, Blayney et al 1983b). However, infection with HTLV-I or HTLV-II has been reported in a number of cases that conform to the case definition of MF/SS (Kapanski et al 1986, Zucker-Franklin et al 1991, 1992, Bazarbachi et al 1994, Manca et al 1994). These results are part of a spectrum of evidence implicating HTLV-I-related retroviruses in T cell neoplasia distinct from ATL in HTLV-I non-endemic regions (Capésius et al 1991, D Incan et al 1992, Lapis et al 1992,
Lisby et al 1992, Bazarbachi et al 1993, Chan et al 1993, Whittaker and Luzatto 1993, Pancake et al 1995). However, the conflicting results of many of these studies indicate the need for stringent criteria to confirm that positive findings are genuine and have aetiological significance. Such criteria include establishing unequivocal case definitions for MF/SS and other CTCLs to enable them to be distinguished from ATL. The concept of HTLV-I positive MF/SS distinct from ATL remains controversial and may depend on strict pathological definitions.

Classification systems for T cell neoplasia are continually being updated and caution should be exercised in the interpretation of studies that have used different criteria for definition of cases (Willemze et al 1997, 1999, Fink-Puches et al 2002). The classification of LGL leukaemia, which has T cell and natural killer (NK) cell forms, is still developing (Semenzato et al 1997). The relationship between clonal LGL leukaemia and lymphoproliferative disease of LGLs (LDLGLs) in which clonality cannot be demonstrated remains to be elucidated (Scott et al 1993, Richards et al 1995). Adult ALL is well-defined, but childhood ALL has received more attention in the search for an infectious aetiology because of temporal and spatial clustering (Alexander 1993, Harris et al 1994, Birch et al 2000).

Establishing an association between a virus and a defined clinicopathological entity depends on the acceptability of evidence that the virus is present. Gold standards for diagnosis of HTLV-I infection by serology in patients with ATL may not be applicable to MF/SS or LGL leukaemia if the serological response is altered in patients with these diseases. Variations in patterns of seroreactivity are difficult to interpret in this context, particularly as variant HTLVs have not so far been detected in seroindeterminate patients (Maruyama et al 1989, Ranki et al 1990, Srivastava et al 1990, Nerurkar et al 1992, Madeleine et al 1993, Re et al 1993, Busch et al 2000). Therefore, serological testing was not performed in this study. Molecular techniques are increasingly being used for detection of viral sequences in blood and tissues but the most definitive evidence of retroviral infection remains isolation of infectious virus. In studies testing the hypothesis of a viral aetiology of MF/SS, a goal would be to isolate a new species of exogenous retrovirus or a distinct strain of HTLV-I or HTLV-II.

If the presence of infectious virus or viral sequences is confirmed in patients with T cell neoplasia, then further investigation is required to determine if the virus has an aetiological role in the disease (Evans 1976, Fredricks and Relman 1995). The presence of a retrovirus in patients with CTCL, even if integrated in neoplastic cells, is not proof of a causal role in oncogenesis. Retroviruses may be carried as passengers in neoplastic cells, with infection representing tropism for proliferating T cells rather than an aetiological role. Early isolates of HTLV-II were obtained from T cell variants of hairy cell leukaemia (HCL) and CD8 T cell lymphocytosis in patients with B cell HCL, but HTLV-II does not appear to be aetiologically involved in HCL (Rosenblatt et al 1987, Hjelle et al 1991a). Similarly, cases of LGL leukaemia in which HTLV-I and HTLV-II have been demonstrated may reflect coincidental infection in patients from populations harbouring these viruses (Heneine et al 1994, Loughran et al 1994b).
5.2 Detection of retroviruses in human T cell leukaemias and lymphomas by cell culture

In the first part of this study attempts were made to isolate HTLV-I or other retroviruses in cell culture from patients with cutaneous lymphoid infiltrates. HTLV-I was first detected in a CD4\(^+\) T cell line established from a patient with ATL (Poiesz et al 1980a, b) and HTLV-II was first identified in a CD8\(^+\) T cell line derived from a patient with a T cell variant of HCL (Kalyanaraman et al 1982b). HTLV-I-infected T cell lines can be readily established from ATL patients using mitogens, interleukin (IL) 2, conditioned medium (CM) and by cocultivation of peripheral blood mononuclear cells (PBMCs) with umbilical cord blood mononuclear cells (UCMCs) (Gazdar et al 1980, Miyoshi et al 1981a, b, Markham et al 1983). A number of CD4\(^+\) T cell lines have been established from patients with MF and SS using IL2, IL4, IL7 and CM as growth stimulants (Kaltoft et al 1984, 1987, Abrams et al 1991a, b, Crae et al 1991, Starkebaum et al 1991, Kaltoft et al 1992). Although retrovirus-like particles have sometimes been demonstrated by electron microscopy (EM) and reverse transcriptase (RT) activity sometimes detected in cell culture supernatants in these cell lines, there is no definitive evidence of infection with exogenous retroviruses. Two groups have identified HTLV-I or HTLV-II sequences by the polymerase chain reaction (PCR) in lymphocytes cultivated from patients with MF/SS using IL2 and granulocyte-macrophage colony stimulating factor (GMCSF) as growth stimulants but infectious retroviruses have not been purified or propagated (Zucker-Franklin et al 1991, 1992, Ghosh et al 1994). Similar cell culture techniques were adopted in this study in an attempt to propagate T cell lines that might harbour T lymphotropic viruses such as HTLV-I and HTLV-II. Cultivated cells were examined by EM for evidence of retrovirus infection and culture supernatants were assayed for RT activity. Cultivated cells were also tested for HTLV-I and HTLV-II by PCR.

PBMCs and lymphocytes derived from skin and lymph nodes were cultivated from 18 patients with cutaneous lymphoid infiltrates. These were 11 patients with MF, three patients with SS, one patient with CTCL (peripheral T cell lymphoma) and three patients with cutaneous B cell lymphoma (CBCL). Four CD8\(^+\) T cell lines were cultivated for more than 6 months from the skin of three patients with MF. Since these patients had CD4\(^+\) T cell neoplasia, the cultivated cells probably arose from non-neoplastic CD8\(^+\) tumour infiltrating lymphocytes (TILs). It has been shown that most T cell clones propagated from MF lesions are not derived from neoplastic MF/Sézary cells (Ho et al 1990, Harwix et al 2001). One Epstein-Barr virus (EBV)-infected B-lymphoblastoid cell line (B-LCL) was established from a patient with CBCL, consistent with the known B cell immortalising properties of this ubiquitous virus (Pattengale et al 1973). No evidence of retrovirus infection was found in cultured lymphocytes by EM or RT assay and no HTLV-I or HTLV-II sequences were demonstrated by PCR.

Reports of detection of retrovirus-like particles by EM and RT activity in cultured lymphocytes from patients with MF/SS (Kaltoft et al 1984, 1987, Saal et al 1989, Zucker-Franklin et al 1991, Bazarbachi et al 1994) need to be verified by purification and characterisation of the putative virus, demonstration of infectivity and sequencing. None of the studies so far reported have been able to meet these criteria. It is necessary to exclude the possibility that putative virus isolates are the result of expression of endogenous retroviruses in cultured
cells (Löwer et al 1993, Herbst et al 1996). Virus-like particles may be produced following activation of endogenous retrovirus-like elements in the altered environment of neoplastic tissue or in cell cultures. It is also necessary to ensure that propagated viruses have not been derived from laboratory contamination with exogenous or endogenous retroviruses, especially those of non-human primate origin (Reitz et al 1976, Smith et al 1979, Popovic et al 1982). Contamination of cell cultures with non-human primate and murine retroviruses has been a frequent distraction during attempts to isolate retroviruses from human patients with neoplasia. Cell lines established from patients with CTCL should also be characterised by immunophenotyping, karyotyping and molecular analysis as necessary to determine if they are derived from the neoplastic clone. In many studies reporting HTLV-I-related viruses in cell cultures from patients with CTCL the propagated cells have not been confirmed to be of T cell origin (Manzari et al 1987, Hall et al 1991, Zucker-Franklin et al 1991, 1992).

Human T cells infected with HTLV-I and HTLV-II can be immortalised (remaining IL2-dependent) or transformed (growing independently of IL2) in vitro (Miyoshi et al 1981c, Chen et al 1983). Normal human T cells are able to survive in standard cell cultures for 20 to 30 days if unstimulated or 30 to 60 days if activated (Perillo et al 1989). Use of a variety of stimulants and cocultivation techniques can increase the longevity of T cells in culture and result in the selective outgrowth of specific T cell clones, such as the CD8+ T cell lines established in this study. Success in establishing T cell lines could also be due to infection with immortalising viruses but extending the lifespan of propagated cells per se does not necessarily increase the chances of isolating new retroviruses. The cell culture approach to isolation of new viruses should therefore be directed at maintaining an environment in which the in vitro growth of cells is optimised for virus isolation without merely perpetuating the survival of normal T cells. This could be achieved by selective induction of specific T cell types and subtypes and stimulation of virion production from latent proviruses.

5.3 Use of molecular techniques to detect HTLV-related retroviruses in human T cell leukaemias and lymphomas

There is a wide range in reported frequencies of detection of HTLV-I and HTLV-II sequences by PCR in patients with MF/SS (Capésius et al 1991, Hall et al 1991, D'Incan et al 1992, Lapis et al 1992, Lisby et al 1992, Bazarbachi et al 1993, Chan et al 1993, Manca et al 1994, Pancake et al 1995). A number of groups have provided molecular evidence that incomplete or defective genomes of HTLV-I, or possibly variant viruses, are present in some patients (Hall et al 1991, Zucker-Franklin et al 1991, Srivastava et al 1992, Ghosh et al 1994, Pancake et al 1995). HTLV-I pX sequences, especially tax sequences, are detected more frequently than other regions of the genome in these cases. Defective HTLV-I proviruses with preferential retention of the pX region have been detected in a small proportion of patients with ATL (Konishi et al 1984, Korber et al 1991, Ohshima et al 1991, Kubota et al 1995). Similarly, the pX region is retained in defective BLV proviruses integrated in leukaemic B cells of cattle with enzootic bovine leucosis (EBL) (Kettmann et al 1982). Therefore, the hypothesis was tested that incomplete or defective HTLV-I or HTLV-II proviruses are integrated in neoplastic cells of patients with MF/SS and that tax sequences are detected more frequently by PCR than other regions of the viral genome.
In the second part of this study samples from 36 patients with cutaneous lymphoid infiltrates, six patients with LGL leukaemia and six patients with adult ALL were tested for the presence of HTLV-I and HTLV-II using PCR primer sets that amplified sequences from different regions of the HTLV-I proviral genome. Patients with LGL leukaemia and adult ALL were also tested for BLV gag sequences by PCR. An important feature of this study was the careful optimisation of PCR conditions for each primer set and the use of serial dilutions of positive control DNA and cells to quantify the sensitivity of each assay. Sensitivity was monitored in PCRs conducted on each batch of clinical samples to ensure that an adequate limit of detection was achieved. Precautions were taken to minimise PCR contamination. PCR products were amplified from three patients (one MF, one SS and one CBCL) using HTLV-I or HTLV-I/II tax primers. However, the sequences of cloned PCR products were identical to those amplified from C8166 cell line DNA used as a positive control. It was concluded that these PCR products were the result of contamination, despite the care taken in preparing PCR reagents and handling clinical samples.

False positive results due to PCR contamination have been a major problem in attempts to associate HTLV-I or HTLV-II with a number of diseases. HTLV-I sequences detected by PCR were reported in patients with multiple sclerosis (Koprowski et al 1985, Greenberg et al 1989a, Reddy et al 1989) but extensive follow-up studies have been negative (Ehrlich et al 1991). The use of PCR to detect viruses must be conducted with stringent precautions to eliminate contamination from PCR products, plasmids containing cloned viral sequences, positive control DNA samples and infected clinical samples. When there is controversy regarding the veracity of findings, for example when disparate results are obtained by different groups, positive findings must be subjected to high levels of scrutiny. Criteria for accepting a positive result as genuine need to take account of the precautions to prevent contamination at all stages of sample and reagent preparation and analysis.

In molecular studies of the involvement of HTLV-I-related viruses in T cell neoplasia, an acceptable level of proof in some circumstances would be to accept results only when sequences of PCR products differ significantly from reference strains of HTLV-I and HTLV-II, particularly those used as positive controls. It may be not be possible to resolve the issue of whether prototypical strains of HTLV-I are involved in the aetiology of CTCLs, since cases of MF/SS and T cell lymphomas (TCLs) that are HTLV-I-associated could be classified as variants, particularly cutaneous forms, of ATL. It may only be possible to identify a retrovirus as the cause of MF/SS by isolation of a distinct sequence variant of virus that can be classified as a new strain or new virus species. It would then be necessary to demonstrate that this virus is consistently associated with MF/SS.

Since this molecular investigation was conducted, several other PCR-based studies have been published that report no evidence of HTLV-I or HTLV-II infection in MF/SS patients (Bonif et al 1996, Wood et al 1996b, Bazarbachi et al 1997, Fujihara et al 1997, Wood et al 1997, Daliani et al 1998, Kim et al 1998). The carefully controlled study of Wood et al (1996a, b) provides a good model for future molecular studies attempting to identify viral sequences in lesions of patients with a variety of diseases. Similar to the molecular investigations described in this thesis, a PCR system for detection of HTLV-I in paraffin-embedded histological sections was carefully optimised, controlling for specificity by
Southern blot (SB) hybridisation using an oligonucleotide probe and nucleotide sequencing (Wood et al 1996a). The integrity of total DNA was assessed by PCR for the nucleophosmin gene and the integrity of T cell DNA was assessed by PCR for the T cell receptor (TCR). Using PCR and SB hybridisation, HTLV-I pol sequences were initially detected in three (7%) of 42, env sequences in 2 (5%) of 37 and pX sequences in 7 (17%) of 42 patients from the USA with MF/SS (Wood et al 1996b). However, after treatment of samples with ultraviolet (UV) light, only three of 12 previously positive cases remained positive, suggesting that the earlier positive results were due to trace contamination with PCR reagents or sample DNA. Furthermore, pX and pol sequences cloned from one PCR-positive sample were identical to that of the positive control; the same sample was negative for HTLV-I by dot blot hybridisation of genomic DNA and new samples from the two remaining positive cases were negative by PCR. When material from 28 additional cases of MF/SS from the USA was subjected to PCR with the use of UV light to reduce contamination, all were negative for pX sequences. The conclusion from this study was that positive PCR results for HTLV-I in MF/SS patients were due to trace contamination with PCR products (Wood et al 1996b).

