T-CELL RECEPTOR GAMMA GENE REARRANGEMENTS IN THE INVESTIGATION OF IMMUNOPATHOLOGICAL DISORDERS

By

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Dedicated to my parents Abd Karim and Khadeeja

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T-cell Receptor Gamma Gene Rearrangements in the Investigation of Immunopathological Disorders

Summary

Infiltration of the tissues by T-lymphocytes is a prominent feature of many human diseases including infections, autoimmune disorders, various poorly understood chronic inflammatory conditions, T-cell lymphomas and leukaemias and tumours of other tissues. Very little is known about the infiltrating T-cell clones in these conditions, apart from the T-cell lymphomas and leukaemias most of which are monoclonal, derived from a single transformed cell.

The object of this thesis was to devise a sensitive method for the detection of T-cell clones in small tissue samples and to use the method in pilot studies on examples of human diseases whose lesions are infiltrated with T-cells.

T-cell clones are distinguished from one another by their antigen receptors. Each clone arises in the thymus from a founder cell in which germline genes coding for antigen receptor molecules are altered (rearranged) in a particular way to give that cell an antigen-receptor of unique specificity, much clonal diversity being generated by the variety of possible rearrangements of the germline genes and deletions and random insertions of uncoded nucleotides at hypervariable (N) regions within these rearrangements.

The polymerase chain reaction (PCR) has been adapted to amplify rearrangements of the human T-cell receptor gamma (TCRγ) genes and high resolution polyacrylamide gel electrophoresis has been used to analyse the molecular size of the amplified products which include the hypervariable N regions. Since the N region in different clones varies in size by 40 or so nucleotides, in theory 1280 subsets of rearranged genes (clonotypes) can be distinguished by this method, if a total of 32 PCRs are performed to amplify most of the possible rearrangements.

The method has been developed and validated with model systems employing DNA from cultured T-cell lines whose γ gene rearrangements were already known from the literature. With appropriate primer combinations, PCR with as little as 1 nanogram of DNA from monoclonal T-cell lines gave a positive reaction, a dense dominant electrophoretic band of the expected molecular size and the dominant band could be demonstrated in the presence of 20-100 parts of polyclonal DNA from reactive (hyperplastic) lymph nodes. By itself polyclonal DNA produced electrophoretic smears (sometimes with some minor bands) which reflect the presence of multiple clones with a range of N region sizes.

A battery of 8 PCRs for the most common TCRγ gene rearrangements successfully demonstrated dominant clonal rearrangements in 24 of 36 (67%) cases of malignant T-cell lymphoma in contrast to 1 of 12 cases of reactive lymph node hyperplasia, and none of 12 skin samples from 5 normal controls. Evidence was obtained suggesting that the dominant clonotype detected in plaques of cutaneous T-cell lymphoma is also present in smaller amounts in clinically unaffected skin but not in blood from the same patient. The latter finding and observations on blood and skin from normal subjects are consistent with the existence of a subset of T-cell clones which selectively home to the skin.

The rest of the study investigated a recent modification of Burnet's hypothesis that forbidden clones of autonomously functioning neoplastic T-cells at the benign end of spectrum are the underlying cause of vitiligo, psoriasis and rheumatoid arthritis - poorly understood immunopathological disorders whose lesions are infiltrated by T-cells.

A dominant clonal band was demonstrated in only 1 of 28 vitiligo lesions from 10 patients and in none of 29 psoriatic lesions from 13 patients. Possible examples of minor TCRγ gene rearrangements restricted to the lesions of vitiligo or psoriasis were found respectively in 1 and 3 of 4 patients with both diseases. Dominant bands possibly reflecting the presence of latent low grade T-cell neoplasia were detected in DNA from peripheral blood in 3 of 8 patients with vitiligo and 2 of 12 patients with psoriasis. It is unlikely that these are of pathogenic importance since

similar dominant bands were also found in the blood in 2 of 5 normal control subjects.

In rheumatoid arthritis dominant bands were detected in diseased joints in 4 of 20 cases. Direct evidence was obtained that in vitro culture in Interleukin-2 (IL-2) has significant effects on the relative abundance of T-cell clones in samples of synovial fluid and that investigations based on analysis of cultured synovial lymphocytes are likely to give misleading results.

The finding of dominant rearrangements in the lesions of only a minority of cases of vitiligo, psoriasis and rheumatoid arthritis does not support the hypothesis that T-cell neoplasia at the benign end of the spectrum is the underlying cause of any of these diseases but the possibility of involvement of small (non-dominant) benign neoplastic T-cell clones which selectively localise to the lesions in these conditions cannot be excluded.

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Abbreviations

AIL	Angioimmunoblastic lymphadenopathy
bp	Base pair
CL	Cutaneous lymphoma case
Con A	Concanavalin A
CTCL	Cutaneous T-cell lymphoma
DNA	Deoxyribonucleic acid
E.B.Virus	Epstein-Barr virus
EATL	Enteropathy associated T-cell lymphoma
HLA	Human leukocyte antigen
IL	Interleukin
JRA	Juvenile rheumatoid arthritis
Kb	Kilobase
L	Lymphoma case
LGL	Large granular lymphocyte
MB	Multiple bands
MHC	Major histocompatibility complex
NC	Normal control subject
ND	Not done
NK	Not known
NR	No reaction
P	Psoriasis case
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PLEVA	Pityriasis lichenoides et varioliformis acuta
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RN	Reactive lymph node case
S	Smear
SB	Southern blot
SF	Synovial fluid
SIg	Surface immunoglobulin
SLE	Systemic lupus erythematosus
SM	Synovial membrane
T-NHL	T-cell non-Hodgkin's lymphoma
TCRγ	T-cell receptor gamma
V	Vitiligo case
VP	Vitiligo plus psoriasis case

CHAPTER 1

Introduction

Immunological disorders such as rheumatoid arthritis, the organ-specific autoimmune diseases and psoriasis affect 5-10 percent of people by the time they reach old age and are the cause of much suffering and disability. T-lymphocytes infiltrate the lesions and are thought to be important in the pathogenesis of these conditions but little is known about the T-cell clones which are specifically involved. In this thesis an attempt is made to study this question using T-cell receptor gamma gene rearrangements which have been amplified by the polymerase chain reaction as clonal markers.

1.1 Historical Background

Clonal Selection Theory of Acquired Immunity (Burnet 1959)

In 1958 Burnet postulated that different lymphocytes have different antigen receptors because of random somatic changes which occur in the receptor genes during development of the immune system, and that exposure to antigen later in life induces specific immunity by causing selective proliferation of lymphocytes with receptors which happen to bind that antigen. Each resulting clone consists of a family of cells all with the same antigen receptor as the lymphoid cell from which the clone was originally descended. Since the changes in the receptor genes in the developing immune system are random some lymphocytes have receptors for self antigens, but clones arising from self reactive cells are eliminated or suppressed so that there is normally a state of "immunological tolerance" towards potential autoantigens.

Burnet also suggested that lymphatic leukaemia and malignant lymphomas are abnormal clonal proliferations "freed by somatic mutation from some of the normal growth controls" and that clones derived from neoplastic lymphocytes "approaching or reaching malignancy" are a likely cause of immunopathological disorders. Forbidden (autoreactive) clones

of neoplastic lymphocytes which have escaped from the control of self tolerance may, for example, be the cause of autoimmune tissue damage such as the autoimmune haemolytic anaemia which sometimes accompanies lymphatic leukaemia and malignant lymphoma.

Many of Burnet's ideas have been confirmed. The clonal selection theory is now generally accepted (Roitt 1988, Alberts et al. 1983). The evidence is summarised in Section 1.2 below. Tolerance to self antigens has been shown to be due to deletion or anergy of potentially autoreactive clones (Section 1.3). It is also well established that most lymphoid neoplasms are monoclonal and likely that they are derived from cells with acquired (somatic) changes in growth-controlling genes (Section 1.4).

Burnet's suggestion that autoimmunity is commonly due to unregulated growth and function of forbidden clones of neoplastic autoreactive lymphocytes has received little support. Lymphoid neoplasms are found in only a minority of patients with autoimmune disorders (Holme, Blomgren & Löwhagen 1985, Kinlen 1992) and monoclonal autoantibodies (which would be expected in B-cell neoplasia) are frequently present in only two, chronic cold haemagglutinin disease (Worlledge, Hughes & Bain 1982) and mixed essential cryoglobulinaemia (Gorevic et al. 1980) both of which are uncommon. A number of genes and immune mechanisms have been implicated in the breakdown of self tolerance in man and experimental animals (Section 1.5) but in spite of much research the initiation of the naturally occurring autoimmune diseases and their persistence over long periods of time are still poorly understood. T-lymphocytes are thought to be implicated because of their presence in lesions and their importance as helper/inducer cells in immune responses (Kumar et al. 1989).

Benign T-cell Neoplasia Hypothesis

In 1989 Goudie and Lee revived Burnet's hypothesis of inappropriate functional activity of neoplastic clones in the pathogenesis of autoimmune and other obscure immunopathological disorders. They postulated the

existence of benign T-cell neoplasms which have gone unrecognised in the past because they are morphologically indistinguishable from reactive T-cell hyperplasias, but which might be provisionally identified by their monoclonality (Section 1.6).

The aim of the work presented in this thesis was to develop a large set of sensitive and discriminating clonal markers based on the amplification of T-cell receptor gamma (TCRγ) gene rearrangements (Section 1.7) by the polymerase chain reaction (PCR) (Section 1.8) and to use these markers to study the clonal origin of T-cells in the lesions of three immunopathological skin disorders, cutaneous T-cell lymphoma (Paterson & Edelson 1987), psoriasis (Baker & Fry 1992) and the organ-specific autoimmune disease, vitiligo (Al Badri et al. 1993) and in the affected joints of the non-organ-specific autoimmune disease rheumatoid arthritis (Roitt 1988).

1.2 Clonal Selection and Acquired Immunity

B-lymphocytes provide the best evidence of clonal selection. The nature of the B-cell receptor is reviewed in detail by Warner (1974). Surface immunoglobulin (SIg) can be found in large amounts on most B-lymphocytes. Specific antigens such as flagellin bind to the surface of a small proportion of (polyclonal) B-lymphocytes of non-immune individuals. Antigen binding is blocked by treatment of the lymphocytes with anti-immunoglobulin antibody suggesting that SIg is the B-cell antigen receptor (Ada 1970).

Immunisation with a particular antigen leads to a great increase in the number of lymphocytes binding that antigen and in the number of cells producing the corresponding antibody (Ada 1970, Jerne et al. 1974). Immunisation with two different antigens leads to increase in two distinct populations of antigen-binding cells (Nossal & Lederberg 1958). Absence of cells which combine with both antigens favours clonal proliferation and is against the other possibility, non-specific binding of circulating antibody by Fc receptors on the B-cell surface. Passive transfer of affinity-purified lymphocytes from an immune donor restores the ability of irradiated

animals to produce large amounts of the appropriate specific antibody and confirms the involvement of specific antigen-binding B-cells in B-cell immunity (Ada 1970). Single affinity-purified lymphocytes which bind to a particular antigen can be shown to undergo clonal proliferation when cultured in vitro in the presence of that antigen (Vaux, Pike & Nossal 1981). (For thymus-dependent antigens helper T-cells must also be present in the culture). In vitro antigen binding leads to clustering of all the SIg molecules at one pole of the cell ("capping"), further evidence that SIg is the antigen receptor and that the SIg molecules on a given cell have identical antigen-binding sites (Raff, Feldman & de Petris 1973). Crosslinking of SIg by anti-immunoglobulin antibody (but not by monovalent antibody fragments) also leads to capping (de Petris & Raff 1973) and a proliferative response by B-cells associated with tyrosine-kinase activation (Reth 1992).