Measures to prevent contamination in many studies may not have been sufficiently stringent to prevent false positive results. Associations between diseases and viruses should be viewed with scepticism if they are based on PCR results without other supporting evidence. If a retrovirus is involved in CTCL, it may not be related to HTLV-I or HTLV-II, it might be present in the skin in quantities below the detection limit of PCR or it might have been eliminated from the skin before clinical lesions develop (Lisby et al 1992). The best evidence to date, including the results described in this thesis, thus indicate that HTLV-I and HTLV-II are not involved in CTCL (Lessin et al 1996, Wood et al 1996b).

Supporting these observations, no HTLV-I sequences were detected by PCR in PBMCs or skin lesions of 127 patients with CTCL (108 with MF/SS) from Europe and the USA using HTLV-I and HTLV-II gag, pol, env, pX and LTR specific primers (Bazarbachi et al 1997). No HTLV-I sequences were detected by PCR in 50 Swiss and German patients with CTCL (Böni et al 1996) or 16 HTLV-I seronegative patients with MF/SS from north eastern USA (Fujihara et al 1997). Similarly, PCR analyses conducted since this study have not shown any evidence of HTLV-I or HTLV-II in cases of LGL leukaemia and T prolymphocytic leukaemia in non-endemic regions (Pawson et al 1997).

However, some groups have continued to report detection of HTLV-I sequences by PCR in patients with CTCL (Zucker-Franklin 2001). Pancake and Zucker-Franklin (1998) detected HTLV-I tax sequences by in situ PCR in infiltrating lymphocytes, as well as keratinocyte-like cells, in the skin of 11 of 12 patients with MF. The skin PCR negative patient had HTLV-I sequences demonstrable by PCR and Southern blot (SB) hybridisation in PBMCs (Khan et al 1996). HTLV-II tax and pol sequences were detected by PCR in tissues from one (5%) of 22 patients with CTCL in the USA, although clonal HTLV-II integration could not be detected by SB hybridisation in PBMCs of this patient; all patients were negative for HTLV-I tax or pol sequences (Li et al 1996). Kikuchi et al (1997a) reported that no HTLV-I sequences were detected in 50 HTLV-I seronegative Japanese patients with CTCL, then later reported that HTLV-I gag, pol, env and/or pX sequences were detected by PCR in two HTLV-I seronegative patients with CTCL, although monoclonal integration of HTLV-I provirus could...
not be confirmed in these patients by SB hybridisation (Kikuchi et al 1997b). Similarly, HTLV-I gag, pol, env, pX and LTR sequences were detected by PCR in PBMCs of an HTLV-I seronegative Japanese patient with SS, but no monoclonal integration of HTLV-I provirus could be detected by SB hybridisation (Miyoshi et al 1998). Twelve of 28 patients with CTCL (27 MF, one SS) had antibodies against HTLV-I by WB ELISA and HTLV-I sequences were amplified by PCR from PBMCs of six patients (Shohat et al 1999). An HTLV-II associated CTCL has been identified in an HIV-1-infected AIDS patient (Poiesz et al 2000).

Differences between studies in associating HTLV-I with MF/SS have been attributed to differences in the methods of processing samples for detection of HTLV-I sequences (Pancake and Zucker-Franklin 1996, Zucker-Franklin and Pancake 1998b). Zucker-Franklin and Pancake (1998b) showed that the use of whole-cell lysates instead of DNA extracts and the use of fresh instead of cultured cells for PCR increased the ability to detect HTLV-I tax sequences in samples from MF patients. They also observed that positive PCR products from clinical samples are more likely when tax primers are used than primers from other regions of the HTLV-I genome, when there are more than 30 cycles of amplification and when SB hybridisation is applied to PCR products (Ghosh et al 1994, Pancake et al 1995b, Pancake and Zucker-Franklin 1996). However, contamination is also more likely with extended PCR cycles using highly sensitive primer sets. Since it is difficult to assess precautions taken by other groups to prevent PCR contamination, independent studies are necessary to verify positive findings. The only study to have used a double blind method for confirmation of PCR results from the same patients at different times of collection reported positive results for HTLV-I pol and tax sequences in 10 (34%) of 29 patients with MF from Italy (Manca et al 1994). The need for carefully controlled independent studies will continue while ever there are claims of positive associations between HTLV-I or related viruses and CTCL.

In 50 MF patients positive for HTLV-I tax DNA and mRNA by PCR, 83% had antibodies against HTLV-I Tax, whereas only 5% had reactivity to structural proteins in standard HTLV-I serological assays (Pancake et al 1996a). Although anti-Tax antibodies and tax sequences detected by PCR have been reported by some groups in 8 to 11% of normal blood donors and 4 to 33% of intravenous drug users (IVDUs) negative for HTLV-I in standard serological assays (Ehrlich et al 1989a, Pancake et al 1996a, Zucker-Franklin and Gorman 1997, Zucker-Franklin et al 1997, Zucker-Franklin and Pancake 1998a) these findings have not been supported in an independent, blind, multicentre study (Cowan et al 1999).

DNA extracted from skin samples of patients with MF usually contains only a small percentage of DNA from neoplastic T cells and therefore it is important to determine the sensitivity of detection of HTLV-I and HTLV-II sequences by PCR. The use of PCR to indicate the presence of incomplete HTLV-I or HTLV-II proviruses needs to be critically evaluated, since most studies have not adequately monitored the sensitivity of each primer set. A negative PCR result for some but not other regions of the HTLV genome could be due to poor sensitivity of particular primer sets in a panel. Alternatively, positive results could be due to contamination. It is notable that many tax primer sets appear to be more sensitive than primer sets used for PCR from other regions of the HTLV-I genome. The presence of HTLV-I or HTLV-II with variant sequences could also result in the failure to amplify products.
by PCR. The possibility of variant HTLV-I sequences was taken into account in this study by using primers covering multiple regions of prototypical HTLV-I and by use of consensus primers. Also, the $pX$ regions of HTLV-I and HTLV-II have sufficient similarity to cross-hybridise in SB hybridisation analyses, so the HTLV-I probe (pMT2j) used in these experiments would be expected to hybridise to PCR products amplified using HTLV-I/II primers (Shaw et al 1984).

To verify that a patient with CTCL is infected with HTLV-I or a related virus, it will be necessary to obtain a substantial length of sequence, preferably of the entire provirus, for comparative analysis. Sequencing of a fragment derived from a PCR reaction is inadequate unless the sequence can be shown to be substantially different from sequences of any known HTLV-I or HTLV-II strain and different from any positive control DNA used for the same study. Small differences in the sequences of PCR products could be due to errors introduced by *Taq* polymerase during amplification. This possibility could be excluded by sequencing PCR products from multiple independent reactions or by the use of proof reading enzymes with higher fidelity. Otherwise, even with the use of positive and negative controls, contamination cannot be excluded with the stringency required for declaration of a new virus or variant given the level of controversy in this field. The lack of adequate sequence data is the major flaw of all studies so far reported that have attempted to associate HTLV-I, HTLV-II or related viruses with CTCL.

Regions of sequence similarity in the LTR and *gag* regions of HTLV-I, HTLV-II and BLV were used to design consensus PCR primers for the HTLV/BLV (*Deltaretrovirus*) group of viruses. However, these primers were shown to produce non-specific PCR products under a variety of conditions using HTLV-I and BLV DNA as templates. Further optimisation may improve the specificity of these primer sets or they may have specific applications in customised PCR systems. It would also be necessary to determine the sensitivity of these primer sets. Consensus retroviral PCR primers have been based on alignments of retroviral Pol region amino acid sequences and reverse translation to identify minimum degenerate codons (Mack and Sninsky et al 1988, Donehawer et al 1990). A major problem with these primers is that they will amplify homologous *pol* sequences from endogenous retroviral elements and therefore are only likely to be useful in PCR systems based on cDNA (Nelson et al 1999).

Another approach is to use a modification of representational difference analysis (RDA) to preferentially amplify tester sequences that will not be present in driver DNA, thus limiting amplification of endogenous sequences (Listyse et al 1993). This allows selective amplification of exogenous retroviruses from the tester DNA, which will not have partners in the driver DNA. RDA could also be applied to cDNA to search for retroviral RNA genomes (Hubank and Schatz 1994, Geng et al 1998).

A third HTLV-related retrovirus lineage, known as primate T lymphotropic virus type L (PTLV-L or PTLV-3), has been identified in baboons (*Papio hamadryas*) (Goubau et al 1994, Van Brussel et al 1997). There is sufficient similarity between *tax* sequences of PTLV-L and HTLV-I/II for this divergent group to be identified using generic *tax* primers (Busch et al 2000). As discussed above, no variant PTLV-L *tax* sequences were detected by PCR in 269 HTLV-I/II seroindeterminate blood donors in the USA (Busch et al 2000).
Although there is little evidence for the involvement of EBV in most human primary CTCLs, including MF/SS (Kanavaros et al 1994, Anagnostopoulos et al 1996, Angel et al 1996), an EBV-related virus, HVvmne, has been associated with MF in a pig-tailed macaque (Macaca nemestrina) (Rivadeneira et al 1999). PCR using specific, degenerate and consensus primers for detection of herpesviruses could be applied to CTCLs (MacKenzie et al 2001, Gallagher et al 2002).

This project did not produce evidence that HTLV-I, HTLV-II or BLV are associated with MF/SS, LGL leukaemia or selected cases of adult ALL in the United Kingdom. The available reports attempting to implicate retroviruses in CTCLs similarly have failed to provide conclusive evidence for a primary aetiological role of HTLV-I or HTLV-II in MF/SS (Lessin et al 1994, Woods et al 1996b). Therefore, it is concluded that HTLV-I and HTLV-II are not the cause of CTCLs in HTLV non-endemic regions.

5.4 The role of HTLV-I pX gene products in apoptosis

The third part of this project examined the ability of HTLV-I pX gene products to inhibit apoptosis induced by c-Myc in Rat1 fibroblasts. When cells proliferate in response to the activation of oncogenes such as c-Myc, the apoptosis pathway is switched on concurrently and these cells die when survival factors such as those present in serum are withdrawn (Evan et al 1992). In models of oncogenesis there is a requirement for apoptosis to be blocked in order to enable proliferating cells to survive. Several oncogenic viruses produce proteins that inhibit apoptosis (Rao et al 1992, White et al 1992, Henderson et al 1993).

In this study it was hypothesised that HTLV-I pX region gene products with currently unknown functions might inhibit apoptosis. The system of c-Myc induction by 4-hydroxytamoxifen (4-HT) in Rat1 cells stably transfected with a chimaeric c-myc-modified receptor (ER™) gene provided an elegant model to test the anti-apoptotic properties of HTLV-I pX proteins (Evan et al 1992, Littlewood et al 1995). However, expression of Rex, p21\textsuperscript{\textit{\textsc{iv}}}, p30\textsuperscript{\textit{\textsc{v}}} (Tof), p13\textsuperscript{\textit{\textsc{iv}}} or p12\textsuperscript{\textit{\textsc{v}}} did not inhibit c-Myc-induced apoptosis in Rat1 cells under conditions of serum deprivation. Furthermore, Rat1 cells expressing Tax underwent apoptosis when serum was withdrawn, indicating that Tax might trigger apoptotic pathways at the same time as it activates pathways leading to cell proliferation. Other workers similarly have demonstrated that Tax induces apoptosis in cells following withdrawal of cell survival signals (Sakurai et al 1992b, Yamada et al 1994, Fujita and Shiku 1995). Expression of Rex, p21\textsuperscript{\textit{\textsc{iv}}}, Tof, p13\textsuperscript{\textit{\textsc{iv}}} or p12\textsuperscript{\textit{\textsc{v}}} alone had no effect on Rat1 cells in the absence of serum. Co-expression of these pX proteins with Tax did not inhibit Tax-induced apoptosis compared to expression of Tax alone and there was no evidence of a cooperative effect.

Although no inhibition of c-Myc or Tax-induced apoptosis by other HTLV-I pX proteins was observed in this experimental system, further studies should be undertaken to explore the functions of HTLV-I pX gene products in other systems for detecting anti-apoptotic effects. It would be highly desirable to develop an inducible HTLV-I tax gene expression system such as a cell line stably transfected with a tax-ER™ plasmid construct. This would allow interactions between tax and other HTLV-I pX proteins to be explored in a more tightly controlled system.
5.5 Conclusions

The studies reported in this thesis have demonstrated no evidence for infection with HTLV-I or related viruses in patients with MF/SS or other CTCLs in Scotland using PCR and cell culture. PCR studies also showed no indication of HTLV-I, HTLV-II or BLV infection in cases of LGL leukaemia in the UK or in a cluster of ALL in butchers from the Cardiff region. The HTLV-I Tax protein was shown to induce apoptosis in rodent fibroblasts upon withdrawal of serum, whereas other HTLV-I pX gene products failed to inhibit Tax or c-Myc-induced apoptosis.
References


Asadullah K, Döcke WD, Haeußler A, Sterry W and Volk HD (1996a) Progression of mycosis fungoides is associated with increasing cutaneous expression of interleukin-10 mRNA. *J Invest Dermatol* 107:833-837


Asadullah K, Haeußler A, Friedrich M, Siegling A, Olaizola-Horn S, Trefzer U, Volk HD and Sterry W (1996b) IL-7 mRNA is not overexpressed in mycosis fungoides and pleomorphic T-cell lymphoma and is unlikely to be an autocrine growth factor in vivo. *Arch Dermatol Res* 289:9-13


Bamford RN, Battista AP, Burton JD, Sharma H and Waldmann TA (1996) Interleukin (IL) 15/IL-T production by the adult T-cell leukaemia cell line HTU-102 is associated with a human T-cell lymphotrophic virus type I R region/IL-15 fusion message that lacks many upstream AUGs that normally attenuate IL-15 mRNA translation. Proc Natl Acad Sci USA 93:2997-2992


Bangham CRM, Daenke 8, Phillips RE, Cruickshank JK and Bell JI (1988) Enzymatic amplification of exogenous and endogenous retroviral sequences from DNA of patients with tropical spastic paraparesis. EMBO J 7:4179-4184


Béraud C, Lombard-Platet G, Michal Y and Jalinot P (1991) Binding of the HTLV-I Tax1 transactivator to the inducible 21 bp enhancer is mediated by the cellular factor HEB1. EMBO J 10:3795-3803