At the molecular level study of immunoglobulin from different B-cell clones has shown that the amino acid sequences of the N terminal regions of the heavy and light chains are highly variable and that these regions are involved in antigen recognition and binding (Capra & Kehoe 1975). The genetic mechanisms responsible for immunoglobulin diversity have been demonstrated by recombinant DNA technology (Tonegawa 1983). In the germline and cells which do not produce immunoglobulin, genetic information for an immunoglobulin chain is encoded by multiple gene segments scattered along a chromosome. These include clusters of different V (variable), J (joining) and in the case of heavy chains, D (diversity) segments coding for the variable N-terminal region of the polypeptide chain. In the developing B-cell a complete functional gene is formed by somatic recombination with joining of one V, (one D) and one J segment. Receptor diversity is determined by the selection of V, (D) and J segments from the germline repertoire (combinatorial diversity), variation in recombination breakpoints (junctional site diversity) and insertion of uncoded nucleotides (junctional insertion diversity). A high rate of somatic mutation of individual bases adds to the diversity. These processes are antigen-independent and lead to the generation of a large number ($>10^6$) of different B-cells each with one functional heavy and one functional light chain gene and a unique SIg receptor which determines if the cell will be selected for clonal proliferation following exposure to a particular antigen.

Evidence for clonal selection of T-cells is similar to that of B-cells but less complete. Four genes (α, β, γ and δ) code for T-cell antigen receptors. They are homologous to the immunoglobulin genes with V,J and in some cases D gene segments which recombine during thymic development with marked combinatorial and junctional diversity (Moss, Rosenberg & Bell 1992). (Details of the TCRγ genes and their rearrangements are given in Section 1.7). T-cell receptors are transmembrane polypeptide heterodimers composed either of $\alpha\beta$ or $\gamma\delta$ chains with highly variable N terminal regions which project outwards from the cell surface (Fig 1.1) and are responsible for antigen binding (Moss, Rosenberg & Bell 1992). In vitro T-cell clones have been isolated which show a proliferative response to antigen stimulation, provided that macrophages or other suitable antigen presenting cells are present in the culture (Fathman & Frelinger 1983). Unless antigen is presented in the context of appropriate major histocompatibility (MHC) molecules it combines very weakly with the T-cell receptor (Fathman & Frelinger 1983). The binding of labelled antigen by individual T-cells cannot therefore be studied as it can with B-cells. Another difficulty is that in T-cells there is no equivalent of antibody secretion which has been very helpful in the study of B-cell clones.

Conclusion

The above findings confirm Burnet's prediction that "the development of specific antibody producing capacity is something characteristic not so much of a cell as of a clone of cells" and shows that the same principle applies in T-cell mediated immunity. They also justify the use of the variable regions of antigen receptors (and the receptor gene rearrangements which code for them) as B and T-cell clonal markers.

1.3 Self-tolerance

Because of difficulty in detecting and following the fate of individual cells or clones of cells with high affinity receptors for self antigens among the great diversity of lymphocytes in the normal immune system there has until recently been no direct evidence how the normal condition of absent or diminished immunological reactivity to self antigens comes about

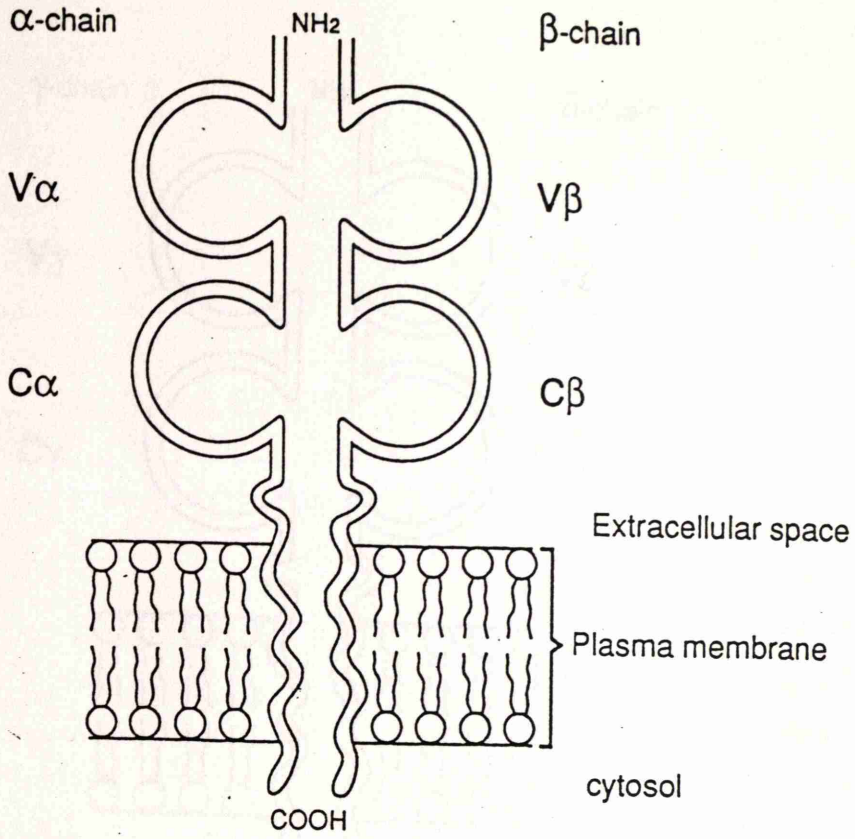


Figure 1.1 (a) Schematic representation of a T-cell receptor heterodimer composed of an α and a β polypeptide chain. Each chain has a constant (C) portion, extending from the inside of the cell through the membrane, to the outside. The outermost part is the variable (V) region.

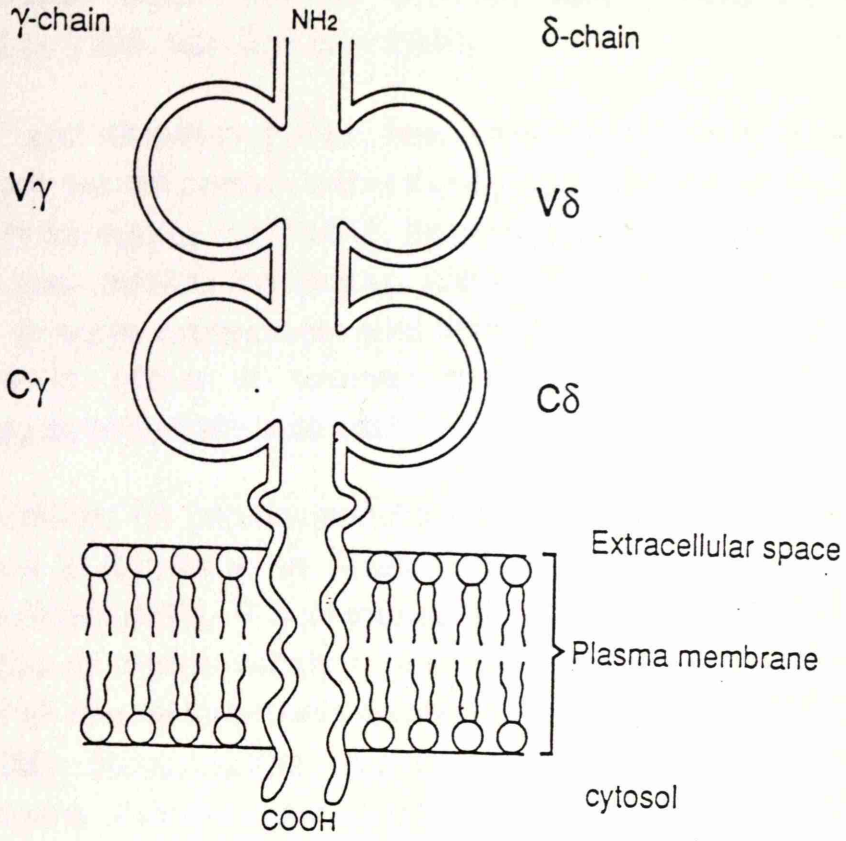


Figure 1.1 (b) Schematic representaion of a T-cell receptor heterodimer composed of a γ and a δ polypeptide chain. Each chain has a constant (C) portion, extending from the inside of the cell through the membrane, to the outside. The outermost part is the variable (V) region. (Adapted from Lefranc & Rabbitts 1989).

(Goodnow 1992). Direct evidence of intrathymic deletion of self reactive cells has now been obtained in normal mice (Kappler et al. 1987, Kappler et al. 1988) and in transgenic animals in which a large proportion of T- or B-cells have productively rearranged genes coding for a high affinity antiself receptor which can be detected with monoclonal antibody (KisieLow et al. 1988, Von Boehmer 1990).

Miller and Morahan (1992) have recently reviewed tolerance to antigens which are not present within the thymus. Most of the studies have been done in transgenic mice which express "foreign" antigens at specific anatomical sites outside the thymus under the control of tissue-specific promoters. In some experiments post thymic tolerance was due to clonal anergy and in others it seemed that extrathymic antigens went unrecognised by potentially autoreactive T-cells.

Experiments on immunoglobulin transgenic mice have shown that self tolerance in B-cells as in T-cells may be due to clonal deletion or anergy (Goodnow 1992). It is of interest that large amounts of low affinity autoantibodies to a wide variety of autoantigens can be demonstrated in the blood of all normal individuals and present no threat to health (Casali & Notkins 1989). Many studies have been done on acquired tolerance to foreign antigens (Nossal 1983, Nossal 1989). As in self tolerance, tolerance to foreign antigens is sometimes due to clonal deletion and sometimes to clonal anergy. There are also many reports of the involvement of suppressor or anti-idiotypic T-cells which prevent activation of otherwise competent B and T-cells (Dorf & Benacerraf 1984, Moller 1988). Tolerance to foreign antigens is more easily induced in young animals and in immature lymphoid cells and is related to antigen dosage. Two signals, cross linking of receptors by antigen plus help from activated T-cells or antigen-presenting cells, are usually required for the induction of immunity while crosslinking of antigen receptors without the help of a second signal seems to favour the development of tolerance.

Conclusion

The generation of receptor diversity in the developing immune system produces potentially autoreactive lymphocytes which are either eliminated or rendered anergic or do not encounter the corresponding autoantigens in an immunogenic form. Breakdown of any of these mechanisms of self tolerance in cells with high affinity receptors for self antigens are possible causes of harmful autoimmune responses.

1.4 B and T-cell Neoplasms are Monoclonal

By definition all the cells of a particular lymphoid clone are the descendants of a single precursor cell and share the same antigen receptor and receptor gene rearrangements which in effect distinguish that clone from all other clones. Monoclonal lymphoid cells therefore have homogeneous antigen receptors and receptor gene rearrangements whereas polyclonal lymphoid cell populations are heterogeneous. Antibody responses even to simple antigens such as dinitrophenyl are usually heterogeneous (Pink & Askonas 1974).