Berneman ZN, Ablashi DV, Li G, Eger-Fletcher M, Reitz MS Jr, Hung CL, Brus I, Komaroff AL and Gallo RC (1992a) Human herpesvirus 7 is a T-lymphotropic virus and is related to, but significantly different from, human herpesvirus 6 and human cytomegalovirus. *Proc Natl Acad Sci USA* **89**:10552-10556


Birnboim HC and Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acids Res 7:1513-1523


Chen ISY, Quan SG and Golde DW (1983) Human T-cell leukemia virus type II transforms normal human lymphocytes. *Proc Natl Acad Sci USA* 80:7006-7009


Dense D, Mikovits J and Ruscetti F (1997) X-I and X-II open reading frames of HTLV-I are not required for virus replication or for immortalization of primary T-cells. in vitro Virology 237:123-128


Fujiura H, Tsuchiya H, Meng X-B and Seiki M (1996) c-Jun, c-Fos and their family members activate the transcription mediated by three 21-bp repetitive sequences in the HTLV-I long terminal repeat. Intervirology 38:221-228


Goldstein DJ, Andresson T, Sparkowski JJ and Schlegel R (1992) The BPV-1 E5 protein, the 16 kDa membrane pore-forming protein and the PDGF receptor exist in a complex that is dependent on hydrophobic transmembrane interactions. EMBO J 11:4851-4859


Good LF, Magginwar SB and Sun S-C (1996) Activation of the IL-2 gene promoter by HTLV-I Tax involves induction of NF-AT complexes bound to the CD28-responsive element. EMBO J 15:3744-3750


Hall AP, Irvine J, Blyth K, Cameron ER, Onions DE and Campbell MEM (1998) Tumours derived from HTLV-I tax transgenic mice are characterized by enhanced levels of apoptosis and oncogene expression. J Pathol 186:209-214


Harrington EA, Bennett MR, Fanidi A and Evan GI (1994a) c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J* 13:3286-3295


Harwix S, Gunzl HJ, Blaschke V, Zachmann K and Neumann C (2001) Inability to culture the dominant T-cell clone from the skin of primary cutaneous T-cell lymphoma as proven by TCR γ-chain gene sequencing. *Arch Dermatol Res* 293:139-146


Inoue J-I, Yoshida M and Seiki M (1987) Transcriptional (p40x) and post-transcriptional (p27xH) regulators are required for the expression and replication of human T-cell leukemia virus type I genes. Proc Natl Acad Sci USA 84:3653-3657


Kawahara A, Minami Y and Taniguchi T (1994) Evidence for a critical role for the cytoplasmic region of the interleukin 2 (IL-2) receptor gamma chain in IL-2, IL-4, and IL-7 signalling. Mol Cell Biol 14:5433-5440


Kazanji M (2000) HTLV type 1 infection in squirrel monkeys (Saimiri sciureus): a promising animal model for HTLV type 1 human infection. AIDS Res Hum Retroviruses 16:1741-1746


Lane DP and Crawford LV (1979) T antigen is bound to a host protein in SV40-transformed cells. Nature 280:261-263


Loughran TP Jr, Starkebaum G and Ruscetti FW (1988b) Simitar rearrangements of T-cell receptor β gene in cell lines and uncultured cells from patients with large granular lymphocyte leukemia *Blood* 72:513-515


Lowe SW and Ruley HE (1993) Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis *Genes Dev* 7:535-545


Macatonia SE, Cruickshank JK, Rudge P and Knight SC (1992) Dendritic cells from patients with tropical spastic paraparesis are infected with HTLV-1 and stimulate autologous lymphocyte proliferation. *AIDS Res Hum Retrov* 8:1699-1705


McDougall JK (2001) "Hit and run" transformation leading to carcinogenesis. Dev Biol (Basel) 106:77-82


Mills GB, Arima N, May C, Hill M, Schmandt R, Li J, Miyamoto NG and Greene WC (1992) Neither the ick nor the fyn kinases are obligatory for IL-2-mediated signal transduction in HTLV-I-infected human T cells. *Int Immunol* 4:1233-1243


Nimer S (1991) Tax responsiveness of the GM-CSF promoter is mediated by mitogen-inducible sequences other than \( ^{x} \)B. New Biol 3:997-1004


Ohshima K, Hashimoto K, Izumo S, Suzumiya J and Kikuchi M (1996) Detection of human T lymphotropic virus type I (HTLV-I) DNA and mRNA in individual cells by polymerase chain reaction (PCR) in situ hybridization (ISH) and reverse transcription (RT)-PCR ISH. Haematol Oncol 14:91-100


Pancake BA, Zucker-Franklin D, Marmor M and Legler PM (1996b) Determination of the true prevalence of infection with the human T-cell lymphotropic viruses (HTLV-I/II) may require a combination of biomolecular and serological analyses. Proc Assoc Am Physicians 108:444-448


European Organization for Research and Treatment of Cancer Cutaneous Lymphoma Project Group. 
*J Clin Oncol* 13:1343-1354


Preston GA, Lang JE, Maronpot RR and Barrett JC (1994) Regulation of apoptosis by low serum in
cells of different stages of neoplastic progression: enhanced susceptibility after loss of a senescence


and malignant hypereosinophilic syndrome. Lancet ii:569-570

Proudfoot NJ and Brownlee GG (1976) 3' non-coding region sequences in eukaryotic messenger RNA.
Nature 263:211-214

Pui C-H, Frankel LS, Carroll AJ, Raimondi SC, Shuster JJ, Head DR, Crist WM, Land VJ, Pullen DJ,
Steuber CP, Behm FG and Borowitz MJ (1991) Clinical characteristics and treatment outcome of
childhood acute lymphoblastic leukemia with the t(4;11)(q21;q23): a collaborative study of 46 cases.
Blood 77:440-447

Pyra H, Böni J and Schönbach J (1994) Ultrasensitive retrovirus detection by a reverse transcriptase
assay based on product enhancement. Proc Natl Acad Sci USA 91:1544-1548

stimulated DNA binding of Myc, Jun, and novel Myc-like proteins in cutaneous T-cell lymphoma cells.
Blood 93:260-267

Quackenbush SL, Work TM, Balazs GH, Casey RN, Rovnak J, Chaves A, duToit L, Baines JD, Parrish
CR, Bowser PR and Casey JW (1998) Three closely related herpesviruses are associated with
fibropapillomatosis in marine turtles. Virology 246:392-399

lymphoproliferative disease associated with a novel gammaherpesvirus in a captive population of
common marmosets. Comp Med 50:59-68


proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. Proc Natl Acad Sci
USA 89:7742-7746

Placa M (1993) Human T-lymphotropic virus type I (HTLV-I) provirus-related DNA sequences in
peripheral blood mononuclear cells of a patient, in the absence of a definite serological positivity. New
Microbiol 16:373-379

Amplification and molecular cloning of HTLV-I sequences from DNA of multiple sclerosis patients.
Science 243:529-533

Reeves WC, Levine PH, Cuevas M, Quiros E, Maloney E and Saxinger WC (1990) Seroepidemiology

Reid RL, Linthom PF, Mireskandari A, Dittmar J and Brady JN (1993) Stabilization of wild-type p53 in
human T-lymphocytes transformed by HTLV-I. Oncogene 8:3029-3035

cells represent a subset of normal human blood lymphocytes. J Immunol 150:2081-2089


Rosenblatt JD, Cann AJ, Slamon DJ, Smealberg IS, Shah NP, Fuji! J, Wachsman W and Chen ISY (1988a) HTLV-II transactivation is regulated by the overlapping tax/ret nonstructural genes. Science 240:916-919


Sarin PS, Rodgers-Johnson P, Sun DK, Thornton AH, Morgan OS, Gibbs WN, Mora C, McKhann G


Saxon A, Stevens RH, Quan SG and Golde DW (1978b) Immunologic characterization of hairy cell leukemias in continuous culture. J Immunol 120:777-782


Shaw GM, Gonda MA, Flickinger GH, Hahn BH, Gallo RC and Wong-Staal F (1984) Genomes of evolutionarily divergent members of the human T-cell leukemia virus family (HTLV-I and HTLV-II) are highly conserved, especially in pX. *Proc Natl Acad Sci USA* 81:4544-4548


Shen Y, Zhang H and Shenk T (1997) Human cytomegalovirus IE1 and IE2 proteins are mutagenic and mediate "hit-and-run" oncogenic transformation in cooperation with the adenovirus E1A proteins. *Proc Natl Acad Sci USA* 94:3341-3345


Shirono K, Hattori T, Matsuoka M, Matsushita S, Asou N and Takatsuki K (1988) Adult T cell leukemia cell lines that originated from primary leukemic clones also had a defect of expression of CD3-T cell receptor complex. *Leukemia* 2:728-733


Suzuki T, Hirai H, Fujisawa J, Fujita T and Yoshida M (1993b) A trans-activator Tax of human T-cell leukemia virus type 1 binds to NF-xB p50 and serum response factor (SRF) and associates with enhancer DNAs of the NF-xB site and CAfG box. *Oncoogene* **8**:2391-2397


Suzuki T, Hirai H, Fujisawa J, Fujita T and Yoshida M (1993b) A trans-activator Tax of human T-cell leukemia virus type 1 binds to NF-xB p50 and serum response factor (SRF) and associates with enhancer DNAs of the NF-xB site and CAfG box. *Oncoogene* **8**:2391-2397


Tabor S and Richardson CC (1987) DNA sequence analysis with a modified T7 DNA polymerase. *Proc Natl Acad Sci USA* 84:4767-4771


Tensen CP, Vermeer MH, van der Stoop PM, van Beek P, Schepers RJ, Boorsma DM and Willemze R (1998) Epidermal interferon-gamma inducible protein-10 (IP-10) and monokine induced by gamma-interferon (Mig) but not IL-6 mRNA expression is associated with epidermotropism in cutaneous T cell lymphomas. J Invest Dermatol 111:222-226


Voevodin AF, Lapin BA, Yakovleva LA, Ponomaryeva TI, Oganyan TE, Razmadze EN (1985) Antibodies reacting with human T-lymphotropic retrovirus (HTLV-I) or related antigens in lymphomatous and healthy hamadryas baboons. Int J Cancer 36:579-584


Yoshimura T, Fujisawa J-I and Yoshida M (1990) Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain. EMBO J 9:2537-2542


Young RA and Davis RW (1983) Efficient isolation of genes by using antibody probes. Proc Natl Acad Sci USA 80:1194-1198


Zucker-Franklin D, Hooper WC and Evatt BL (1992) Human lymphotropic retroviruses associated with mycosis fungoides: evidence that human T-cell lymphotropic virus type II (HTLV-II) as well as HTLV-I may play a role in the disease. *Blood* **80**:1537-1544


Zucker-Franklin D, Pancake BA and Friedman-Kien AE (1994) Cutaneous disease resembling mycosis fungoides in HIV-infected patients whose skin and blood cells also harbor proviral HTLV type I. *AIDS Res Hum Retroviruses* **10**:1173-1177


Appendices

Appendix 1: Nucleotide and peptide sequences of HTLV-I pX region gene products.

Tax

1 ATGCCCACTCCCGAGGGTGGACACAGATCTCTCTTTCTCGATAACCATCTT
   M A H F P G F G Q S I L L G Y F V
51 CTAGGTGTGGAACTGAGTAACGAGAAGCAGCTGCGGCCAGTCCTGCG
   Y V G D C V Q G D W C P I S G
101 GACATGGTCTGCCGCGGGCACATGTCACGCCCTACTGCGACCTCGCA
   G L C S A R L H R H A L L A T C D
151 GAGGACACAGAATCAGGACACCGACATCAGACGACGCTGACGACGACG
   E H Q I T W D P I D E R V I G S A
201 TCTGACTGCTATCTCCGTGCGCTCCGACCCACGCCTGACCCGAGACG
   L Q F L I P R L P S P F T Q R T
251 CTAGACAGCCAGTGGCTGCGAGAAGCAGCTGCGGCCAGTCCTGCG
   S K T L K V L T P P I T H T T P N
301 ATGCCGCTGCTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   I P S S P L Q A M K K Y S F F R N
351 TGCATACCTGAGCCACCCACCCCCATGCTGCTGCTGCTGCTGCTGCTG
   G Y M E P T L G Q C H L P T L S F
401 CAGGACCCCGAATCAGGACACCGACATCAGACGACGCTGACGACGACG
   P D P G L R P Q N L Y T L W O G S
451 GTTTTGTGGAGACATACGCCGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   V C M Y L Y Q L S P I T W P L
501 CTTGCCGCCGCGTGGATCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   L P H V T E C H P G Q L G A F L
551 CCAACTCCCTACACGAGGATGGAAGACATCCCTCCTATACACATCCTCCT
   T N V P T Y K R I B E L L Y K I S L
601 ACCAAGGGGGGCTACACGAGGATGGAAGACATCCCTCCTATACACATCCTCCT
   T T G A L I I I L P H D C L P T L
651 TTGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   F C O P A R A F V T L T A W Q N G
701 TCCCTCCGCTTCCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   L L D P H S T L L T T F G L I W T P
751 ACCAAGGGGGGCTACACGAGGATGGAAGACATCCCTCCTATACACATCCTCCT
   T D G T P M I S G F C P D U G Q P
801 ACCAAGGGGGGCTACACGAGGATGGAAGACATCCCTCCTATACACATCCTCCT
   S L V L Q S S S F F H K F Q T
851 AGCCCTACGCACGCTACATCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   K A Y H P S F L L S R G L I Q Y S
901 TCCTTGCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   S F H S L L Y L P E E Y T N I F I
951 TCTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   S L L F N E K B A D D D N D H P
100 TCCTTGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
   A A A T V V V V C C C G G G G C T T A G A G C C I L C C A C T G O A A A A A C A T T T C G Q A A A
   Q I S P O G L E P P S E X H F R E
1051 ACAGAGGTCTGGA
   T E V *
Appendix 1: (continued).

Rex

1  MTIDCCCAAGGGCTCAGAAACCCCGGCATCCCGGAAGAAAGAAGCCTCCG
   MPTKRRPBRPSQGKR

51  AACACCAGGCCCACTTCTCCAGGGGTTTCTGGACAGAGTCTTCTTTTCG
   TPSQGGLDVRFPSSD

101  CCGCCGCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCC
   TQSTCLETVYXATGAP

151  CTGGGGCGACTCCGCTCCCGCCTACATCGTCACGCCCTACTGGCA
   LGDYYYRTPAYEVTFTYWP

201  TGCCACAGACCTAGATCCAGCTGGGACCCCATCGATGCGTTATCGC
   VQSIERSPQGTSNSDAL

251  CTGGGCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCC
   AQLYSSLSDSSPSPPR

301  GACGGCCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCC
   ZPLRPSRLPSLRQSLIQF

351  CCGCCGCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCC
   PTFHPSRSSRPCANTPP

401  CCGCTACTGCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCC
   SEMDTWNPPPLGSTSQFC

451  CGTCTCCGCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCC
   LFQTPDSGPKTCCTPSGE

501  GGCCGCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCTCC
   APLSACCTSTSFPSP

551  GCCGCCGCTACAGTTGTGGAGACTCTGTAACGCGACCTCTCTCTCTCTCC
   GFSCPT*
Appendix 1: (continued).

p21

1 ATGGACGCGTTATCGGCTCAGCTCTACAGTTCCTTATCCCTCGACTCCC MDALSAQLYSSLSLDS

51 TCCTTCCCCCAGGAGAAACCTAAAGACCCCTAGGTCCTTACCCCG CC PSPPREPLRPERSLPR

101 AATCACTCTACAACCCCCAACATTCCACCCTCCTTCCTCCAGGCCATG QSLIQPFTFHPSSRRC

151 GCAAAATCTCCCGCTTCGGAAATGGATACATGGAAACCCACCCTTGGGCA ANTPESXNDTNFPPLS

201 CACCTCCCAACCCTGTCTTTTCCAGACCCCGGACTCCGGCCCCAAAACC TSQPCLFQFQTPHDGFKT

251 GTACACCTCTCCGGGSSCGCTGGTTGACTCTACGCTCTTCTACGTCTTT CTSPGERAPLSACTSTSF

301 CCCCCCCCCATACCTGGGCCCCCCTAGGCGGCGGCTTAA PPSFGFSCPT
Appendix 1: (continued).

p30\textsuperscript{II} (Tof)

\begin{verbatim}

1  ATGGCACTATGCTGTTTCGCCTTCTCAGCCCCTTGTCTCCACTTGCGCTC
   MALCCFAFSAAPCLHLRS

51  ACGGCGCTCCTCGCTCTTTCCTGCTTTCTCCGGGCGACGTCAGCGGCCTTCT
   RSSCSRSCFLRATSAAF

101  TCTCCGCCCGCCTCCTGCGCCGTGCCTTCTCCTCTTCCTTTTCAAAT
   FSARLLRRAYSFFSLFK

151  TACGGACAGTCTGCTTTTCTCTCTCTTCCTCCTCTTCTCTCTCTCCGCCG
   YSAILPSSSFRRSRPFLS

201  TACTCAGCAATCTGCTTTTTCCTCCTCTTTCTCCCGCTCTTTTTTTCGCTT
   YSAICFSSSFSRFRSFRLS

251  CCTCTCGGCTCGCCGGCCGGCTGCGGTGGCTCCGATGCGTTTCCCCCGAG
   LPDSSARRCRRSCVRSPRS

301  GTGGCGCTTTCTCCCCTGGAAGGCCCCGCTGCGCGGCGCAGTCTCGCCAG
   GGAPSPGRPRRSSRPRLS

351  TCTTCTAAGGRTAGGAAACGTCAAGCAGTCTGCTCTCTCTCTCTCTGTC
   SSKDSKPSSTASSSSLS

401  CTCTAAGCTCCTGCTCCAGAAGGATAAGGGCTCCTGACAGACCAGCTCCAC
   FINSSSKDANSPTMTST

451  GCAAGTCTCCGCGCCACGACGACGACCTCAAGATGGAACCTGCTGCTGAT
   SRSSEHTGKURRSNPTD

501  ACAGAAGCTTACAGAGTTATTATACAGCGCCACCTCCGGGCTTGGACACA
   TKLTMILIISSPLPRVTWTE

551  GCTCTCTTGCCGATACGCTACGCTACGCTACGCTACGCTACGCTACGCTAC
   SSFRIFFSLRVRWRLCTR

601  CACGGGCTGGCGGGATACGCTACGCTACGCTACGCTACGCTACGCTACGCTAC
   RVPHLWGTMTFGPPTSS

651  CGCCCGCTACTCGGCGGCTGGGCTGGGCGGCTGGGCGGCTGGGCGGCTGGGCG
   RTPGHLRSASDHLPFR

701  ARGSGACGGGTTTGCGCTCACTCACTCACTCACTCACTCACTCACTCACTCACT
   WTRRELSTSSVPFPSTP

751  TCCTTCCGAGAGGACCCCTCTTRA
   LLFHPFNLL*

\end{verbatim}
Appendix 1: (continued).

p13**

1 ATGCTTATTATGCTGCGTACCTGCGAGGCTTGGGACCGAGCTTCTTTTATGCTTATTATGCTGCGTACCTGCGAGGCTTGGGACCGAGCTTCTTTTACCTGCGAGG
 MLIISPLPRVWTESSFR

51 GATACCCAGTCTACGTGTTTGGAGACTGTGTACAAGGCGACTGGTGCCGATACCCAGTCTACGTGTTTGGAGACTGTGTACAAGGCGACTGGTGCC
 IPSLVRVWRLCTRKLVE

101 ATCTCTGGGGGACTATGTTCGGCCCGCCTACATCGTCACGCCCTACTGGCACTCTGGGGGACTATGTTCGGCCCGCCTACATCGTCACGCCCTACTGGC
 HLWGTMFGPTSSRPTG

151 CAACCTGCAGAGCATCAGATCACCTGGGACCCCATCGATGGACGCGTTCAACCTGCAGAGCATCAGATCACCTGGGACCCCATCGATGGACGCGT
 HLSRASDHLGPHRWTRY

201 TCGGCTCAGCTCTACAGTTCCTTATCCCTCGACTCCCCTCCTTCCCCACCTCGGCTCAGCTCTACAGTTCCTTATCCCTCGACTCCCCTCCTTCC
 RLSTVYPSTPLLP

251 CAGAGACCTCTAA
 FENL *
Appendix 1: (continued).

p12

MLFRLLSPLSPLALTAL

CLLFDVSGCLLRL

PPPAPCLLLLFLPPQILS

NLLLFLLLFLDLLPSSLPLL

LSFPSLPFTKAFAPRWR

FLPWKAPSPQPAALFLY*

The nomenclature for incompletely specified bases in nucleic acid sequences is also depicted. (Nomenclature Committee of the International Union of Biochemistry 1985).

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## Appendix 2: (continued).

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Appendix 3: Sources of frequently used materials.

A & J Beveridge
Alpha Labs, UK
Alta Bioscience, University of Birmingham
Amersham, Little Chalfont
Applied Biosystems, Warrington
AT Biochem, Malvern, Pennsylvania
BDH (Merck), Poole, Dorset
Beckman Instruments, High Wycombe
Becton Dickinson, Cowley
J Bibby, Stone
Bio-Rad, Hemel Hempstead
Boehringer Manheim, Lewes
British Biotechnology, Abingdon
BRL, Bethesda, Maryland
Cambio, Cambridge
Costar, High Wycombe
Coulter, Luton
Dako, Glostrup, Denmark
Difco, East Molesey
Fisons Scientific Equipment, Loughborough
Fotodyne, New Berlin, Wisconsin
Genetic Research Instrumentation, Dunmow
Genosys, Cambridge
Greiner Labortechnik, Dursley
Hybaid, Teddington
ICN Biomedicals, Thame
International Laboratory Services, UK
Invitrogen, San Diego, California
J.Bio, Les Ulis Cedex, France
Leo Labs, UK
Life Technologies, Gibco BRL, Paisley
Millipore, Watford
MSE, supplied by Philip Harris, Paisley
Bando Chemical Industries, Nippon Shoji Kaisha, Japan
Nunc, supplied by Philip Harris, Paisley
Unipath, Basingstoke
Perkin-Elmer, Beaconsfield
Pharmaclia LKB Biotechnology, St Albans
Philip Harris Scientific, Clydebank
Polaroid, St Albans
Promega, Southampton
Qiagen, Chatsworth, California
Appendix 3: (continued).

Rathburn Chemical Company, UK
Sarstedt, Beaumont Leys Schleicher & Schuell, West Germany
Sarstedt Bioscience, Coatbridge
Sigma Chemical Company, Poole
Stratagene, Cambridge
Surgikos, Johnson & Johnson, Livingston
Techne, Cambridge
UVP, San Gabriel, California
Vector Laboratories, Burlingame, California
Whatman, Maidstone
Appendix 4: List of commonly used reagents, stock solutions and buffers for cell culture, immunophenotyping and reverse transcriptase assay.

A4.1 Tissue culture disposable plastic-ware

50 cm², 75 cm² and 250 cm² screw-top tissue culture flasks (Costar)
96-well microtitre tissue culture plate (Costar)
12-well tissue culture plate (Costar)
1, 5, 10 and 25 ml disposable filter-plugged polycarbonate pipettes (Costar)
5, 15 and 50 ml screw-top polypropylene centrifuge tubes (Falcon and Greiner)
0.6 and 1.5 ml flip-top and screw-top polypropylene microcentrifuge tubes (Elkay, Treff and Eppendorf, supplied by Scotlab)
20 ml screw-top polycarbonate Universal tube (Scotlab)
5 ml screw-top polycarbonate bijou (Scotlab)
1, 2.5, 5, 10 and 20 ml syringes (Scotlab)
0.2 and 0.45 μm sterile filters (Acrodisc, Gelman Sciences, supplied by Scotlab)
250 and 500 ml bottle filter systems with 0.2 or 0.45 μm cellulose acetate filter (Costar, Nalgene)
Cell scraper (Costar)
Sterile Petri dish, non-tissue culture grade (Scotlab)
Sterile Petri dish, tissue culture grade (Nunc, Nunc)
Sterile plastic bulb pastettes (Scotlab)
Cryotubes (Nunc)
30 ml Corex glass centrifuge tubes (Sarstedt)

A4.2 Media for handling and culturing cells

A4.2.1 Standard tissue culture medium

A4.2.1.1 RPMI 1640 + 10% hi-FBS + 2ME

500 ml RPMI 1640 (Life Technologies)
50 ml heat-inactivated FBS (hi-FBS) (final concentration: nominal 10%; actual 8.7% FBS)
5 ml 200 mM (29.2 g/L) L-glutamine (final concentration: nominal 600 mg/L, 4 mM; actual 296 mg/L, 2 mM)
20 ml 1 × 10⁶ U/ml penicillin (final concentration: nominal 400 U/ml; actual 348 U/ml) and 1 × 10⁴ μg/ml streptomycin (final concentration: nominal 400 μg/ml; actual 348 μg/ml)
500 μL 5 × 10⁻² M 2 (β) mercaptoethanol (2ME) (final concentration: nominal: 5 × 10⁻⁵ M; actual 4 × 10⁻⁵ M)
Stored at 4°C
Appendix 4: (continued).

A4.2.1.2 RPMI 1640 + 20% hi-FBS + 2ME

500 ml RPMI 1640
100 ml hi-FBS (final concentration: nominal 20%; actual 16% FBS)
5 ml 200 mM L-glutamine (final concentration: nominal 4 mM; actual 3.2 mM)
20 ml 1 x 10^4 U/ml penicillin (final concentration: nominal 400 U/ml; actual 320 U/ml) and 1 x 10^4 µg/ml streptomycin (final concentration: nominal 400 µg/ml; actual 320 µg/ml)
500 µL 5 x 10^-2 M 2ME (final concentration: nominal: 5 x 10^-5 M; actual 4 x 10^-5 M)
Stored at 4°C

A4.2.1.3 Dulbecco’s Modified Eagle’s Medium (DMEM) + 10% hi-FBS + 2ME

Dulbecco's Modified Eagle Medium with sodium pyruvate and 1 x 10^3 mg/L glucose (Life Technologies)
50 ml charcoal-dextran-stripped (cds)-hi-FBS (final concentration: nominal 10%; actual 8.7% FBS)
5 ml 200 mM L-glutamine (final concentration: nominal 4 mM; actual 2 mM)
20 ml 1 x 10^4 U/ml penicillin (final concentration: nominal 400 U/ml; actual 348 U/ml) and 1 x 10^4 µg/ml streptomycin (final concentration: nominal 400 µg/ml; actual 348 µg/ml)
500 µL 5 x 10^-2 M 2ME (final concentration: nominal: 5 x 10^-5 M; actual 4 x 10^-5 M)
Stored at 4°C

A4.2.2 Transport medium

500 ml RPMI 1640
100 ml hi-FBS (final concentration: nominal 20%; actual 15% FBS)
5 ml 200 mM L-glutamine (final concentration: nominal 4 mM; actual 1.5 mM)
20 ml 1 x 10^4 U/ml penicillin (final concentration: nominal 400 U/ml; actual 300 U/ml) and 1 x 10^4 µg/ml streptomycin (final concentration: nominal 400 µg/ml; actual 300 µg/ml)
5 ml 250 µg/ml amphotericin B (final concentration: 2 µg/ml)
7.5 ml 10 mg/ml gentamicin (final concentration: 100 µg/ml)
12.5 ml 1 M N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES) buffer (final concentration: 20 mM)
500 µL 5 x 10^-2 M 2ME (final concentration: nominal: 5 x 10^-6 M; actual 4 x 10^-5 M)
300 µL 1 x 10^3 U/ml heparin (final concentration: 0.45 U/ml)
Stored at 4°C

A4.2.3 Additives for tissue culture medium

Heat-inactivated foetal bovine serum (hi-FBS) (Foetal calf serum, Life Technologies)
Heat-inactivated at 56°C for 30 min
Stored at -20°C
Appendix 4: (continued).

200 mM L-glutamine 200 (Life Technologies)
292 mg in 10 ml ultrapure water
Stored at -20°C

1 x 10⁶ U/ml penicillin (Life Technologies)
Stored at -20°C

1 x 10⁶ µg/ml streptomycin (Life Technologies)
Stored at -20°C

10 mg/ml gentamicin (Life Technologies)
Stored at 4°C

250 µg/ml amphotericin B (Fungizone, Life Technologies)
2.5 g in 50 ml deionised water
Filter sterilised
Stored at -20°C

50 mM 2-(β)-mercaptoethanol (2-ME) (Sigma)
39 mg in 10 ml HBSS
Stored at room temperature

1 x 10⁵ U/ml preservative-free heparin (Leo Labs)
Stored at 4°C

1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer (Life Technologies)
Stored at room temperature

100 mg/ml α-methylmannoside (methyl α-D-mannopyranoside) (Sigma)
1 g in 10 ml RPMI 1640 + 20% hi-FBS + 2ME
Filter sterilised
Stored at -20°C

Dimethyl sulfoxide (DMSO) (Sigma)
Stored at 4°C

0.2% w/v trypan blue (Sigma)
20 mg in 10 ml PBS 1x with 3 mM NaN₃
Stored at room temperature
Appendix 4: (continued).