Monoclonality was first suspected in B-cell neoplasia when it was recognised that the sharp peak found on electrophoresis of serum from patients with multiple myeloma might be due to a high concentration of unusually homogeneous immunoglobulin molecules secreted by the malignant plasma cells (Putnam 1957). This has been confirmed by the successful sequencing of the amino acids of the variable regions of individual myeloma proteins (Capra & Kehoe 1975) which is technically possible only if they are homogeneous. Serological analysis with anti-light chain antibodies shows that individual myeloma proteins are either type kappa or type lambda unlike normal serum immunoglobulin which is polyclonal and a mixture of kappa and lambda molecules (Mannik & Kunkel 1963). Immunocytochemical staining with anti-light chain antibodies provides direct evidence for the monotypia and monoclonality of the neoplastic cells in multiple myeloma (Hitzman, Li & Kyle 1981), B-cell lymphoma (Picker et al. 1987) and B-cell leukaemia (Levy et al. 1977). This can also be shown by in situ hybridisation of light chain mRNA with

kappa or lambda-specific oligonucleotide probes (Akhtar et al. 1989). Analysis of immunoglobulin heavy or light chain gene rearrangements by Southern blotting (Griesser et al. 1986, Foroni et al. 1984) or the polymerase chain reaction (McCarthy et al. 1990) produces disproportionately large bands consistent with the presence of a single dominant neoplastic clone.

The presence of large homogeneous monoclonal T-cell populations can be detected immunocytochemically with antibodies specific for the products of particular V gene families. The method has been used successfully to demonstrate the monoclonality of T-cell leukaemias and lymphomas when antibodies of appropriate specificity are available (Clark et al. 1986, Gledhill et al. 1990). Diagnostic tests for T-cell neoplasia based on the detection of monoclonality by Southern blot or PCR analysis of T-cell receptor gene rearrangements usually give positive results (Table 1.1). Negative findings are presumably due to technical factors, loss of rearranged chromosomes by malignant cells or errors in diagnosis of T-cell malignancy.

Further evidence of monoclonality of lymphoid neoplasms comes from the presence of identical chromosomal abnormalities in all of the malignant B or T-cells but not in the non-neoplastic cells from the same individual. It is likely that many of these chromosomal abnormalities contribute directly to the neoplastic transformation (Rabbitts & Boehm 1991). In nearly all cases of Burkitt's lymphoma there is translocation between one of the immunoglobulin heavy or light chain genes (on chromosomes 14, 2 and 22) and chromosome band 8q 24, the location of the proto-oncogene c-myc (Boehm & Rabbitts 1989). Following translocation the proto-oncogene is thought to be activated inappropriately by the immunoglobulin promoters and enhancers which are active in B-cells. Somatic mutations are also often present in addition in the first exon of the translocated c-myc gene (Morse et al. 1989). Another well known example is the (14:18) translocation in cases of follicular B-cell lymphoma. This translocation involves the immunoglobulin heavy chain and bcl-2 genes (Tsujimoto et al. 1985) and is thought to immortalise the cell by making it resistant to death by apoptosis (Hockenberry et al. 1990).

TABLE 1.1 Reported frequency (No. of postive cases/No. of cases tested) of dominant TCRβ and γ gene rearrangements in T-cell malignancy

Reference	Method	T-cell leukaemia	T-cell lymphoma	Cutaneous T-cell lymphoma	Sezary syndrome
Bertness et al. (1985)	Southern blot β	2/2	5/5	7/8	12/12
O'Connor et al. (1985)	Southern blot β	6/6	15/18	1/1	
Waldmann et al. (1985)	Southern blot β	9/9			5/5
O'Connor (1987)	Southern blot β	16/16	33/37	7/19	5/5
Tkachuk et al. (1988)	Southern blot β Southern blot γ		19/19 19/19		
Gledhill et al. (1990)	Southern blot β Southern blot γ		5/6 4/6		
McCarthy et al. (1991)	Southern blot β	6/6	2/2		1/1

Table 1.1 (continued)

Reference	Method	T-cell leukaemia	T-cell lymphoma	Cutaneous T-cell lymphoma	Sezary syndrome
Bourguin et al. (1990)	PCRγ	5/5	7/7		
Taylor et al. (1991)	PCRγ	24/28			
McCarthy et al. (1991)	PCRβ	6/8	6/8		1/1
Bahler et al. (1992)	PCRβ			23/24	4/4

Comparable chromosomal abnormalities occur in T-cell neoplasia. A T-cell equivalent of the Burkitt's lymphoma translocation has been described between c-myc and the TCRα locus (Bernard et al. 1988). In a quarter of cases of acute T-cell leukaemia there is a 90Kb deletion in the chromosomal region 1p32, the site of the gene tal/SCL/TCL5 which codes for a regulatory DNA binding protein (Brown et al. 1990). This gene is sometimes translocated with TCRα/δ in acute T-cell leukaemia (Chen et al. 1990).

Many of the chromosomal abnormalities in lymphoid neoplasia involve receptor genes and are due to error by the recombinase enzyme system in catalysing receptor gene rearrangements in developing B and T-cells (Croce 1987).

Conclusion

Monoclonality and acquired chromosomal abnormalities are features which distinguish lymphoid neoplasia from normal or hyperplastic lymphoid tissue.

1.5 Pathogenesis of Autoimmunity

Little is known about the mechanisms responsible for the breakdown of self tolerance in the naturally occurring autoimmune diseases. Concordance rates for clinical autoimmunity in monozygotic twins (Barnett et al. 1981, Silman et al. 1993) suggest that heredity and environment are both involved. The following is a brief review of factors known or thought to be implicated in the initiation of autoimmunity in man and animals.

Major Histocompatibility Complex (MHC)

Compared with the general population the frequency of certain alleles of the MHC is abnormal in patients with various autoimmune diseases (linkage disequilibrium). Examples are shown in Table 1.2. Disease associations are stronger with MHC class II (HLA-D) antigen than with class I (HLA-A, -B, -C) (Weetman 1992, Nepom & Erlich 1991). MHC identical siblings of children with the organ-specific autoimmune disease

TABLE 1.2 Some significant HLA associations with immunopathological disorders in caucasians (data from Tiwari & Terasaki 1985)

Disease	No. of studies	Frequency in patients (%)	Frequency in controls (%)	Average relative risk
Rheumatoid arthritis				
DR4	17	68	25	3.8
Ankylosing spondylitis	40	89	9	69.1
B27				
Juvenile diabetes mellitus				
DR3	13	46	22	3.3
DR4	12	51	25	3.6
Graves' disease				
DR3	4	56	25	3.7
Psoriasis vulgaris				
CW6	7	56	15	7.5
DR7	5	48	23	3.2

Type I diabetes mellitus develop the disease more often than MHC non-identical siblings (Gorsuch et al. 1982). The presence of the amino acid asparagine at position 57 of the HLA DQB chain protects against the development of Type 1 diabetes (Todd, Bell & McDevitt 1987). MHC is also linked to susceptibility to the autoimmune diabetes which develops spontaneously in BB rats (Colle, Guttmann & Seemayer 1981) and NOD mice (Hattori et al. 1986) and in the induction of experimental allergic encephalomyelitis and thyroiditis by injection of autoantigen (Fritz et al. 1985, Weetman 1991). The association of MHC with a wide range of autoimmune diseases suggests that T-cells have an important role in the development of autoimmunity. Antigen can only be recognised by CD4 (helper/inducer) T-cells in the context of self MHC class II molecules on the surface of an antigen presenting cell and by CD8 (cytotoxic) T-cells in the context of self MHC class I molecules (Schwartz 1985). The MHC alleles which an individual inherits influence the T-cell receptor repertoire and the efficiency with which different antigens are presented to T-cells (Kumar et al. 1989).

Other Hereditary Factors

Breeding experiments show that in addition to MHC three other unlinked loci contribute to the susceptibility of NOD mice to diabetes mellitus (Todd et al. 1991). The function of none of these genes is known. Severe T-cell lymphopenia, inherited as an autosomal recessive, correlates closely but not completely with the development of diabetes in BB rats (Jackson et al. 1984).

lpr and gld are two single gene mouse models of a non-organ-specific autoimmune disease resembling systemic lupus erythematosus (Cohen & Eisenberg 1991). It is accompanied by lymph node enlargement due to the presence of large numbers of phenotypically abnormal T-lymphocytes which are polyclonal.

Abnormal Antigen Stimulation

Autoimmunity can be induced in experimental animals by injection of normal tissue components such as myelin basic protein (Zamvil &

Steinman 1990) or thyroglobulin (Weetman 1991). The autoimmune response and the development of autoimmune tissue damage are greatly increased if the antigen is injected with dead tubercle bacilli and mineral oil (Freund's adjuvant) which appears non-specifically to enhance immune responsiveness and activate autoreactive lymphocytes. The autoimmune thyroiditis produced in this way is often transient (Jones & Roitt 1961, Flax 1963).

Molecular mimicry of self antigens by foreign antigens associated with bacteria (Shoenfeld & Isenberg 1988), viruses (Oldstone 1987) and diet (Karjalainen et al. 1992) are thought to have a role in the development of autoimmunity. Helper T-cells activated by the foreign components of these antigens may induce an autoimmune response in lymphocytes with receptors for the epitopes which mimic self.

Inappropriate Expression of MHC class II Antigens

The observation that MHC class II molecules are inappropriately expressed by thyrocytes in autoimmune thyroid disease and pancreatic beta cells in autoimmune (Type I) diabetes (Bottazzo, Pujol-Borrell & Hanafusa 1983, Foulis & Farquharson 1986) has led to the hypothesis that these cells may function as antigen presenting cells capable of stimulating autoreactive T-cells by presentation of endogenous autoantigens. Inappropriate MHC class II expression may have a role in perpetuating autoimmune disease but it seems unlikely to be the initiating event since it may be dependent on the local presence of activated T-cells producing gamma interferon (Weetman 1992).

Non-antigenic Lymphoid Stimulation

Polyclonal activation by substances such as bacterial lipopolysaccharide (Fournié, Lambert & Miescher 1974) or viruses such as E.B virus (Fong et al. 1981) may stimulate anergic potentially autoreactive lymphocytes and lead to autoimmunity independently of antigenic stimulation. Neoplastic lymphoid cells may also proliferate independently of antigenic stimulation. This may explain the presence of autoantibodies which bind antigen most strongly far below normal body temperature in the

two autoimmune diseases frequently associated with B-cell neoplasia, chronic cold haemagglutinin disease and mixed essential cryoglobulinaemia (Goudie & Lee 1989). The possible relationship between lymphoid neoplasia and autoimmunity is considered further in Section 1.6.

Failure of Normal Immunoregulation

There are many reports of helper (CD4) suppressor (CD8) T-cell imbalance in the blood and lesions of patients with autoimmune diseases but the abnormalities may be the result rather than the cause of the disease (Bach & Bach 1981, Weetman 1991). It is now thought that suppressor functions are not restricted to a particular T-cell subset (Moller 1988). Networks of anti-idiotypic antibodies and T-cells are believed to regulate immune responses to foreign antigens and may also regulate responses to autoantigens (Rossi, Deitrich & Kazatchkine 1989, Roitt & Cooke 1986). Depletion of regulatory T-cells may account for the accelerated development of several spontaneously occurring autoimmune diseases in neonatally thymectomised animals such as NZB/NZW mice (lupus nephritis) (Helyer & Howie 1963) and obese strain chickens (autoimmune thyroiditis) (Wick, Kite & Witebsky 1970). In others such as BB rats occurrence of disease (autoimmune diabetes mellitus) is reduced by neonatal thymectomy (Like et al. 1982).