Tissue culture grade distilled water (Life Technologies)
Stored at room temperature

**A4.2.4 Solutions for suspending viable cells**

Phosphate-buffered saline (PBS) 1x (Dulbecco's Phosphate-Buffered Saline without Mg^{2+} or Ca^{2+}, Life Technologies)
Stored at room temperature

Phosphate-buffered saline (PBS) 10x (Dulbecco's Phosphate-Buffered Saline pH 7.4 without Mg^{2+} or Ca^{2+}, Life Technologies)
Stored at room temperature

Hanks' balanced saline solution (HBSS) + 2% hi-FBS
500 ml Hanks' balanced saline solution (Life Technologies)
10 ml hi-FBS (final concentration: 2%)
Stored at 4°C

Tris-NaCl-EDTA (TNE) 1x
0.1 M NaCl
10 mM Tris.Cl
1 mM ethylenediaminetetraacetic acid (EDTA)
Adjusted to pH 8.0
Stored at room temperature

**A4.2.5 Solutions for dissociating cells and tissues**

Trypsin-EDTA
0.25% crude trypsin (Sigma)
1 M EDTA
Stored at -20°C

100 U/ml dispase II (Boehringer Manheim)
0.5 g (250 U) in 10 ml PBS 1x without Ca^{2+} or Mg^{2+}
Filter sterilised
Stored at -20°C

**A4.2.6 Mitogens and cytokines**

0.5 mg/ml phytohaemagglutinin-P (PHA-P, Sigma)
Filter sterilised
Stored at -20°C
Appendix 4: (continued).

0.5 mg/ml concanavalin A (ConA, Sigma or Boehringer Mannheim)
5 mg in 10 ml RPMI 1640 + 20% hi-FBS + 2ME
Filter sterilised
Stored at -20°C

5 U/μl interleukin 2 (IL2, Interleukin-2, human recombinant, Amersham)
50 μg (1.5 x 10⁵ U) dissolved in 30 ml RPMI 1640 + 20% hi-FBS + 2ME
Stored at -20°C

5 μg/ml interleukin 4 (IL4, hIL-4, recombinant interleukin 4, human sequence, R & D Systems, British Biotechnology)
5 μg (2 x 10⁵ to 5 x 10⁵ U) dissolved in 1 ml PBS 1x containing 0.1% BSA
Stored at -20°C

1 μg/ml interleukin 7 (IL7) (hIL-7, recombinant interleukin 7, human sequence, R & D Systems, British Biotechnology)
5 μg (2 x 10⁵ to 5 x 10⁵ U) dissolved in 5 ml PBS 1x containing 0.1% BSA
Stored at -20°C

1 x 10⁴ U/ml granulocyte-macrophage colony stimulating factor (GMCSF, GM-CSF, human recombinant, Boehringer Mannheim) in PBS 1x containing 0.1% BSA
Stored at -20°C

A4.3 Reagents for immunophenotyping cultured lymphocytes by flow cytometry

A4.3.1 Stock solutions

10% sodium azide (Sigma)
10 g in 100 ml sterile ultrapure water
Filter sterilised
Stored at 4°C

PAB
100 ml PBS 10x (final concentration: PBS 1x)
10 ml 10% sodium azide (final concentration: 0.1%)
2 g bovine serum albumin (final concentration: 2% w/v)
Made up to 1 L with deionised water
Filter sterilised
Stored at 4°C
Appendix 4: (continued).

20% rabbit serum in PAB
100 µl rabbit serum (Dako). Heat inactivated at 56° for 30 min. Filter sterilised. Stored at -20°C
400 µl PAB

1% paraformaldehyde in PBS 1x
10 g paraformaldehyde (BDH) in 450 ml deionised water (heated to 70°C)
Adjusted to pH 7.4 with 1 M NaOH
50 ml PBS 10x
Filter sterilised

20% paraformaldehyde in PBS 1x
10 g paraformaldehyde (BDH) in 20 ml deionised water (heated to 70°C)
Adjusted to pH 7.4 with 1 M NaOH
5 ml PBS 10x
Filter sterilised

A4.3.2 Monoclonal antibodies

Mouse anti-Aspergillus niger glucose oxidase IgG1-FITC κ chain (X927, Dako) 100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA (negative control)

Mouse anti-Aspergillus niger glucose oxidase IgG1-FITC + IgG1-RPE κ chain (X932, Dako) in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA (negative control)

Mouse anti-CD2-FITC + anti-CD19-RPE IgG1 κ chain (FR894, Dako) in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA

Mouse anti-CD3-FITC + anti-CD4-RPE IgG1 κ chain (FR885, Dako) in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA

Mouse anti-CD15-FITC IgM κ chain (F830, Dako) 75 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA

Mouse anti-CD30-FITC IgG1 κ chain (F849, Dako) 100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA

Mouse anti-CD33-FITC IgG1 κ chain (F832, Dako) 100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA

Mouse anti-CD45 (LCA)-FITC IgG1 κ chain (F796, Dako) 100 µg/ml in 0.05 M Tris.HCl pH7.2, 15 mM NaN₃ and 1% BSA
Appendix 4: (continued).

A4.4 Reagents for conventional reverse transcriptase assay

A4.4.1 Stock solutions

0.1 M Dithiothreitol (DTT) (Sigma)
1.54 g in 100 ml ultrapure water
Stored at room temperature

2% Nonidet P-40 (NP-40) (Sigma)
2 ml plus 98 ml ultrapure water
Stored at room temperature

0.8 M Tris pH 8.1 (Trizma-8.1, Sigma)
5.59 g in 50 ml ultrapure water
Stored at room temperature

3 M NaCl (Sigma)
17.53 g in 100 ml ultrapure water
Stored at room temperature

Tris pH 8.1, 0.15 M NaCl
2.80 g Trizma-8.1
1.25 ml 3 M NaCl
Made up to 25 ml with ultrapure water
Stored at room temperature

1 M KCl (Sigma)
7.46 g in 100 ml ultrapure water
Stored at room temperature

20 mM MnCl₂ (Sigma)
396 mg in 100 ml ultrapure water
Stored at room temperature

0.1 M MgCl₂ (Sigma)
952 mg in 100 ml ultrapure water
Stored at room temperature

100% trichloroacetic acid (TCA) (BDH)
500 g in 500 ml ultrapure water
Stored at room temperature
Appendix 4: (continued).

10% TCA, 1% w/v sodium pyrophosphate (NaPP)₄
50 ml 100% TCA
5 g NaPP (Fisons)
Made up to 500 ml with ultrapure water
Stored at room temperature

5% TCA, 1% w/v tetrasodium pyrophosphate (Na₈P₂O₁₀, NaPP₁₀)
25 ml 100% TCA
5 g NaPP (Fisons)
Made up to 500 ml with ultrapure water
Stored at room temperature

5% TCA
50 ml 100% TCA
Made up to 1 L with ultrapure water on day of use
Cooled to 4°C before use

5 U/ml Poly(A).p(dT)₁₂₋₁₈ (Pharmacia)
5 U precipitate (15.7 Å₂₆₀ units/mg) in 1 ml ultrapure water
Stored at -20°C

5 U/ml Poly(dA).p(dT)₁₂₋₁₈ (Pharmacia)
5 U precipitate (16.0 Å₂₆₀ units/mg) in 1 ml ultrapure water
Stored at -20°C

Ethanol 95% (Absolute alcohol, BDH)

A4.4.2 Virus disruption buffer

20 ml 0.1 M DTT
5 ml 2% NP-40
5 ml 0.8 M Tris pH 8.1
5 ml 1 M KCl
Made up to 95 ml with ultrapure water
Stored at -20°C

A4.4.3 Tritiated thymidine triphosphate

Tritiated thymidine triphosphate (³H-TTP) [methyl-³H] thymidine 5’-triphosphate, ammonium salt (Amersham) 37 MBq/ml (1 mC/ml) dissolved in 50% ethanol and 50% water
Stored at 4°C
Appendix 4: (continued).

A4.5 Reagents for eucaryotic expression

100 mg puromycin (P-7255, Sigma)

5 g geneticin (G-418 sulphate, Life Technologies)

1 mM 4-hydroxytamoxifen (Tamoxifen, T-5648, Sigma)
100 mg dissolved in 27 ml absolute alcohol
Stored at -20°C

10 μM hydroxytamoxifen
50 μl 1 mM hydroxytamoxifen
450 μl absolute alcohol

A4.6 Reagents for propidium iodide staining

Acetic acid-alcohol fixative
5 ml glacial acetic acid
95 ml ethanol
Stored at room temperature

4 M HCl
34.5 ml concentrated HCl (specific gravity 1.18)
Made up to 100 ml with ultrapure water

0.1 M sodium borate
3.8 g sodium borate
Made up to 100 ml with ultrapure water

500 μg/ml propidium iodide
5 mg propidium iodide (Propidium I, Sigma)
Made up to 10 ml with PBS 1x

50 mg/ml RNase A (Sigma) in PBS 1x

Propidium iodide/RNase A staining solution
50 μl 500 μg/ml propidium iodide (final concentration: 5 μg/ml)
10 μl 50 mg/ml RNase A (final concentration: 100 μg/ml)
Made up to 5 ml with PBS 1x
Appendix 4: (continued).

A4.7 Reagents for mycoplasma testing

Hoescht 33258 [2-(4-hydroxyphenyl)-5-[5-(4-methylpiperazine-1-yl)-
benzimidazol-2-yl]benzimidazole](bisbenzimide) (Sigma)
1 mg/ml in HBSS without phenol red or bicarbonate

Methanol:glacial acetic acid (3:1)
Appendix 5: List of commonly used reagents, stock solutions and buffers for molecular biology.

A5.1 Disposable plasticware

0.1-2 μl, 0.5-10 μl, 2-200 μl and 50-1,000 μl micropipettes (Gilson, Sealpette)
2-25 μl and 10-50 μl positive (direct) displacement pipettes (Gilson)
0.1-2 μl, 0.5-10 μl, 2-200 μl and 50-1,000 μl micropipette tips (Greiner)
0.1-2 μl, 0.5-10 μl, 2-200 μl and 50-1,000 μl filter-plugged micropipette tips (Greiner)
2-25 μl and 10-50 μl positive (direct) displacement tips (Gilson)
5-60 μl fine gel-loading pipette tips (Greiner)
1, 5, 10 and 25 ml disposable filter-plugged polycarbonate pipettes (Costar)
5, 15 and 50 ml screw-top polypropylene centrifuge tubes (Falcon and Greiner)
0.6 and 1.5 ml flip-top and screw-top polypropylene microcentrifuge tubes (Elkay and Treff)
20 ml screw-top polycarbonate Universal tube (Scotlab)
5 ml screw-top polycarbonate bijou (Scotlab)
1, 2.5, 5, 10 and 20 ml syringes (Scotlab)
Narrow-bore polypropylene bulb pastette (Alpha Labs)
Wide-bore polypropylene bulb pastette (Alpha Labs)
14 ml (17 mm x 100 mm) polypropylene 2059 tubes (Falcon)
Petri dishes (Fisons)

A5.2 General laboratory solutions and buffers

Tris-EDTA (TE) 1x
10 mM Tris.HCl pH 8.0
1 mM EDTA

Tris-boric acid-EDTA (TBE) 1x
90 mM Tris.Cl
90 mM boric acid
2.25 mM EDTA
Adjusted to pH 8.0

Tris-acetic acid-EDTA (TAE) 1x
40 mM Tris.Cl pH 8.0
20 mM sodium acetate
20 mM sodium chloride
2 mM EDTA
Adjusted to pH 8.0
Appendix 5: (continued).

Tris-NaCl-EDTA (TNE) 1x
10 mM Tris.Cl
100 mM NaCl
1 mM EDTA
Adjusted to pH 8.0

20% sodium dodecyl sulphate (SDS)

A5.3 Reagents for restriction endonuclease digestion of DNA

A5.3.1 Restriction endonucleases

Restriction endonucleases (Life Technologies). 10 or 40 U/µl
BamHI
EcoRI
EcoRV
HindIII
PstI
SstI
XbaI
Xhol

A5.3.2 Additives for restriction endonuclease digests

100 mM (5 mg/ml) Sonicated salmon sperm DNA, GeneBloc, International Laboratory Services)
Boiled for 5 min then placed on ice before adding to RE digests

Restriction buffers 10 x (Life Technologies)

50 mg/ml RNase A (Sigma) in PBS 1x

A5.4 Reagents for the polymerase chain reaction

10 x dNTPs
10 mM each deoxynucleoside triphosphates (dATP, dGTP, dCTP, dUTP or dTTP) (Cambio)

10 x PCR buffer (Perkin-Elmer)
15 mM MgCl2
500 mM KCl
100 mM Tris pH 8.2
0.5% Nonidet P-40 (NP-40)
Appendix 5: (continued).

*Taq* polymerase
5 U/µl *Thermus aquaticus* (*Taq*) thermostable DNA polymerase (Amplitaq DNA polymerase, Perkin-Elmer)

25 mM MgCl$_2$ (Perkin-Elmer)

**A5.5 Reagents for processing cells and tissues for the polymerase chain reaction**

Non-ionic detergent buffer (NIB)
PCR buffer 1x (1.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris pH 8.2, 100 µg/ml gelatin) (Perkin-Elmer)
0.45% Nonidet P-40 (NP-40) (Sigma)
0.45% Tween-20 (polyoxyethylene (20)-sorbitan monolaurate, BDH)

1 µg/µl proteinase K (Sigma)
10 mg in 10 ml ultrapure water
Stored at -20°C

10 µg/µl proteinase K (Sigma)
10 mg in 1 ml ultrapure water
Stored at -20°C

**A5.6 Reagents for gel electrophoresis**

**A5.6.1 DNA Size Markers**

100 ng/µl PhiX174 bacteriophage DNA digested with *Hae*III (ΦX174 RF DNA/*Hae*III Frag, Life Technologies) (1353, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp)

Lambda bacteriophage DNA digested with *Hind* III (λ*Hind*III, Life Technologies) (23100, 9400, 6600, 4400, 2300, 2000, 560 bp)

**A5.6.2 Loading buffer/dye and ethidium bromide**

Gel loading buffer/dye 5x
0.42% bromophenol blue
0.42% xylene cyanole
50% glycerol
Made up to 100% with deionised water

10 mg/ml ethidium bromide (Sigma)
Appendix 5: (continued).