Conclusion

It is clear that autoimmune diseases in man and animals are heterogeneous and multifactorial. Genetic predisposition is important and several immunological mechanisms may be involved. It is still unknown what actually initiates the proliferation of autoreactive clones in the naturally occurring autoimmune diseases. Why, for example, does childhood autoimmune (Type I) diabetes mellitus frequently affect only one of a pair of monozygotic twins who inherit the same genes and share a similar environment ?

1.6 The Benign T-Cell Neoplasia Hypothesis

The benign T-cell neoplasia hypothesis (Goudie & Lee 1989) is a recent modification of Burnet's original idea that uncontrolled functional activity of clones of neoplastic lymphocytes "approaching or reaching malignancy" are a likely cause of autoimmune and other obscure immunopathological disorders.

Benign neoplasms are recognised in most tissues of the body (MacSween & Whaley 1992). They are characterised by excessive and apparently "purposeless" growth of well differentiated cells which resemble those of the tissue of origin on microscopic examination and form sharply circumscribed tumour masses which do not invade the surrounding tissues or metastasise to distant sites. Clinically they usually present as swellings and may cause mechanical problems but in the case of endocrine neoplasia they may present with the effects of inappropriate hormone production such as the bone lesions of hyperparathyroidism before the tumour mass is clinically apparent. In some tissues, colonic mucosa for example, benign tumours have a significant tendency to progress to malignancy but in others like parathyroid or myometrium this hardly ever happens. Benign tumours are sometimes multiple and may be familial. Like most neoplasms they are usually monoclonal (Fialkow 1976) and some have acquired chromosomal abnormalities (Mitelman, Kaneko & Trent 1991).

Benign lymphoid neoplasia is not included in standard tumour classifications and is scarcely mentioned in the scientific literature. Goudie and Lee suspect that it may be quite common but that it is either overlooked or misdiagnosed as reactive lymphoid hyperplasia because the neoplastic cells are morphologically indistinguishable from normal lymphocytes and do not form sharply circumscribed tumour masses because of their tendency to migrate and mingle with other cells such as normal lymphocytes.

Several possible examples of immunological abnormalities due to benign lymphoid neoplasia have been suggested. Roitt suspects that the symptomless monoclonal gammopathies which affect about 3 percent of

elderly subjects are due to otherwise inapparent "benign tumours of the lymphocyte - plasma cell series". If the monoclonal immunoglobulin happens to be autoantibody it may lead to autoimmune tissue damage as in the autoimmune haemolytic anaemia of chronic cold haemagglutinin disease or the glomerulonephritis and arteritis which results from immune complexes formed by the monoclonal anti-immunoglobulin autoantibody in mixed cryoglobulinaemia. Approximately 20 percent of patients with these conditions are associated with frank B-cell malignancy. The remainder are presumably examples of benign B-cell neoplasia. Examples of clinically inapparent benign T-cell neoplasia are found in ataxia telangiectasia, an autosomal recessive disorder characterised by neurological and vascular abnormalities and a probable DNA repair defect leading to a high incidence of T-cell leukaemia and lymphoma. Some patients with this condition have a large clone of mature but cytogenetically abnormal T-cells which form as much as half of the peripheral blood lymphocytes. Some of these are preleukaemic but others never progress to frank malignancy (Rabbitts & Boehm 1991).

Benign neoplasms of CD8 T cells with receptors for self antigens may produce cytotoxic effects (Fig 1.2). Pityriasis lichenoides et varioli formis acuta (PLEVA) which was previously thought by some authorities to be an inflammatory disorder may be an example. The condition is characterised by recurrent crops of necrotic skin lesions which are infiltrated by mature T-cells with homogeneous T-cell receptor gene rearrangements (Weiss et al 1987). Some cases progress to cutaneous T-cell lymphoma (Black 1982). The pathogensis of the epidermal necrosis is not known.

Another possible example is chronic T-cell lymphocytosis with neutropenia. In this condition the blood contains large numbers of mature large granular CD8+ lymphocytes which in many cases are monoclonal (Loughran et al 1988). Associated conditions include neutropenia which is often associated with antileukocyte autoantibodies and seropositive rheumatoid arthritis (30 percent of cases) (Newland et al 1984). Chromosomal abnormalities have been detected in the T-cells in some cases (Brito-Babapulle et al. 1986).

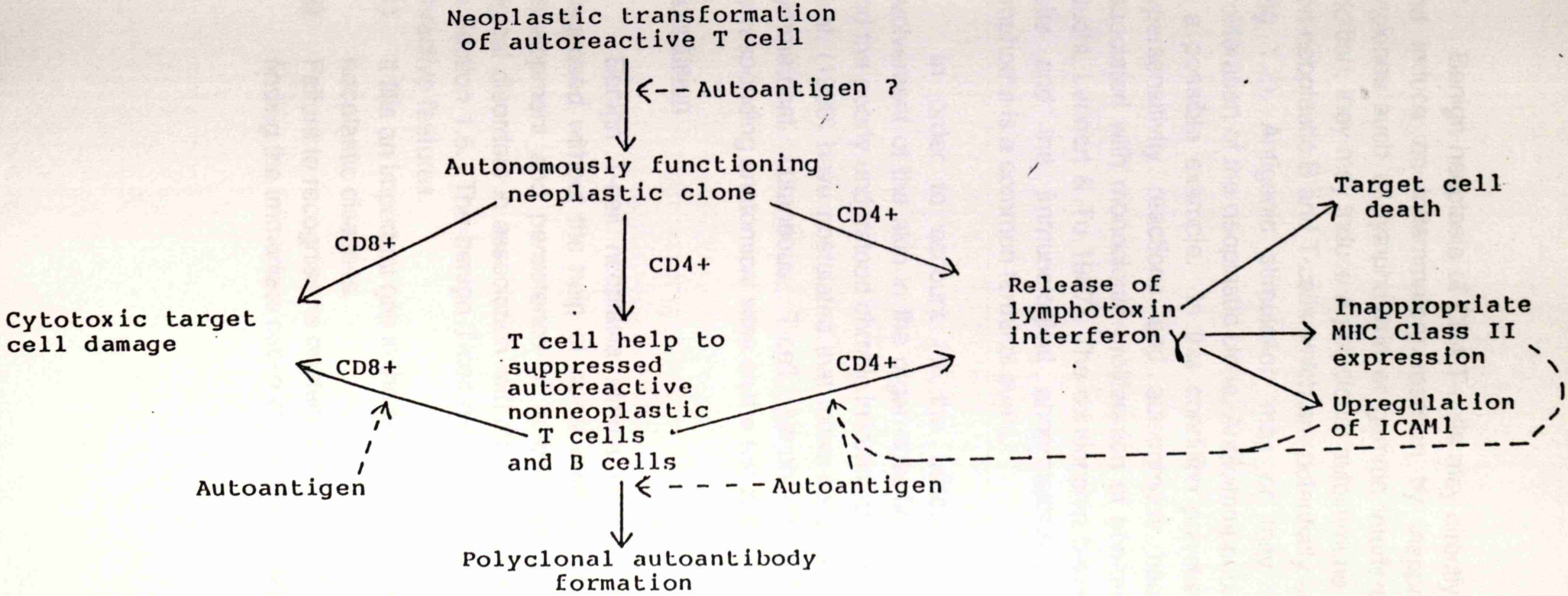


Figure 1.2 Suggested role of benign T-cell neoplasia in the pathogenesis of autoimmune disease.

Benign neoplasia of CD4 T-cells may directly cause tissue damage and induce an inflammatory reaction by inappropriate production of cytokines such as lymphotoxin or gamma interferon. Alternatively, or in addition, they may induce a polyclonal autoimmune response by activating non-neoplastic B and T-cells which are potentially autoreactive but anergic (Fig 1.2). Antigenic stimulation may or may not contribute to the proliferation of the neoplastic clone. Angioimmunoblastic lymphadenopathy is a possible example. In this condition polyclonal gammopathy, drug hypersensitivity reactions and autoimmune haemolytic anaemia are associated with monoclonal proliferation of non-malignant T-lymphocytes (Suchi, Lennert & Tu 1987). The relationship between the monoclonal T-cells and the immunological abnormalities is unknown. Malignant lymphoma is a common terminal event.

In order to account for the curious patchy and symmetrical involvement of the skin in the organ-specific autoimmune disease vitiligo and the poorly understood chronic inflammatory disorder psoriasis, Goudie et al. (1990) have postulated that these conditions are benign variants of symmetrical cutaneous T-cell lymphoma which similarly affects corresponding anatomical sites on the two sides of the body (Fig 1.3).

Conclusion

Benign T-cell neoplasia almost certainly occurs but cannot be diagnosed without the help of clonal markers. It may contribute to the development and persistence of autoimmune and other immunopathological disorders in association with other factors such as those discussed in Section 1.5. The benign T-cell neoplasia hypothesis has several other attractive features.

(1) It fills an important gap in the systematic classification of neoplastic diseases.

(2) Failure to recognise its existence may account for past difficulty in finding the immediate cause of "spontaneously "occurring

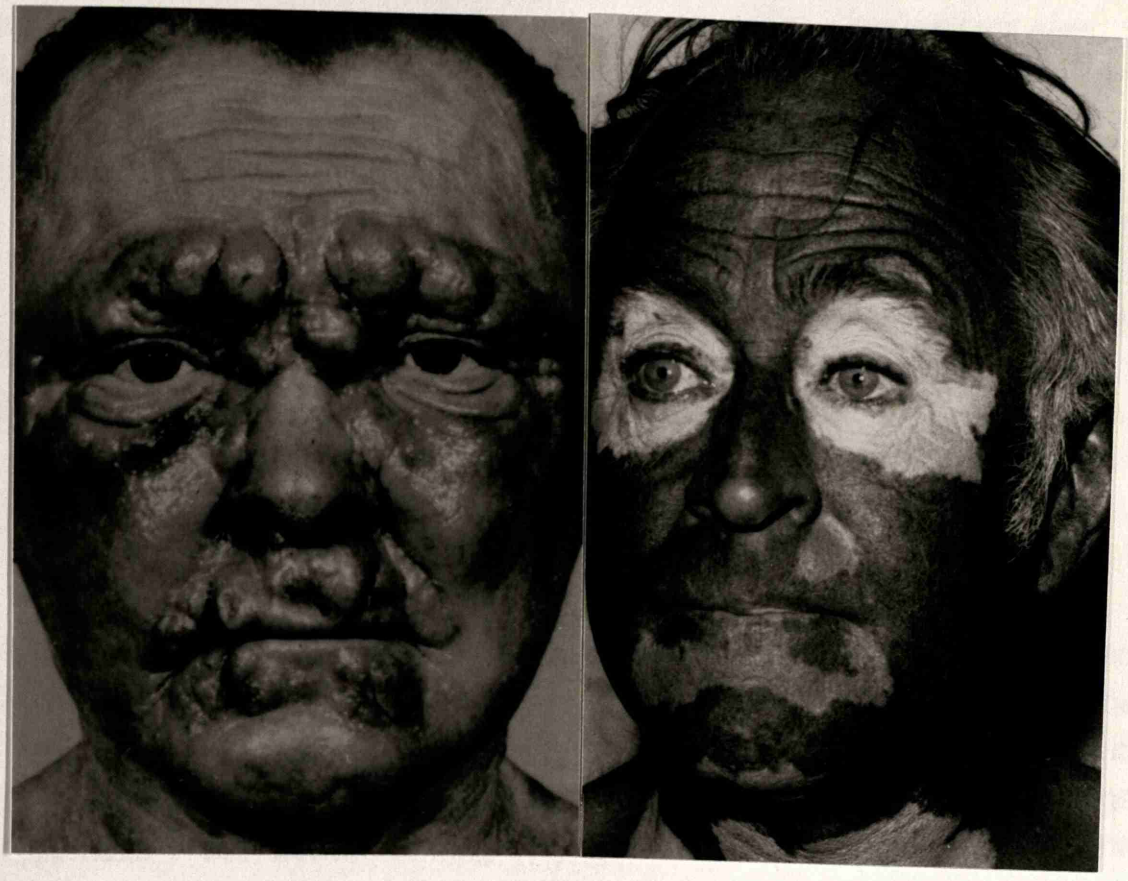


Figure 1.3 Facial lesions in symmetrical cutaneous T-cell lymphoma (left) and depigmentation in vitiligo (right), showing similar patchy anatomical distribution (Goudie et al. 1990).

autoimmune and other poorly understood immunopathological disorders.

(3) Random neoplastic transformation of potentially pathogenic T-cells may explain the occurrence of monozygotic twins who share the same environment but are discordant for autoimmune disease.

(4) The patchy and symmetrical anatomical distribution of the lesions of vitiligo, psoriasis and other poorly understood T-cell associated skin diseases may be an expression of inappropriately functioning symmetrical cutaneous T-cell lymphomas at the benign end of the spectrum.

(5) The hypothesis predicts the presence of a dominant (putatively neoplastic) T-cell clone associated with lesions of the relevant diseases. This prediction can be tested given suitable clonal markers.

When the present project started immunocytochemistry and Southern blot analysis had been used successfully to demonstrate the monoclonality of malignant lymphomas and leukaemias of T-cell origin (Section 1.4). Neither method seemed suitable for the study of benign neoplastic T-cell clones forming only a minority of the cells present in the small samples of diseased tissue likely to be available for examination. For Southern blot analysis a relatively large amount of high molecular weight DNA (10μg) is required to demonstrate a clonal T-cell receptor gene rearrangement constituting 1-5% of the DNA in the specimen (Arnold et al. 1983, Greaves et al. 1986). Immunocytochemistry could detect the products of only a minority of V gene families because of the limited range of available antibodies (Moss, Rosenberg & Bell 1992). It was therefore decided to develop a new method based on the amplification of T-cell receptor gene rearrangements by the PCR. The TCRγ gene locus was selected on the advice of Dr. T.H. Rabbitts, of the MRC Molecular Biology Laboratory, Cambridge.

1.7 T-cell Rearranging Gamma (γ) Genes

T-cell receptors (TCR) are heterodimers consisting of α and β or γ and δ polypeptide chains (Fig 1.1). The carboxy-terminal portion of each

chain is constant (the same in all cells in which it is expressed) and extends from the inside of the cell, through the membrane where it is complexed with CD3, to the outside of the cell. The outermost N-terminal portion consists of a highly variable region which differs in different clones and in conjunction with the variable region of the paired receptor polypeptide forms the antigen binding site (Davis & Bjorkman 1988). Approximately 95 percent of peripheral blood T-cells express αβ receptors and 0.5-10 percent γδ (Raulet 1989) but TCRγ gene rearrangements are present in most T-cells (Lefranc 1986) and are therefore generally applicable as T-cell clonal markers. TCRγ genes are also frequently rearranged in immature (Pre-B) cells of acute B-lymphoblastic leukaemia but not in chronic lymphoid malignancies composed of mature B-cells (Chen et al. 1987).

The human TCRγ locus has been mapped to chromosome 7 (Rabbitts et al. 1985) at band 7p15 (Murre et al. 1985). It is well characterised consisting of 14 V (variable), 5 J (joining) and two C (constant) gene segments (Lefranc, Forster & Rabbitts 1986, Lefranc et al. 1986, Forster et al. 1987, Lefranc & Rabbitts 1985). Fig 1.4 is a schematic representation of the locus.

The Vγ Genes

The rearranging V segments are subdivided into four different subgroups (Forster et al. 1987) whose numbering follows their order in the genome. The subroup Vγl consists of 9 genes five of which are functional (V2, 3, 4, 5 and 8) whereas the others are non-functional pseudogenes (Ψ V1, ΨV5, ΨV6 and ΨV7). The other three groups have one gene, VγII, (V9), VγIII, (V10) and VγIV, (V11) (Lefranc, Forster & Rabbitts 1986, Lefranc et al. 1986, Forster et al. 1987). VA and VB are two other pseudogenes (Forster et al. 1987, Huck, Dariavach & Lefranc 1988). All the V genes differ considerably from one another (Fig.1.5). The pseudogenes seldom rearrange.

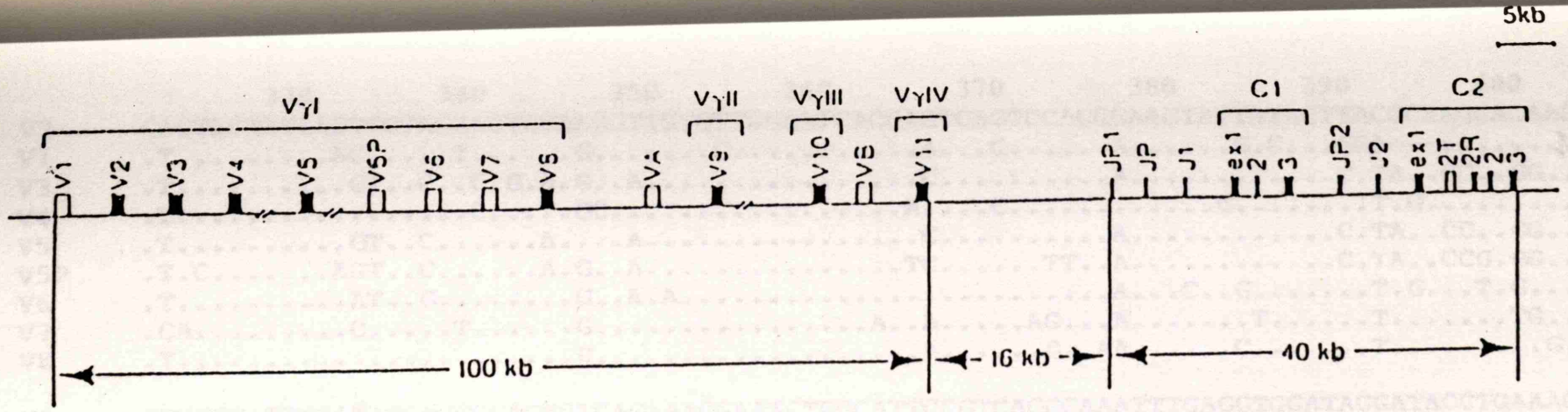


Figure 1.4 Germline organisation of the human TCRγ gene locus (adapted from Lefranc & Rabbitts 1989). The functional V genes and pseudogenes are represented by shaded and unshaded boxes respectively.

```
                330       340       350       360       370       380       390       400       410
V2      CAGTACTATGACTCCTACAACTCCAAGGTTGTGTTGGAATCAGGAGTCAGTCCAGGGAAGTATTATACTTACGCAAGCACAAGGAACAAC
V1      .T.........AC.....T......G.......C...........A...C......A......G.C...GGA---..........G...T
V3      .T..........GT..C..C.G.A.G..A................C..........A............C.TA..CC..GG...TGG.G.
V4      .T..................C.....GC..................A....C............G........T.G...........G...
V5      .T..........GT..C......A....A................C..........A............C.TA..CC..GG...TGG.G.
V5P     .T.C........AGT..C......A.G..A................TC......TT..A............C.TA..CCG.GG...TGG.G.
V6      .T..........AT..G........G..A.A..............................A...C..G.......T.G...T.G.....TA.G.
V7      .CA.........C.....T......G..................A..A.....AG...A.......T......T.......TG....GG.G.
V8      .T.........................G..................A......G..AA......C.......T.........G....G.G.

V9      GTGTCCATTTCATATGACGGCACTGTCAGAAAGGAATCTGGCATTCCGTCAGGCAAATTTGAGGTGGATAGGATACCTGAAACGTCTACA
V10     TATATTG.C...ACAA.ATC.G.A.CTC..CGCAGCATG..T.AGA.AAGCAA....G.G....CAAGA.A..ATT..C....TCTC..T
V11(a)  TACATGTC...T.GAC.ATCT ...CTCA.G.TTGC..A..TGGGAA.A.TAAG...C........AAG..AA.ATG..C.C..T..C..T

                420       430       440       450       460       470       480       490
V2      TTGAGATTGATACTGCGAAATCTAATTGAAAATGACTCTGGGGTCTATTACTGTGCCACCTGGGACGGGCACAGTGATTC
V1      .G..AT....G.....A..........A.......T......T.........................A.............
V3      .G..T.....G.....A..................T.................................A.............
V4      ......A................T..................A.........................T....
V5      .G..T.........A.....................T.................................A.............
V5P     .G..AT....G.....A..................T.................................G.A.............
V6      .G..A...T....CT.C...A....A........C.................................A....A....G........
V7      .G..A...........A..................T.....A.........................A.............
V8      C.T.A...T......GA.............CG.....................................TA.............

V9      TCCACTCTCACCATTCACAATGTAGAGAAACAGGACATAGCTACCTACTACTGTGCCTTGTGGGAGGTGCACAGCAGCAG
V10     ..A.TC..T.....CA.GTCC.........G.A.....G..CGTT...........TGC....TG....GC..CATA.TA
V11(a)  ......T.G.AA..AA.GTTCT........G.A..TGAG.TGGTG...C.........GC...ATTAG....CA...TGT
V11(b)                                          .TGGTG...CCA.......GTCA.ATCC.
```

Figure 1.5 Nucleotide sequences of human Vγ genes. Heptamer sequences are overlined. Only the full sequence of Vγ2 and Vγ9 are given; positions of similarity in the other genes are indicated by a dot, and the differences are shown by the relevant nucleotide change. (Lefranc et al. 1986, Font et al. 1988, Forster et al. 1987, Quertermous et al. 1986, Dariavach & Lefranc 1988).

The Jγ Genes

Five Jγ segments have been described (Fig 1.4). J1 and J2 (Lefranc, Forster & Rabbitts 1986) were first identified 4Kb upstream of the C1 and C2 genes as well as a third J segment JP upstream of J1 (Lefranc et al. 1986). More recently two additional J gene segments JP1 and JP2 have been located upstream of J2 and JP (Huck & Lefranc 1987, Quertermous et al. 1987). The sequences are all different except those of J1 and J2 which are identical (Fig 1.6). All 5 J genes are potentially functional (Huck, Dariavach & Lefranc 1988).

The Cγ Genes

The two Cγ genes (C1 and C2) are linked to each other and are 16Kb apart (Lefranc & Rabbitts 1985, Lefranc, Forster & Rabbitts 1986). Overall their sequences are similar.

Size of the Human TCRγ Locus

Eighty five Kb of DNA containing all the V genes have been cloned and with the exception of the V9 and V10 genes most of the V genes have been linked (Lefranc et al. 1986, Forster et al. 1987). The size of the entire locus has been estimated at 160Kb with its 14V genes spanning 100Kb the two C and five J segments covering less than 40Kb, 16Kb separating the most 3' end TCRγ V genes from the most 5' end TCR J segment (Lefranc & Rabbitts 1989).