A5.6.1 Polyacrylamide gel electrophoresis

8% polyacrylamide gels
26 ml 30% acrylamide (Northumbria Biologicals)
13 ml 2% bis-acrylamide (Northumbria Biologicals)
10 ml TBE 10x
2.2 ml 3% ammonium persulphate (APS)
30 µL N,N,N',N'-tetramethylene diamine (TEMED)
Made up to 100 ml with water

Elution buffer
0.5 M ammonium acetate
1 mM EDTA

A5.6.2 DNA agarose gel electrophoresis

Agarose (Sigma)
Low melting point agarose (Nusieve)
Diethylaminoethyl (DEAE) membrane (Schleicher & Schuell)
1 U/ml Gelase (Cambio)
5 M ammonium acetate

A5.7 Solutions for electroblotting and Southern blotting

Alkali (denaturing) buffer
438.3 g NaCl (final concentration: 1.5 M)
100 g NaOH (final concentration: 0.5 M)
Made up to 2 L with deionised water

Neutralising buffer pH 8.0
303.5 g Tris base
876.6 g NaCl
165 ml HCl
Made up to 5 L with deionised water
Adjusted to pH 8.0
Appendix 5: (continued).

Sodium saline citrate (SSC) 20x
3 M NaCl
0.3 M trisodium citrate
Adjusted to pH 7.0 with 10 M or 0.1 M NaOH

Transfer buffer SSC 10x
500 ml SSC 10x
Made up to 1 L with water

SSC 24x
1 kg NaCl (final concentration: 3.6 M)
500 g sodium citrate (final concentration: 0.36 M)
Made up to 4.7 L with water

Transfer buffer SSC 10x (9.6x)
400 ml SSC 24x
Made up to 1 L with water

Rinse buffer SSC 3x
250 ml SSC 24x
Made up to 1 L with water

A5.8 Reagents for radiolabelling probes

A5.8.1 Random priming

Multiprime Kit (RPN.1601Y, Amersham)
Multiprime buffer solution/nucleotide mix. dATP, dGTP and dTTP in concentrated buffer solution containing Tris.HCl pH 7.8, MgCl₂ and 2-mercaptoethanol (2ME)
Primer solution/primer-BSA containing random hexanucleotides and bovine serum albumin (BSA)
Enzyme solution/Klenow 1 U/μl containing 1 U/μl DNA polymerase I "Klenow" fragment in 50 mM potassium phosphate pH 6.5, 10 mM 2ME and 50% glycerol

α³²P dCTP (R82075, Amersham)
370 MBq/ml (10 mCi/ml) α³²P dCTP. Specific activity 30 TBq/mmol (800 Ci/mmol)
Appendix 5: (continued).

A5.8.2 5' end-labelling

T4 kinase buffer 10x
500 mM Tris.HCl pH 7.5
100 mM MgCl₂
50 mM dithiothreitol (DTT)
1 mM spermidine
1 mM EDTA pH 8.0

10 U/µl T4 polynucleotide kinase

γ³²P ATP (R85132, ICN)
370 MBq/ml (10 mCi/ml) γ³²P ATP. Specific activity 111 MBq/mmol (3000 Ci/mmol)

A5.9 Reagents for hybridisation

A5.9.1 Hybridisation solution

Standard hybridisation solution (50% formamide, SSC 3x)
500 ml 100% formamide (final concentration: 50%)
50 ml Denhardt’s solution 100x (final concentration: 5x)
5 ml 20% SDS (final concentration: 0.1%)
50 ml 1 M Tris pH 7.4 (final concentration: 50 mM)
20 ml 0.5 M EDTA (final concentration: 10 mM)
125 ml SSC 24x (final concentration: SSC 3x)
250 mg Sonicated salmon sperm DNA (GeneBloc, International Laboratory Services). Boiled for 5 min then placed on ice
Made up to 1 L with deionised water

Denhardt’s solution 100x
2% Ficoll
2% bovine serum albumin (Fraction V)
2% polyvinylpyrolidone

A5.9.2 Wash solutions

SSC 3x, 0.1% SDS
30 ml SSC 24x
1.2 ml 20% SDS
Made up to 240 ml with water
Appendix 5: (continued).

SSC 2x, 0.1% SDS
100 ml SSC 20x
5 ml 20% SDS
Made up to 1 L with water

SSC 0.5x, 0.1% SDS
25 ml SSC 20x
5 ml 20% SDS
Made up to 1 L with water

A5.10 Media and reagents for cultivation of bacteria

A5.10.1 Media

Luria-Bertani (LB) broth
10 g tryptone (Unipath)
5 g yeast extract (Unipath)
10 g NaCl (BDH)
Made up to 1 L with water
Sterilised by autoclaving

LB agar
10 g tryptone (Unipath)
5 g yeast extract (Unipath)
10 g NaCl (BDH)
15 g bacteriological agar (Number 1, Unipath)
Made up to 1 L with water
Sterilised by autoclaving

SOC medium
20 g tryptone
5 g yeast extract
500 mg NaCl
200 mg KCl
10 ml 1 M MgCl$_2$
10 ml 1 M MgSO$_4$
1 ml 2 M glucose
Made up to 1 L with water
Sterilised by autoclaving
Appendix 5: (continued).

A5.10.2 Antibiotics

50 mg/ml ampicillin
12 mg/ml tetracycline
100 mg/ml chloramphenicol
20 mg/ml kanamycin

A5.10.3 Additives for blue-white screening

0.2 M isopropyl-1-thio-β-D-galactoside (IPTG)
40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal)
100 mg dissolved in 2.5 ml dimethyl formamide
Protected from light
Stored at -20°C

A5.10.4 Additives for bacterial transformation

500 mM 2-(β)-mercaptoethanol (2-ME) (Sigma)
5 μl 1.12 g/ml (14.3 M) 2-ME stock solution
138 μl sterile ultrapure water

A5.11 Reagents for extraction of plasmids from bacteria

A5.11.1 Modified alkaline lysis

Solution I
50 mM glucose
25 mM Tris.Cl pH 8.0
10 mM EDTA pH 8.0

Solution II
0.2 M NaOH
1% SDS

Solution III
3 M potassium acetate
Appendix 5: (continued).

3 M potassium acetate pH 4.8
80 ml 5 M potassium acetate
11.5 ml glacial acetic acid
Make up to 100 ml with water

A5.11.2 Boiling method

Sucrose-Triton X-100-EDTA-Tris (STET)
8% sucrose
0.5% Triton X-100
50 mM EDTA pH 8.0
10 mM Tris pH 8.0

A5.12 Reagents for sequencing

A5.12.1 Sequencing primers

M13  5' > GTAAAACGACGGCCAGT < 3'

T3  5' > AATTAACCTCACTAAAGG < 3'

T7  5' > G2ATACGACCTCACTTAGGGC < 3'

SP6  5' > ATATACGACGTACATAATGAA < 3'

A5.12.2 T7 Sequencing Kit (Pharmacia)

T7 DNA polymerase 8 U/μl in buffered glycerol

Sequencing mixes ('A', 'C', 'G' or 'T' mix-short) in 40 mM Tris.HCl pH 7.6 and 50 mM NaCl
'A' mix-short: 840 μM each dCTP, dGTP and dTTP, 93.5 μM dATP, 14 μM ddATP
'C' mix-short: 840 μM each dATP, dGTP and dTTP, 93.5 μM dCTP, 17 μM ddCTP
'G' mix-short: 840 μM each dATP, dCTP and dTTP, 93.5 μM dGTP, 14 μM ddGTP
'T' mix-short: 840 μM each dCTP, dGTP and dGTP, 93.5 μM dTTP, 14 μM ddTTP

Universal primer
5 μM M13

Annealing buffer
1 M Tris.HCl pH7.6
100 mM MgCl₂
160 mM DTT
Appendix 5: (continued).

Enzyme dilution buffer
20 mM Tris.HCl pH 7.5
5 mM DTT
100 µg/ml bovine serum albumin (BSA)
5% glycerol

Labelling mix-dATP
1.375 µM each dCTP, dGTP and dTTP
333.5 mM NaCl

Stop solution
97.5% formamide
10 mM EDTA pH 7.5
0.3% bromophenol blue
0.3% xylene cyanol

A5.12.3 $^{35}$S-labelled dATP radioisotope

$\alpha^{35}$S dATP (SJ1304, Amersham)
9.25 MBq/µl (250 µCi/µl) $\alpha^{35}$S dATP. Specific activity 37 TBq/mmol (1000 Ci/mmol)

A5.12.4 Polyacrylamide sequencing gel

5% sequencing gel mix (non-setting)
10 ml Long Ranger DNA sequencing gel solution Hydro-Link (AT Biochem) 50% concentrate
6 ml TBE 10x
42 g urea
Make up to 100 ml with deionised water

5% polyacrylamide gel mix (setting)
100 ml 5% sequencing gel mix
500 µl 10% APS
50 µl TEMED
Appendix 5: (continued).

A5.13 Northern blot hybridisation

A5.13.1 Reagents for RNA extraction

0.05% diethyl pyrocarbonate (DEPC) in ultrapure water

EDTA-SDS-DEPC
1 mM EDTA pH 7.0
0.5% SDS
Made with 0.05% DEPC in ultrapure water

A5.13.2 Reagents for RNA gel electrophoresis

3-(N-morpholino)-2-hydroxypropane sulphonic acid (MOPS) buffer 10x
41.8 g MOPS
6.8 g sodium acetate (final concentration: 0.05 M)
3.7 g EDTA (final concentration: 5 mM)
Made up to 1 L with water
Adjusted to pH 7.0 with 10 M NaOH

1% agarose gel with MOPS 1x and 8% formaldehyde
2 g agarose dissolved in 147 ml water by boiling
20 ml MOPS 10x
33 ml 37% formaldehyde (Analar). Warmed to 37°C

Formamide buffer
500 µl MOPS 10x
830 µl 37% formaldehyde
2.5 ml formamide

RNA loading buffer/dye
50% glycerol
1 mM EDTA
0.4% bromophenol blue
Appendix 6: Complete nucleotide sequence of human T lymphotropic virus type I proviral DNA.

GenBank Accession number J02029 (9068 bp) (Seiki et al. 1983). Oligonucleotide primer sequences for PCR are underlined (overlined, shaded or emboldened where overlapping) and annotated at right.

```
1  ggaaaaaactt  ggaagtgtag  tcggatcag  accatggcc  ccaaatatcc  cccggggcct
61  tagagcctcc  cagtgaaaaa  catttccgag  aaacagaagt  ctgaaaaggt  cagggcccag
121  actaaggctc  tgacgfcctcc  ccccggaggg  cagctcagca  ccggctcggg  ctaggccctg
181  acgtgtcccc  ctgaagacaa  atcataagct  cagacctccg  ggaagccacc  aagaaccacc
241  catttcctcc  ccatgtttgt  caagccgtcc  tcaggcgttg  acgacaaccc  ctcacctcaa
301  1  aaaacttttc  atggcacgca  tatggctcaa  taaactagca  ggagtctata  aaagcgtgga
361  gacagttcag  gagggggctc  gcatctctcc  ttcacgcgcc  cgccgcccta  cctgaggccg
421  ccatccacgc  cggttgagtc  gcgttctgcc  gcctcccgcc  tgtggtgcct  cctgaactgc
481  gtccgccgtc  tagggtaagtt  taaagctcag  gtcgagaccg  ggcctttgtc  cggcgctccc
541  ttggagccta  cctagactca  gccggctctc  cacgctttgc  ctgaccctgc  ttqctcaact
601  ctacgtcttt  gtttcgtttt  ctgttctgcg  ccgttacaga  tcgaaagtttc  cacccctttc
661  cctttcattc  acgactgact  gccggcttgg  cccacggcca  agtaccggcg  actccgttgg
721  ctcggagcca  gcgacagccc  atcctatagc  actctcagga  gagaaattta  gtacacagtt
781  gggqgctcgb  ccgggatacg  agcgcccctt  tattccctag  gcaatgggcc  aaatcttttc
841  ccgtagcgct  agccctattc  cgcgaccgcc  ccgggggctg  gccgctcatc  actggcttaa
901  cttcctccag  gcggcatatc  gcctagaacc  cggtccctcc  agttacgatt  tccaccagtt
961  aaaaaaattt  cttaaaatag  ctttagaaac  accggctcgg  atctgtccca  ttaactactc
1021  cctcctagcc  agcctactcc  caaaaggata  ccccggccgg  gtgaatgaaa  ttttacacat
1081  actcatccaa  acccaagccc  agatcccgtc  ccgtcccgcg  ccaccgccgc  cgtcatcccc
1141  cacccacgac  cccccggatt  ctgatccaca  aatcccccct  ccctatgttg  agcctacggc
1201  cccccaagtc  cttccagtca  tgcatccaca  tggtgctcct  cctaaccatc  gcccatggca
1261  aatgaaaqac  ctacaggcca  ttaagcaaga  agtctcccaa  gcagcccctg  ggagccccca
1321  gtttatgcag  accatccggc  ttgcggtgca  gcagtttgac  ccoactgcca  aagacctcca
1381  agacctcctg  cagbacctbt  gctcctccct  cqtggcttcc  cbccabcacc  agcagcbaga
1441  bagccbbaba  bcagaggccg  aaacccgagg  b  ba  acag gb  babaacccab bagccggbcc
1501  ccbccgbgbc  caagccaaca  atccacaaca  acaaggabta  agg cgagaab  accagcaact
1561  cbggcbcgcc  gccbbcgccg  cccbgccggg  gagbgccaaa  gacccbbccb  g ggccbcbab
1621  cctccaaggc  cb ggaggagc  cbbaccacgc  cbbcgbagaa cgccbcaaca bagcbcbbga
1681  caabgggcbg  ccagaaggca  cgcccaaaga  ccccabcbba cgbbccbbag ccbacbccaa
1741  tgcaaacaaa  gaabgccaaa  aabtactaca  ggcccgagga  cacacbaaba gccctctagg
1801  agababgbbg cg g g cb b g b c agacctggac  ccccaaagac  aaaccaaag  tgttagbbgb
1861  ccagccbaaa  aaaccccccc caaatcagcc  gbgcbbccgg  bgcgggaaag  caggccacbg
1921  g agbcgggac bgcacbcagc  cbcgbccccc  ccccgggcca  bgcccccbab gbcaagaccc
1981  aacbcacbgg  aagcgagact  gcccccgccb  aaagcccacb abcccagaac cagagccaga
2041  ggaagabgcc cbccbattag  accbccccgc bgacabccca cacccaaaaa acbbcabagg
2101  g g g g a g g b b  baaccbcccc ccccacabba cagcaagtcc ttccbaacca agacccagca
2161  tctabtcbgc cagbbabacc gbbagabccc gcccgbcggc ccgbaabbaa agcccaggbb
2221  gacacccaga ccagccaccc aaagacbabc gaagcbbbac bagabacagg agcagacabg
2281  acagbccbtc cgabagccbb gbbcbcaagb aabacbcccc bcaaaaabac abccgbabba
2341  g gg gcag gg g  gccaaaccca agabcacbbb aagcbcaccb cccbbccbgb gcbaabacgc
2401  cbcccbbbcc ggacaacgcc babbgbttba acabctbgcc b ag b b g ab ac caaaaacaac
2461  baggccabca b ag g b cg b g a bgccbbacaa caabgccaag gcgbcctgba ccbcccbgag
2521  gcaaaaaggc cgccbgbaab cbbgccaaba caggcgccag ccgbccbbgg gcbagaacac
2581  cbcccaaggc cccccgaaab cagccagbbc ccbbbaaacc agaacgccbc caggccb b g c
2641  aacactbggb ccggaaggcc cbggaggcag gccababcga acccbacacc gggccaggga
2701  ataacccagb abbcccagbb aaaaaggcca abggaaccbg gcgabbcabc cacgaccbgc
2761  gggccacbaa cbccbttacc aagtaagctc catatccctc cocoggccgg cccgctcct
2821  cctccacgca taactttaag ccaatccactt acctgctacttacctgctatt
```
Appendix 6: (continued).

6361 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
6321 aattggagaa ggaggtattg cagagccaa cagagccagt gcgctttcgc gaaattaccc
6481 tggcggtgca ctgtctgccg cagcttctca cggctttgct ttttcttttc ccagtgcttt
6601 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
6661 tggcggtgca ctgtctgccg cagcttctca cggctttgct ttttcttttc ccagtgcttt
6721 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
6781 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
6841 ttttttttcct tggccagggc tctgttcgct gcctggacac ggcaccgttt gggtaacggc
6901 tggcggtgca ctgtctgccg cagcttctca cggctttgct ttttcttttc ccagtgcttt
6961 ttttttttcct tggccagggc tctgttcgct gcctggacac ggcaccgttt gggtaacggc
7021 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7081 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7141 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7201 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7261 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7321 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7381 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7441 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7501 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7561 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7621 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7681 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7741 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7801 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7861 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7921 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
7981 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8041 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8101 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8161 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8221 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8281 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8341 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8401 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8461 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8521 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8581 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8641 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8701 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8761 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8821 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8881 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
8941 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
9001 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
9061 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc
9121 aactactaca aaattgccga gtaagctgcg cagacagcag gaggccgtga tctctggttc

Appendix 7: Complete nucleotide sequence of human T lymphotropic virus type II proviral DNA.

GenBank Accession number M10060 (8952 bp) (Shimotohno et al 1985). Oligonucleotide primer sequences for PCR are underlined (shaded or emboldened where overlapping) and annotated at right.

```
1 ttgacaatgga gactagcctc caacggccag cctccaggag ggtcagctga ccacaaaggt
61 cgggactgct ccaagccaca atacccagct caggtcgaga ccagacgcgt ctacccgcac
121 ccaatggaac ccagccctga ggtcctccac ctagaagaaa acagaggtcttg cgacattac
181 ccacccgcocaa taaaaagctgg cacacttata cagagccaga ctagccttata cagagccaga
241 gcagctgtagc gggggtcctg ggtcagcctg gggtccctgc tggggtccgg ctagccttata
301 ctaaggacct ggtcctcaggg tggcagcctg ggtcctcaga ggtcctgctt gggggtcctg
361 ggtcctgctt gggggtcctg ggtcctcaga ggtcctgctt gggggtcctg ggtcctcaga
421 gggggtcctg ggtcctcaga ggtcctgctt gggggtcctg ggtcctcaga ggtcctgctt
481 gggggtcctg ggtcctcaga ggtcctgctt gggggtcctg gggcgggtcctg ggtcctcaga
541 gggggtcctg ggtcctcaga ggtcctgctt gggggtcctg ggtcctcaga ggtcctgctt
601 gggggtcctg ggtcctcaga ggtcctgctt gggggtcctg ggtcctcaga ggtcctgctt
```

Appendix 7: (continued).

2941 tggaaccgcgg tccacaggg gttttaaaca acgtccaccc tcttgaacac acaatttagaa
3001 cgatctcaac aacccatagg gaaaggtttt tccacatcgc acgttccca acacactggat
3061 ctgactcaat ctagcagcgg cccatagcgg gaaatcaca gcacccactc acaatcctcctc
d121 cccggaagcg cggccacttt tctattcag ccccaataaat ccccaaccc cgggcaagc
3181 cactccaggt tcttgaacac gttactctac ctaatcctc tttataactg gacagcaacc
3241 actataacca taataacact cggcatctgg aatgcatctc agctgagact ggacagagac
3301 caagtggatg clcaagaggg aaccaactcg gaaatcaca acacctcctc acacactggat
3361 cttcaggggt accgggccc aagagctgtg aatccctcacc cccaccaccc acacactggat
3421 ttacattgca tcttgaacac gttataaact acacgtccct ccccgccaca acacactgga
3481 ccttctcgct gctctctcct gttaatcact cacttacact ctccctcactt cccaccaccc
3541 acacactgca agccctcggg tttgctcaac accccaaccc ctccagacag aagacacttac
t010 tggcggacgg caaacgcggt gccggaaggt gtttcg tccgcagt cccgcaagcc
3601 cccggaagcg cggccacttt tctattcag ccccaataaat ccccaaccc cgggcaagc
3661 ggcgagatgc cccactccat ccccaaccaac attgcagaa cggcctcttg gccatctggat
3721 aggacactcc ccttcaacag tccggccgat cttattcact cccgcaagcc aacactctgct
3781 ctttccgacgg aacgcggtgg gcggagaggt gtttcact cgggacactt cccgcaagcc
3841 gggcgacact ccctctagggg tggctcgtgc aacgatgtac cggtcgagag aacactctgct
3901 ctgctctggag cccgtctctc cctccggtgg cccgcaagcc aacactctgct cccgcaagcc
3961 cccggaagcg cggccacttt tctattcag ccccaataaat ccccaaccc cgggcaagc
4021 cctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4081 cttagctac/a cctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc
4141 acctcgccgg atccactcc ccctctggtt cctccggtgg cccgcaagcc aacactctgct
4201 ctactctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4261 tctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4321 atctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4381 tctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4441 gctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4501 gactctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4561 tctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4621acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4681 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4741 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4801 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4861 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4921 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
4981 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5041 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5101 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5161 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5221 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5281 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5341 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5401 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5461 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5521 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5581 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5641 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5701 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5761 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5821 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5881 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
5941 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
6001 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
6061 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
6121 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
6181 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
6241 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
6301 acctctctcct cctccggtgg cccgcaagcc aacactctgct cccgcaagcc aacactctgct
Appendix 7: (continued).

681  gcaagtgtgc  tctctcaaa  tcagtaacg  tcagtgatcc  gctccgaag  aagagccccc
691  tcttgaaaa  cgtcttcg  cccgttgtgg  aatactctgg  gatctcggc  ttctccatgg
701  ggcagagaa  gcctccag  ccagataac  ctatttgcct  ctctctctc  tcgcctcttatt
711  gttgtgcct  gctctgact  gggaaaac  gcgttctca  cagctctcag  aaaaacgcga
721  taaccgctat  tcocullcct  acaccaaaaa  cgctcttaaa  tagaagctct  agtttctgtg
731  ggcctccatct  cgctctgatt  ctctctctt  tgcctcaaaa  tcctctctct  tagcttctct
741  acctccgct  caaccttccc  cttaagagg  tcttctctcc  ccaccaaat  ttgaacactac
751  ctggaggcct  gtctacaatac  ccagggccct  tccagctctc  cctcaataat  ttgagctgat
761  gtctctctc  ctctctgagg  gccctgtaaa  cagctctcag  cagttctctc  tcggacactc
771  gctgtgctct  cgctcttgag  gccctgtaaa  cagctctcag  cagttctcct  tcggacactc
781  acctccgct  taaccttccc  cttaagagg  tcttctctcc  ccaccaaat  ttgaacactac
791  tccttttctc  ccagggccct  tccagctctc  cctcaataat  ttgagctgat  50
801  taccgctat  cgggattt  aagggccaag  ggttgcccg  tcttgccgg  tcttgccgg  tcttgccgg  288
811  acctccgct  taaccttccc  cttaagagg  tcttctctcc  ccaccaaat  ttgaacactac  cactgggac  3, 209
821  ccagctctc  gaccggctgg  cagccttggc  ccagctctc  gaccggctgg  cagccttggc  cagccttggc  3
831  tctctcttccc  ccagggccct  tccagctctc  cctcaataat  ttgagctgat  81
841  gccctgtaaa  cagctctcag  cagttctctc  tcggacactc  cagctctcag  cagttctctc  tcggacactc
851  ccagctctc  gaccggctgg  cagccttggc  ccagctctc  gaccggctgg  cagccttggc  cagccttggc  3
861  accggggagt  gcagacccgg  aacccggccg  aacccggccg  gcagacccgg  aacccggccg  aacccggccg
871  taccgctat  cgggattt  aagggccaag  ggttgcccg  tcttgccgg  tcttgccgg  tcttgccgg  288
881  taccgctat  cgggattt  aagggccaag  ggttgcccg  tcttgccgg  tcttgccgg  tcttgccgg  288
891  gccctgtaaa  cagctctcag  cagttctctc  tcggacactc  cagctctcag  cagttctctc  tcggacactc

Appendix 8: Complete nucleotide sequence of bovine leukaemia virus proviral DNA.

GenBank Accession number K02120 (8714 bp) (Sagata et al 1985). Oligonucleotide primer sequences for PCR are underlined and annotated at right.

```plaintext
1 tgtatgaag acatgcgga cctaggagcc gccaccgccc cgtaa accag acagagacgt
6 1  
cagctgccag aaaagctggt gacggcagct ggtggctaga atccccgt ac ctccccaact
121 tcccctttcc cgaaaaatcc acaccctgag ctgctgacct cacct gctga taaattaata
181 aaatgccggc cctgtcgagt tagcggcacc agaagcgttc ttctcct gag accctcgtgc
241 tcagctctcg gtcctgagct ctcttgctcc cgagaccttc tggtcggc ta tccggcagcg
301 gtcaggtaag gcaagcacgg tttggagggt ggttctcggc tgagacca cc gcgagctcta
361 tcagctgagt tctggtcG tctgaGGgtG tccacgtgga ctGtctcctt tgcctGct ga GGGcgGgGtc
421 caagggcgtG tggcttgGac ccgcgtttgt ttGCtgtGtt actttctg tt tctcgcggcc
481 cgcgctctct ccttcggcgc cctctagcgg GGaggagaga cGggcaaa ca attgggggGt
541 cqtccgggat tgatcacccc ggaaGGGtaa caactGtctg gacccacc cc ctcggGggca
601 ttttgggtct ctccttGaaa ttatatcatg ggaaattcGc cctccta taa ccccGGGgct
661 ggtatctCGG cctcagaGtg gctcaacctt Gtgcaaagcg cgGaaagg ct caatGGgGga
721 ccGtGtccta gcgattttac Ggatttaaag aattaGatGc attggtt tca taagaGccag
7  8 1 aaaaaccat ggactttcac ttGtggtggc cccaGctGat gtccacc cgg gagattcggc
841 cgggttCGGG tGgtGttggc GaGCctaaaG gaagtactct caaacga agg gggGgcccGg
901 ggtgcatGgg Gcccagaaga aGaacccccc ccttatgacc cGCCGgG Gat tttgccaatc
961 atatctgaag ggaatcgcaa GGgccatGgt gcttgggcac tccqagaa tt acaagatatc
1021 aaaaagaaa ttgaaaataa ggcacGgggt tcgcaagtat ggatacaa ac actacgactt
1081 gGaatcctgc aggGcgaccG tactcGgqct qacctagaac aactttgG Ga atatattgct
1141 tcGGGggtGg accaaaGggG ccatatgacc agcctaacgg Gagcaata gc cgGGgctgaa
1201 gcggcaacac cctccagggt tttaaccGGG aaaaGgggta Gcctaac cca aGaatcagct
1261 Gagcccaacg ccggggatGt tagaagtcaa tatcaaaacc tctggct tca ggGcggaaaa
1321 atGtGGGtac tcqtGcttGa gctacaacct tggtGcacca tGgtccaa gg GGCGgccgaa
1381 agctctgtag agtttgtcaa ccggttaGaa atttGattag Gtgacaac ct tGccgacgga
1441 gtcctaagga acccattatt gaGtGGCtta gttatgcaaa tgctaac aga gagtgtcagc
1501 aaattttgca ggggcgaggc cagtggGcgc ggtggggcaa aaactgG agg GttgcgGaca
1561 attgggcGcc Gaagaatgaa acagcctgca cttctcgtGG aGacGGG agg gcccaagatg
1621 GGcgggGctc ggcaaccggc ccccaaaagg GGtCGCGcag gaccatg cta tGgatgcctc
1681 aaagaaggcc attgggccGg ggattgtCGt accaaggcGa ccggccG acc tccgggacct
1741 tgcccGatat gtaaagatGc ttcGGattgg aaaGgagact gtccaacc ct Gaaatcaaaa
1801 aactaataga ggggggaGtt agcgGGGCcc aaaccataac acctata acg gattctctta
1861 gtgaggccga attagaatgc ttactttcta ttcctctggG tcgcagG Ggt ccctccgtgg
1921 ctgtatacct gtctggcGcc tggctgGagc cGtGtGagaa tcaagccc tc atgcttgtgg
1981 acaccggggc tgaaaataGg gttGtGGcaG aaaattggct ggttcgag at taGccaGgga
2041 tcccGgccgc agtgGtcgga gcagggggag tctcccggaa cagatac aat tggctacaag
2101 gaacgGGtcG aggcccttca agaGctggtc catcgctctc tggaggGa gg ttatatctcc
2161 CGCtgggacg ggccaggcaa taatccagtc ttGCGggtac ggaaaGG aaaa tggcgcctgg
2221 agctctgtag agtttgtcaa ccggttaGaa atttGattag Gtgacaac ct tGccgacgga
2281 gtcctaagga acccattatt gaGtGGCtta gttatgcaaa tgctaac aga gagtgtcagc
2341 ccacattgga ttagaaGatG tgGGCGCccc acGtgaggtg Gctcaatt GC ctttaaaGta
2401 gtcgatttcc tcaacacgc cttctctctt ttcctctctt ttcctctctt ttcctctctt
2461 acaccggggc tgaaaataGg gttGtGGcaG aaaattggct ggttcgag at taGccaGgga
2521 GGcgggGctc ggcaaccggc ccccaaaagg GGtCGCGcag gaccatg cta tGgatgcctc
2581 tcccctttcc cgaaaaatcc acaccctgag ctgctgacct cacct gctga taaattaata
2641 acctaaagac cttctctctt ttcctctctt ttcctctctt ttcctctctt ttcctctctt
2701 gaagctcgc ctaacacgc cttctctctt ttcctctctt ttcctctctt ttcctctctt
2761 gttgggttag attttctttt gttgggttag attttctttt gttgggttag attttctttt
2821 ataatcgttgc gttgggttag attttctttt gttgggttag attttctttt gttgggttag
2881 gtcgatttcc tcaacacgc cttctctctt ttcctctctt ttcctctctt ttcctctctt
2941 gtaaagatGc ttcGGattgg aaaGgagact gtccaacc ct Gaaatcaaaa
3001 gcggtcctgt ctctctcttt ttcctctctt ttcctctctt ttcctctctt ttcctctctt
3061 gtaaagatGc ttcGGattgg aaaGgagact gtccaacc ct Gaaatcaaaa
```
Appendix 8: (continued).