Rearrangements of Human TCR γ Genes

Rearrangement occurs early during thymic maturation to form a potentially active gene (Tault et al. 1985). One of the V genes is rearranged to join with a J segment, resulting in the deletion of the DNA sequence between the V and J segments (Fig 1.7) most likely by a mechanism of loop excision (Forster et al. 1987). Each of the J segments can theoretically be rearranged randomly with any of the pool of TCRγ V genes. The junction of a V to the J2 segment involves the deletion of the downstream V genes as well as that of the C1 and associated J segments

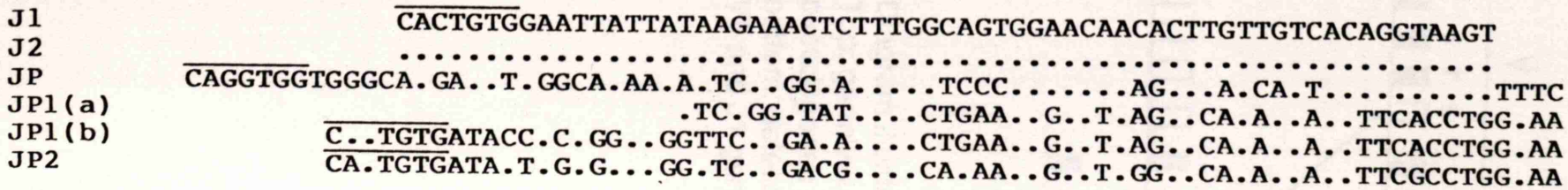


Figure 1.6 Nucleotide sequences of human Jγ genes. Heptamer sequences are overlined. Only the full sequenceof Jγ1 is given; positions of similarity in the other genes are indicated by a dot, and the differences are shown by the relevant nucleotide change (Lefranc et al. 1986, Huck & Lefranc 1987).

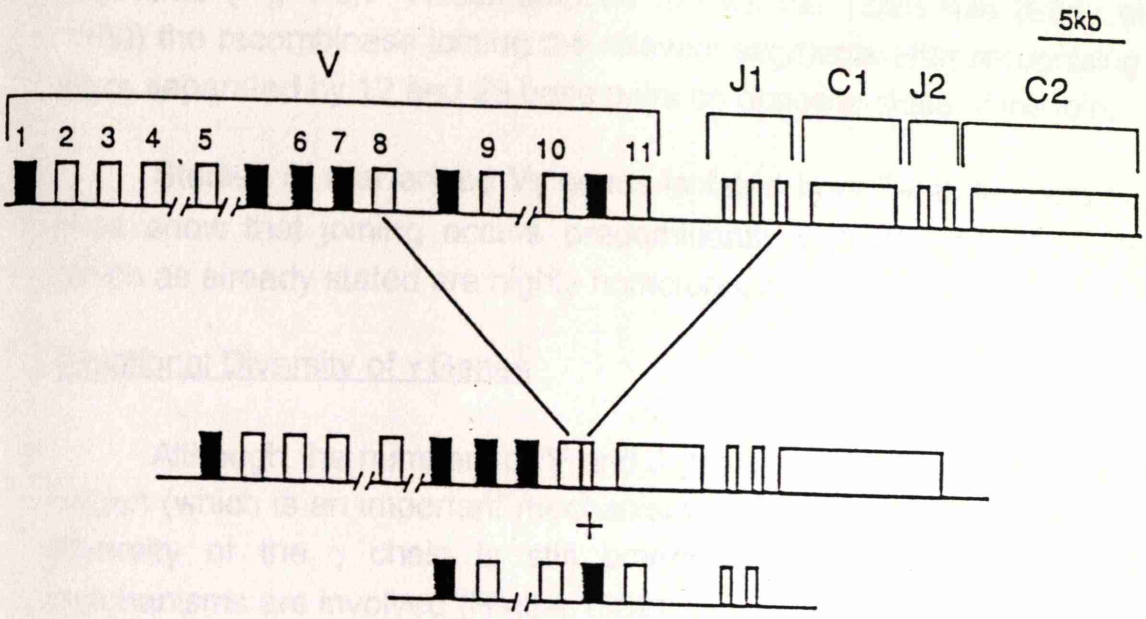


Figure 1.7 Diagram illustrating example of TCRγ gene rearrangement. Top: germline configuration (functional genes unshaded, pseudogenes shaded); middle: V8-J1 rearrangement; bottom: deletion of germline segment between rearranged V8 and J1 genes.

(Murre et al. 1985, Lefranc & Rabbitts 1985). The rearrangements are probably mediated by the conserved heptamer - nonamer sequences located at the 3' end site of the V genes (Fig 1.5) and upstream of the J segments (Fig 1.6). Recombination follows the 12/23 rule (Early et al. 1980) the recombinase joining the relevant segments after recognising 7/9 mers separated by 12 and 23 base pairs on opposite sides of the join.

Studies of rearranged Vγ genes isolated from T-cell tumours or cell lines show that joining occurs predominantly with J1 and J2 segments which as already stated are highly homologous.

Junctional Diversity of γ Genes

Although the numbers of V and J genes are small and there is no D region (which is an important mechanism for diversifying TCRβ and δ) the diversity of the γ chain is still potentially enormous. The following mechanisms are involved (Raulet 1989).

1. Combinatorial diversity. Germline V and J region repertoires are randomly recombined.
2. Junctional site diversity. In many cases the two coding elements are not joined to one another exactly at their ends. A number of bases may be deleted either at the 3' end of the V segment or at the 5' end of the J segment or both.
3. Junctional insertion diversity. A number of non germline encoded (N region) nucleotides may be added at the V-J junction. This process is catalysed by the enzyme terminal dexoyribonucleotide transferase (TdT) in progenitor lymphocytes. It has recently been shown that one or two nucleotides are frequently added to form a palindrome (P nucleotides) with the immediately adjacent germline encoded nucleotides (Lewis & Gellert 1989). Examples of (2) and (3) are shown in Table 1.3.

An analysis of the influence of nucleotide deletions and insertions on the size of sequenced V-J junctions of γ gene rearrangements in 25 T-cell and 7 B-cell acute leukaemias (Macintyre et al. 1990) is shown in Fig 1.8. The hypervariable region varies in size by as many as 40 nucleotides.

TABLE 1.3 Effects of deletions and insertions on sizes (no. of nucleotides) of rearranged Vγ4-Jγ1/2 junctions (examples from sequencing studies in T-cell acute leukaemia (Macintyre et al. 1990))

V deletions	N/P insertions	J deletion	Net effect
- 20	+ 9	0	- 11
- 3	+ 3	- 2	- 2
- 2	+ 9	- 7	0
- 4	+ 7	0	+ 3
0	+ 11	- 8	+ 3
0	+ 26	- 6	+ 20

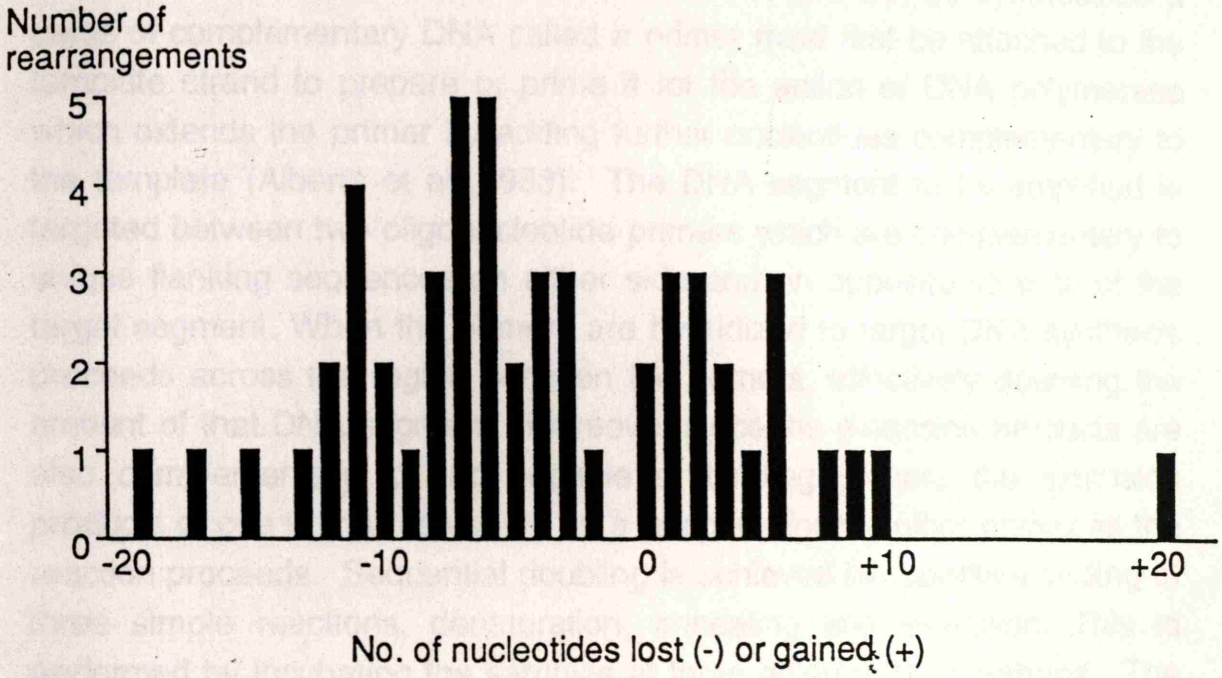


Figure 1.8 Histogram showing effects of deletions and insertions on sizes (no. of nucleotides) of rearranged γ gene V-J junction (data from sequencing studies in 32 acute leukaemia cases (Macintyre et al. 1990)).

1.8 Analysis of TCRγ gene rearrangements by the PCR

The PCR is a rapid and efficient method of amplifying specified segments of DNA by "molecular" cloning. It was invented by Kary Mullis in 1983-84 and the first published account in 1985 reported its application to the prenatal diagnosis of sickle cell anaemia (Saiki et al. 1985). Mullis was awarded the Nobel Prize for the invention in 1993.