3121  gaccccaggg caaacatgca gctttccccg  cagcagctgc  aaggcat  tgc  agagcttcga

3181  caagccctgt  cccacaacgc  aagatctaga  tataacgagc  aagaacc  cct  gctagcctac

3241  gtacacctaa  cccgggcggg  gtccaccctg  gtactcttcc  aaaaggg  cgc  tcaatttccc

3301  ctggcctact  ttcagacccc  cttgactgac  aaccaagcct  caccttg  ggg  cctccttctc

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Appendix 8: (continued).

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Appendix 9: Human β-globin partial sequence.

GenBank Accession number NM_000518 (626 bp) (Proudfoot and Brownlee 1976). Oligonucleotide primer sequences for PCR are underlined and annotated at right.

1 acatttgctt ctgacacaac tgtgttcact agcaacctca aacagacacc atggtgcacc 18 61 lgactcctga ggagaagtct gccgttactg ccctgtgggg caaggtgaac gtggatgaag 52 121 ttggtggtgd ggccctgggc aggctgctgg tggtctaccc ttggacccag aggttctttg 181 aatgtctttgg ggcctgcaac aatcnnnggg ctggttcgga caaacctcaag gtgaagggctc 241 atgcggcagc agcctcctgc gcntttgggc gtaacgctgac ccacctctcgg 301 gcccctttgg gcagcttctg gcctcctgctg gcctctccgg gcctcctgctg gcctctccgg 361 gtccttcctc cncgcttctg gcctcctgctg gcctctccgg gcctcctgctg gcctctccgg 421 ccctctctct gcctctctct gcctctctct gcctctctct gcctctctct gcctctctct 481 acataataat gctgctgctg tcctctctct ctctctctct ctctctctct ctctctctct 541 ctcgctctct gcctctctct gcctctctct gcctctctct gcctctctct gcctctctct 591 taataataa atctctctct gcctctctct gcctctctct gcctctctct gcctctctct 601
Appendix 10: Alignments of conserved regions of retroviral and hepadnaviral Pol amino acid sequences and derived consensus oligonucleotides.

A10.1: Comparison of conserved regions of retroviral and hepadnaviral reverse transcriptase (Pol) amino acid sequences.


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| Endogenous | WW* QFENFSPTDEALKHDLASFAENQOTLLQYVMDLILNGS |
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| Hepadnaviruses |  |  |
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| HerOnDHEV | FRRHPGVQVSLPILHIAPTIALJIESIKRFNWFTTYMDLILNGS |
| HEV | FRRHPGVQVSLPILHIAPTIALJIESIKRFNWFTTYMDLILNGS |
| WHV | FRRHPGVQVSLPILHIAPTIALJIESIKRFNWFTTYMDLILNGS |
| GSHV | FRRHPGVQVSLPILHIAPTIALJIESIKRFNWFTTYMDLILNGS |
### Abbreviations

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<td>SHIAP</td>
<td>Syrian hamster intracellular type A particles</td>
</tr>
<tr>
<td>SIVagm</td>
<td>Simian immunodeficiency virus (African green monkey)</td>
</tr>
<tr>
<td>SIVmac</td>
<td>Simian immunodeficiency virus (rhesus macaque)</td>
</tr>
<tr>
<td>SIVsm</td>
<td>Simian immunodeficiency virus (sooty mangabey)</td>
</tr>
<tr>
<td>SRV1</td>
<td>Simian retrovirus type 1</td>
</tr>
<tr>
<td>SRV2</td>
<td>Simian retrovirus type 2</td>
</tr>
<tr>
<td>Visna</td>
<td>Visna virus</td>
</tr>
<tr>
<td>WHV</td>
<td>Woodchuck hepatitis virus</td>
</tr>
</tbody>
</table>
Appendix 10: (continued).

A10.2 Comparison of nucleotide and amino acid sequences of conserved regions of retroviral reverse transcriptase (*pol*) genes (Donehower et al 1990).

Nucleotides are designated by single letter codes (Appendix 5). The single letter code for each amino acid is centred underneath the three nucleotides that encode it.

<table>
<thead>
<tr>
<th></th>
<th>5' sequence</th>
<th>3' sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'</td>
<td>GACTACCCCAAGGGTTTAAA</td>
<td>GTTCAGTACAGGATGAC</td>
</tr>
<tr>
<td>3'</td>
<td>VLPQGFK</td>
<td>LQYMDD</td>
</tr>
<tr>
<td>5'</td>
<td>GTCCCTGCCAGGGTTTAAA</td>
<td>GTCCATACAGGATGAC</td>
</tr>
<tr>
<td>3'</td>
<td>VLPQGFK</td>
<td>VQYMDD</td>
</tr>
<tr>
<td>5'</td>
<td>GTCTGCGCCAGGGTTTAAA</td>
<td>GTCTCTTCRGAGATGAT</td>
</tr>
<tr>
<td>3'</td>
<td>VLPQGFK</td>
<td>VSXMDD</td>
</tr>
<tr>
<td>5'</td>
<td>GTGCTTCCAGGGTTTAAA</td>
<td>GTGCAATACAGGATGAT</td>
</tr>
<tr>
<td>3'</td>
<td>VLPQGWK</td>
<td>YQYMDD</td>
</tr>
<tr>
<td>5'</td>
<td>TGTTACCCAGGGTTTAAA</td>
<td>TATCAATACAGGATGAT</td>
</tr>
<tr>
<td>3'</td>
<td>CLPQGFV</td>
<td>YQYMDD</td>
</tr>
<tr>
<td>5'</td>
<td>AGACTCCCAAGGGTTTAAA</td>
<td>GTACGTACGGGAGAC</td>
</tr>
<tr>
<td>3'</td>
<td>RLPQGFK</td>
<td>LQYVDD</td>
</tr>
<tr>
<td>5'</td>
<td>GCCTGCGCCAGGGATTGACC</td>
<td>TGGCAATTGAGGATGAT</td>
</tr>
<tr>
<td>3'</td>
<td>VLPQGM</td>
<td>LHYMDD</td>
</tr>
<tr>
<td>5'</td>
<td>GTCTGCGCCAGGGATTGAAA</td>
<td>GTGCAATACAGGATGAC</td>
</tr>
<tr>
<td>3'</td>
<td>VLPQGM</td>
<td>VHYMDD</td>
</tr>
</tbody>
</table>

5' > GTNYTNCCNCARGG < 3'  
5' > RTCRTCCMRTA < 3'  
5' consensus primer  
3' consensus primer
Appendix 10: (continued).

A10.3 Comparison of 5' conserved regions of retroviral and hepadnaviral reverse transcriptase (Pol) amino acid sequences and derivation of consensus sense (forward) oligonucleotide primers for PCR (Mack and Sninsky et al 1988).

Amino acids and nucleotides are designated by single letter codes (Appendix 5). Potential three-nucleotide codons are listed under each amino acid alignment. Consensus nucleotide sequences derived from these reverse translations are shown using the standard nomenclature for incompletely specified bases.

**Group 1 viruses**

<table>
<thead>
<tr>
<th>virus</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTLV-I</td>
<td>L P Q G F K N S P L E</td>
</tr>
<tr>
<td>HTLV-II</td>
<td>L P Q G F K N S P L Y</td>
</tr>
<tr>
<td>BLV</td>
<td>L P Q G F K N S P L E</td>
</tr>
<tr>
<td>MuLV</td>
<td>L P Q G F K N S P L E</td>
</tr>
<tr>
<td>FeLV</td>
<td>L P Q G F K N S P L E</td>
</tr>
</tbody>
</table>

**Group 2 viruses**

<table>
<thead>
<tr>
<th>virus</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMTV</td>
<td>L P Q G M K N S P T L C Q</td>
</tr>
<tr>
<td>SRV-1</td>
<td>L P Q R M A N S P T L C Q</td>
</tr>
<tr>
<td>MPMV</td>
<td>L P Q G M A N S P T L C Q</td>
</tr>
<tr>
<td>SLELV</td>
<td>L P Q G M A N S P T L C Q</td>
</tr>
<tr>
<td>REV</td>
<td>L P Q G M T C S P T L C Q</td>
</tr>
</tbody>
</table>

5' > YTNCCNCARGGRMNWKNSNCNRCNYTNTGYCAR < 3' 
5' > YTNCCNCARGGRMNWKNSNCNRCNCT < 3' 
5' > YTNCCNCAKIG < 3'
### Appendix 10: (continued).

#### Group 3 viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1</td>
<td>LPQGNSKP</td>
</tr>
<tr>
<td>HIV-2</td>
<td>LPQGNSKP</td>
</tr>
<tr>
<td>SIV-1</td>
<td>LPQGNSKP</td>
</tr>
<tr>
<td>EIAV</td>
<td>LPQGEVSP</td>
</tr>
<tr>
<td>GABV</td>
<td>LPQGWSLP</td>
</tr>
<tr>
<td>Visna</td>
<td>LPQGWLP</td>
</tr>
</tbody>
</table>

* * * * *

**TTGCCTCAAGGTAATAAAGGTTCTCCT**

**TTACCCCAGGGCAACAAGGGCTCCCGC**

**CTTCCA GGATGGGTTGGATCACCA**

**CTCCCG GGGTTTGTCGGGTCGCCG**

**CTA TTCGTATTGAGT**

**CTG**

5' > YTNCCCARCAGGWDBRRNDDKWSNCCN < 3'

#### Group 4 viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV</td>
<td>LPMGVGLSPFLLAQFT</td>
</tr>
<tr>
<td>WHV</td>
<td>LPMGVGLSPFLLAQFT</td>
</tr>
<tr>
<td>GSHV</td>
<td>LPMGVGLSPFLLAQFT</td>
</tr>
<tr>
<td>DEHV</td>
<td>LPMGVGLSPFLHLHFT</td>
</tr>
</tbody>
</table>

* * * * *

**TTGCCCTTGGGGTTGGTTTCTCTCTTTCTTTGATGGCGATTACT**

**TTGCCCG GGGCCAGCCTTCCACCA GTCCCTGCATTG ACA**

**CTGCCG GGGGTGGGGCTCTGGCCG CTCCCGGCGTTA AGS**

**CTA CPGAGT GAATCAGATCTT**

**CTG CTGAGC CTGCTGACCTC**

**GCT CTA**

**AIT CIG**

**ATC**

**ATA**

5' > NYNCCCARATGGNGTGGGNYTNNSNCFFTYTNTNYTNNSNYNTTYYACN < 3'

5' > CCRATGGGNGTGGGNYTNNSNCFFTT < 3'
Appendix 10: (continued).

A10.4 Comparison of 3' conserved regions of retroviral and hepadnaviral reverse transcriptase (Pol) amino acid sequences and derivation of consensus antisense (reverse) oligonucleotide primers for PCR (Mack and Sninsky et al. 1988).

Amino acids and nucleotides are designated by single letter codes (Appendix 5). Potential three-nucleotide codons are listed under each amino acid alignment. Consensus nucleotide sequences derived from these reverse translations are shown using the standard nomenclature for incompletely specified bases. The complementary sequences are used to derive antisense (reverse) oligonucleotide primers.

<table>
<thead>
<tr>
<th>Group 1 viruses</th>
<th>MuLV</th>
<th>G Y V D D L</th>
<th>PolV</th>
<th>Q Y V D D L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2 viruses</td>
<td>HTLV-I</td>
<td>Q Y M D D I</td>
<td>HTLV-II</td>
<td>Q Y M D D I</td>
</tr>
<tr>
<td></td>
<td>BLV</td>
<td>S Y M D D I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3 viruses</td>
<td>MNLV</td>
<td>H Y M D D I</td>
<td>SBV-1</td>
<td>H Y M D D I</td>
</tr>
<tr>
<td></td>
<td>NMV</td>
<td>H Y M D D I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHIV</td>
<td>H Y M D D I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RSV</td>
<td>H Y M D D I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4 viruses</td>
<td>HIV-1</td>
<td>Q Y M D D I</td>
<td>HIV-2</td>
<td>Q Y M D D I</td>
</tr>
<tr>
<td></td>
<td>SIV-1</td>
<td>Q Y M D D I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EIAV</td>
<td>Q Y M D D L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAEV</td>
<td>I Y M D D I</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Visna</td>
<td>I Y M D D I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 5 viruses</td>
<td>HBV</td>
<td>S Y M D D V</td>
<td>KBV</td>
<td>A Y M D D L</td>
</tr>
<tr>
<td></td>
<td>GSHV</td>
<td>A Y M D D L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEBV</td>
<td>T Y M D D F</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Consensus: TATGTTGATCAT TAC GCACGAC GTR GTS ** ** ** **

5' > TAYGAYGAY < 3'

5' > TAYGAYGAY < 3'

5' > ATRYANCTRCT < 5'

5' > TCRCTCCTRTA < 3'