The principle is illustrated in Fig 1.9. The specificity of the reaction is based on the fact that before a new DNA strand can be synthesised a piece of complementary DNA called a primer must first be attached to the template strand to prepare or prime it for the action of DNA polymerase which extends the primer by adding further nucleotides complementary to the template (Alberts et al. 1983). The DNA segment to be amplified is targeted between two oligonucleotide primers which are complementary to unique flanking sequences on either side and on opposite strands of the target segment. When the primers are hybridized to target DNA synthesis proceeds across the region between the primers, effectively doubling the amount of that DNA segment. Moreover since the extension products are also complementary to and capable of binding primers the extension products of one primer can serve as a template for the other primer as the reaction proceeds. Sequential doubling is achieved by repetitive cycling of three simple reactions, denaturation, annealing and extension. This is performed by incubating the samples at three different temperatures. The first step in the procedure is heat denaturation of target double stranded DNA. The target DNA melts at a temperature sufficient to break the hydrogen bonds holding the two DNA strands together. The single strands can then anneal to any other DNA that has complementary sequences. The second step of the procedure is performed at lower temperature during which two primers are annealed to complementary sequences on opposite strands of the target DNA. The actual synthesis of new DNA takes place during the third step of the cycle at 70-75ºC, the optimum temperature for the enzyme Taq polymerase. Taq polymerase is a DNA polymerase which is relatively unaffected by the denaturation temperature and does not need to be replenished at each cycle. This simplifies the procedure and makes it amenable to automation. It also improves the overall performance of the reaction by increasing specificity, sensitivity, yield and length of the target

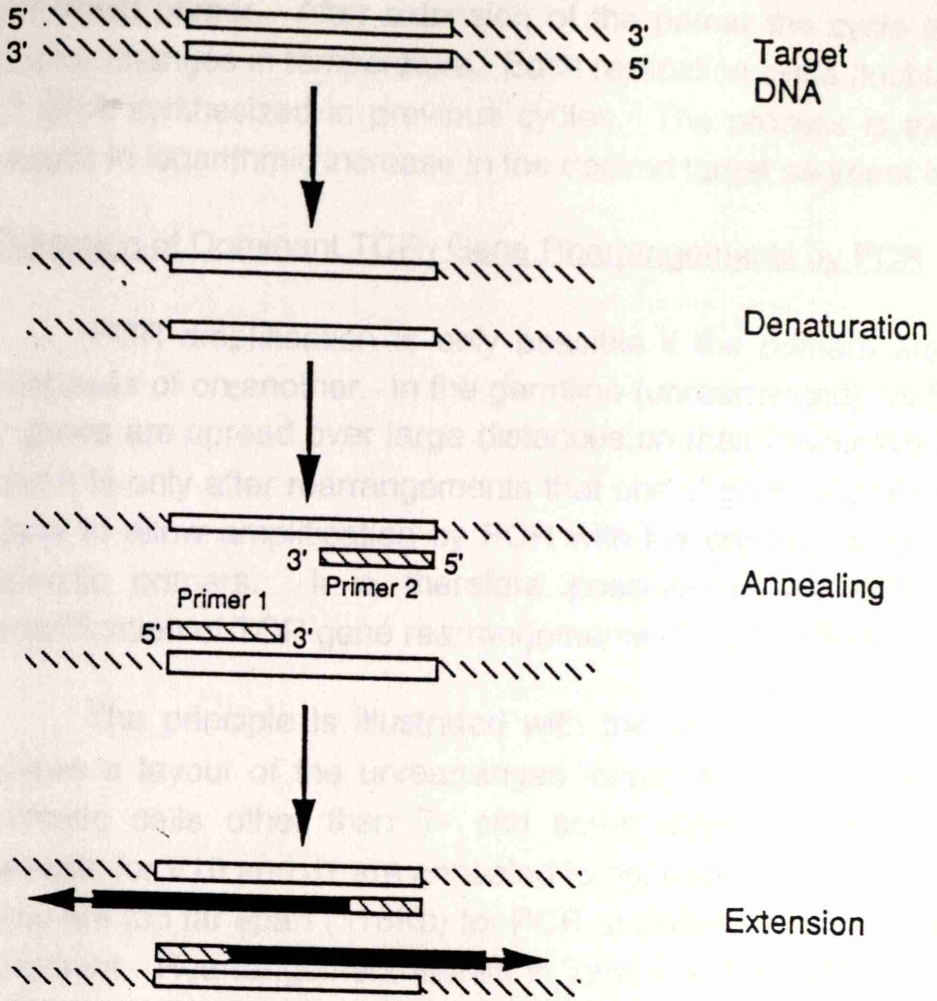


Figure 1.9 Principle of PCR . Part of a sample of double stranded target DNA. In the first step it is separated into single strands (denaturation). Two oppositely directed oligonucleotide primers anneal to complementary sequences on the target DNA (annealing). Enzyme Taq polymerase directs DNA synthesis in opposite directions (extension). The two primers define the limits of the desired site of DNA replication. After the first cycle newly synthesized strands act as templates and lead to a chain reaction (adapted from Eisenstein 1990).

to be amplified. A new single strand of DNA is synthesized for each annealed primer. After extension of the primer the cycle is repeated by further changes in temperature. Each replication cycle doubles the amount of DNA synthesized in previous cycles. The process is exponential and results in logarithmic increase in the desired target segment of DNA.

Detection of Dominant TCRγ Gene Rearrangements by PCR

PCR amplification is only possible if the primers are within a few kilobases of oneanother. In the germline (unrearranged) configuration TCR γ genes are spread over large distances on their respective chromosomes and it is only after rearrangements that one V gene is close enough to a J gene to allow amplification by PCR with the corresponding V and J gene specific primers. It is therefore possible to use PCR for selective amplification of TCR gene rearrangements (Goudie 1989).

The principle is illustrated with the TCRγ locus in Fig 1.10 which shows a layout of the unrearranged locus as found in the germline and somatic cells other than T- and some B-lymphocytes. When primers specific for V10 and J1 are annealed to opposite strands of the double helix they are too far apart (>16Kb) for PCR amplification of the intervening DNA segment. Rearrangement results in joining of V10 and J1 which are now close enough together to be spanned by PCR. With appropriate pairs of V and J primers rearranged genes are amplified but unrearranged loci are not.

By performing PCRs using primers specific for each of the 8 commonly rearranged V genes paired with primers specific for each of the 5 J genes (4 primers since J1 and J2 are identical) it is possible to amplify 32 (8 x 4) sets of TCRγ gene rearrangements from virtually all of the T-cell clones in a sample of blood or tissue. Because of deletions and insertions of nucleotides at the V-J junction (Section 1.7) the PCR products in each of these sets can be divided into 40 subsets by high resolution gel electrophoresis, each subset consisting of one or more amplified V-J segments of identical molecular size and appearing as a single discrete electrophoretic band. Therefore with 8V and 4J primers it is possible to

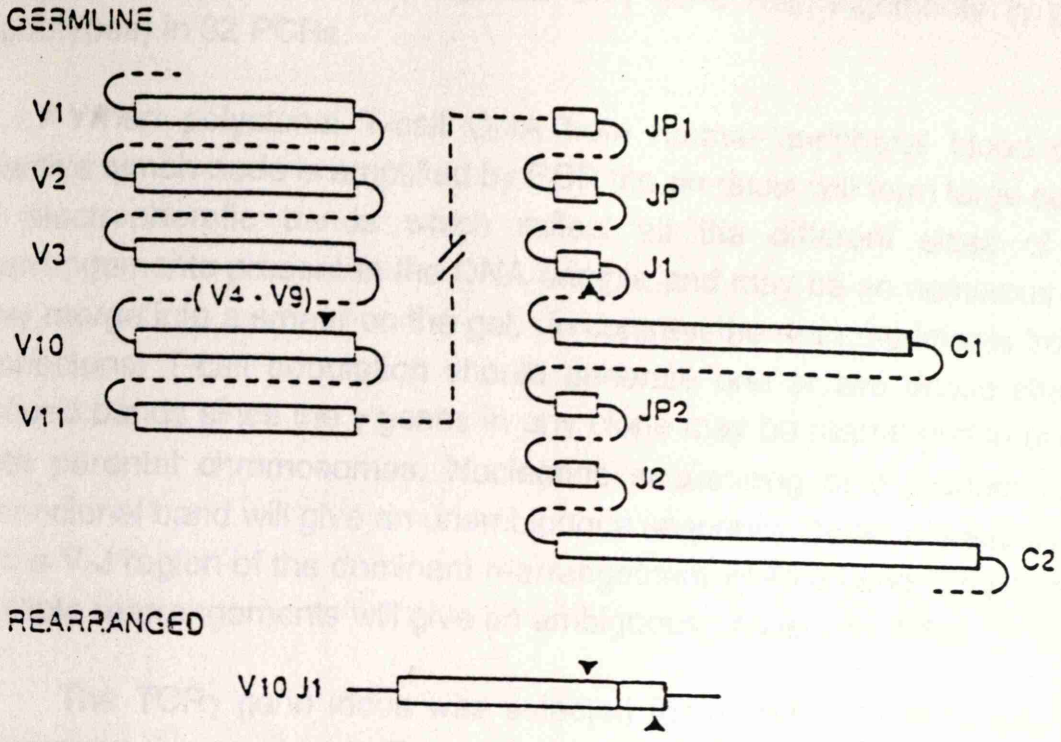


Figure 1.10 Diagram illustrating selective amplification of TCRγ gene rearrangement by PCR (Goudie et al 1991). Only on the rearranged genes are the primers for V10 and J1 (triangles) close enough together for the intervening DNA segment to be amplified.

distinguish 1280 (40 x 32) subsets of γ gene rearrangements (γ gene clonotypes) in 32 PCRs.

When polyclonal T-cell DNA from normal peripheral blood or a reactive lymph node is amplified by PCR the products will form large series of electrophoretic bands which reflect all the different sizes of the rearrangements present in the DNA sample and may be so numerous that they merge into a smear on the gel. In contrast the rearrangements from a monoclonal T-cell population should generate one or two dense sharply defined bands since the γ genes in any clone may be rearranged in one or both parental chromosomes. Nucleotide sequencing of a predominantly monoclonal band will give an unambiguous sequence ladder corresponding to the V-J region of the dominant rearrangement while a band composed of multiple rearrangements will give an ambiguous sequence ladder.

The TCRγ gene locus was selected for amplification because it is rearranged in nearly all T-cells and consists of a small set of well characterised V and J segments whose nucleotide sequences were already known (Section 1.7). The rearrangements in most T-cells can therefore be amplified by PCR with a panel of 8V and 4J gene-specific primers. The use of specific primers which are fully complementary to the target sequences and do not cross react with other V or J segments may be important in detecting relatively small benign neoplastic T-cell clones since any given gene-specific primer combination is likely to maintain the quantitative relationship of the clonotypes in any given DNA sample (Moss, Rosenberg & Bell 1992). In contrast quantitatively misleading results may be obtained with degenerate (partially mismatching) consensus primers (McCarthy et al. 1991) required to cover the great combinatorial diversity associated with the α (50V and 70J gene segments) and β (57V, 2D and 13J gene segments) loci (Moss, Rosenberg & Bell 1992). γ was preferred to the relatively simple δ locus (3V, 3D and 3J gene segments (Moss, Rosenberg & Bell 1992) because δ rearrangements have been found in less than 50% of T-cell neoplasms (Tkachuk et al. 1988).

Layout of Thesis

Chapter 1 explains the background and object of the thesis which is to test the hypothesis that benign T-cell neoplasia may be a common underlying cause of autoimmune and other poorly understood immunopathological disorders of man. If this is true, dominant (putatively neoplastic) disease-associated T-cell clones should be present in the lesions in these conditions. TCRγ gene rearrangements amplified by PCR and analysed by high resolution polyacrylamide gel electrophoresis may be suitable clonal markers to test this hypothesis.

Chapter 2 describes the development of a highly sensitive PCR method in model systems consisting of well characterised cultured monoclonal T-cell lines whose γ gene rearrangements are already known.

Chapter 3 demonstrates the ability of the new method to distinguish non-malignant from malignant T-cell clones in unfixed clinical specimens of reactive (non-neoplastic) lymph nodes and a series of nodal, cutaneous and enteropathy-associated T-cell lymphomas, the biopsies having been obtained for routine diagnostic purposes.

In Chapter 4 TCRγ gene rearrangements present in normal skin are examined. Samples of plaque stage cutaneous T-cell lymphoma (CTCL) and clinically unaffected skin and blood from the same patient are also tested in a small series of cases to determine whether the method can detect dominant clonal rearrangements selectively localised to the lesions before obvious tumour masses develop. A single case of diffuse CTCL with T-cell leukaemia (Sezary syndrome) is reported.

Chapter 5 describes attempts to demonstrate dominant TCRγ gene rearrangements and selective localisation of (disease-associated) minor γ gene rearrangements in the cutaneous lesions of the organ-specific autoimmune disease vitiligo and the poorly understood skin disease psoriasis in which T-cells are though to be of pathogenic importance.

Chapter 6 describes attempts to demonstrate dominant γ gene rearrangements in synovial biopsies and samples of synovial fluid from joints affected by the non-organ-specific autoimmune disease rheumatoid arthritis. The findings in synovial fluid were compared before and after culture in IL-2, a cytokine which leads to proliferation of T-cells with receptors and has been used by others to obtain sufficient number of lymphocytes to examine the TCR gene rearrangements by Southern blot analysis.

Chapter 7 summarises the main findings and conclusions of the work.

CHAPTER 2

Development of Sensitive Method of Screening for Dominant Clonal T-cell Receptor γ Gene Rearrangements by the Polymerase Chain Reaction

This chapter describes the development of a sensitive PCR for the detection of dominant TCRγ gene rearrangements in small samples of DNA. Ideally the PCR should only amplify rearranged DNA segments lying between the V and J primers present in the reaction mixture and there should be enough PCR product for visualisation by high resolution electrophoresis of a rearrangement present in a few cells of the same clone against a background of other (polyclonal) rearrangements of the same V and J genes. To simplify the performance of the test it would be helpful to include all the desired primer combinations in a single reaction mixture and to avoid the use of radio-isotope for visualising the reaction products following electrophoresis. It would also be desirable if purification of extracted DNA was unnecessary prior to the PCR.

The investigation was carried out on model systems consisting of samples of purified DNA extracted from cultured monoclonal T-cell lines whose γ gene rearrangements had previously been identified by Southern blot analysis and reported by other workers, and from unfixed reactive lymph nodes from surgical pathology specimens or normal peripheral blood mononuclear cells (PBMC) as sources of polyclonal T-cell DNA.
For convenience the chapter is divided into four parts:

i. Pilot studies attempting to obtain specific PCR amplification of V-J γ gene rearrangements in RPMI 8402, a monoclonal T-cell line with known rearrangements.
ii. Development of a suitable method of detecting dominant clonal rearrangements among PCR products.
iii. Assessment and validation of chosen method.
iv. Extraction and processing of DNA for PCR.

These studies commenced in October 1989 before publication of the excellent manual "PCR Protocols" and before the amplification of TCR gene rearrangements by PCR had been reported by other workers. The experiments are based on the strategy proposed by Professor R.B. Goudie in 1989 and described in Chapter I of the thesis.

i. Specific amplification of RPMI 8402 V-J γ gene rearrangements

This pilot study involved the choice of suitable V and J gene primers and the finding of appropriate conditions for specific amplification by PCR of the V4-J2 and V10-J2 γ gene rearrangements known to be present in the cultured monoclonal leukaemic T-cell line RPMI 8402. The expected molecular size of the PCR product of the V4-J2 rearrangement (whose V-J junction has not been sequenced) is approximately 201bp (base pairs). The nucleotide sequence of the V10-J/2 rearrangement is known (Forster et al. 1987) and the expected molecular size of the specific V10-J2 PCR product is 92bp (Fig 2.5), with the primer combination used in this part of the study (Figs 2.1 and 2.2, Tables 2.1 and 2.2).

METHODS

Selection of V and J γ gene specific primers

This was done by comparing published sequences of V1-V11 and the five known J genes and choosing 17-21 mer oligonucleotides specific for each (Figs 2.1 and 2.2). The lengths of the various primers were adjusted so that they had similar melting points (Appendix 2.1) and could therefore be used with the same PCR protocol. An attempt was made to locate the primers at different positions on different genes (Figs 2.1 and 2.2 and Table 2.1) in the hope that multiple combinations of primers in the same tube (reaction mixture) would give rise to products whose molecular size differed with each V-J primer pair and so would not overlap following gel electrophoresis (Table 2.2). This approach was later abandoned and new primers were chosen which seemed likely to discriminate best between different genes, especially at the 3' end of the primer where the last three nucleotides are important in determining specificity (McNamara & Stumph 1989). Figs 2.3 and 2.4 and Tables 2.3 and 2.4 give the location and sequences of the modified primers which replaced those used in the original experiments.

DNA Extraction

Cells were recovered from culture suspensions and DNA was extracted, and purified by the standard method of proteinase-K digestion,

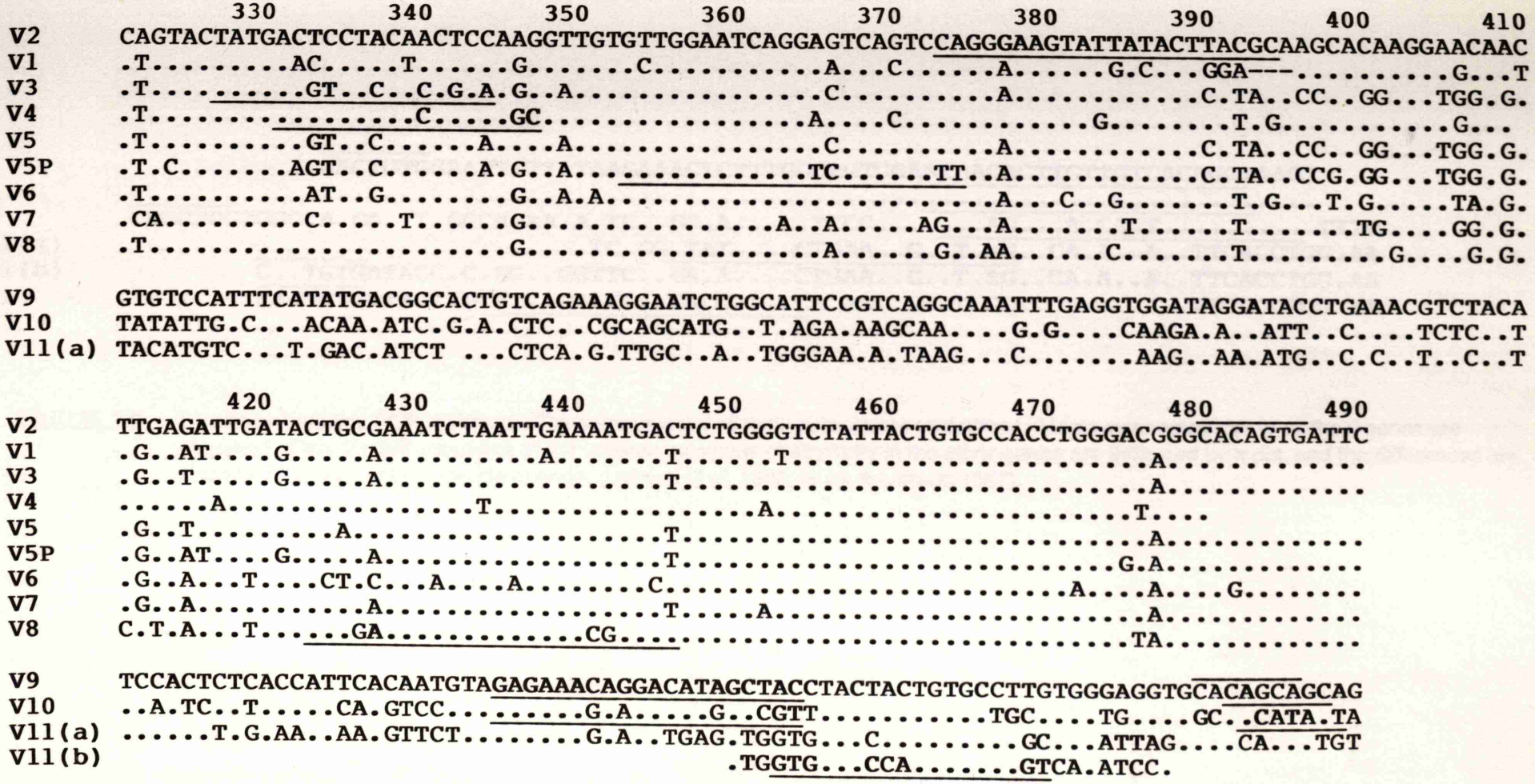


FIGURE 2.1 Location of original PCR V primers. The sequences of all the V primers are underlined. Heptamer sequences of all the V genes are overlined. Only the full sequence of Vγ2 and Vγ9 are given; positions of similarity in the other genes are indicated by a dot, and the differences are shown by the relevant nucleotide change (Lefrance et al. 1986, Font et al. 1988, Forster et al. 1987, Quertermous et al. 1986, Dariavach & Lefranc 1988).

```
J1                CACTGTGGAATTATTATAAGAAACTCTTTGGCAGTGGAACAACACTTGTTGTCACAGGTAAGT
J2                ..................................................................
JP      CAGGTGGTGGGCA.GA..T.GGCA.AA.A.TC..GG.A.....TCCC.....AG...A.CA.T..........TTTC
JP1(a)                                .TC.GG.TAT....CTGAA..G..T.AG..CA.A..A..TTCACCTGG.AA
JP1(b)          C..TGTGATACC.C.GG..GGTTC..GA.A....CTGAA..G..T.AG..CA.A..A..TTCACCTGG.AA
JP2             CA.TGTGATA.T.G.G...GG.TC..GACG....CA.AA..G..T.GG..CA.A..A..TTCGCCTGG.AA
```

FIGURE 2.2 Location of original PCR J primers. The sequences of all the J primers are underlined. Heptamer sequences of all the J genes are overlined. Only the full sequence of Jγ1 is given; positions of similarity in the other genes are indicated by a dot, and the differences are shown by the relevant nucleotide change. (Lefranc et al. 1986, Huck & Lefranc 1987).

TABLE 2.1 Details of original PCR primers

			Length (Nucleotides)	% GC	Tm °C
V2	373	CAGGGAAGTATTATACTTACGC 3'	22	41	67.6
V3	327	TATGACGTCTCCACCGCA 3'	18	55.6	66.8
V4	331	ACTCCTACACCTCCAGC 3'	17	59	65.9
V5P	353	GTTGGAATCAGGTCTCAGTCTT 3'	22	45.5	69.5
V8	423	CTGGAAAATCTAATTGAACGTGAC 3'	24	37.5	68.8
V9	435	GAGAAACAGGACATAGCTAC 3'	20	45	66.2
V10	435	GAGAAAGAAGACATGGCCGT 3'	20	50	68.3
V11	453	GTGTACCCATGTGCCTGT 3'	18	55.6	66.8
J1/2		5' ACCTGTGACAACAAGTGTTGT	21	43	66.9
JP		5' CTTACCTGTAATGATAAGCTTTG	23	35	66.4
JP1		5' CTTAGTCCCTTCAGCAAAATAT	22	36	65.7
JP2		5' GCAAACGTCTTGATCCAATC	20	45	66.2

TABLE 2.2 Estimated sizes of PCR products (base pairs) with original primers assuming no deletions and no N region

	J1/2	JPA	JP1*	JP2
V2	159	174	148	135
V3	205	220	194	181
V4	201	216	190	177
V5P	179	194	168	155
V8	109	124	98	85
V9	97	112	86	73
V10	99	114	88	75
V11*	83	98	72	59

*. Estimate based on rearrangements lacking heptamer sequences.

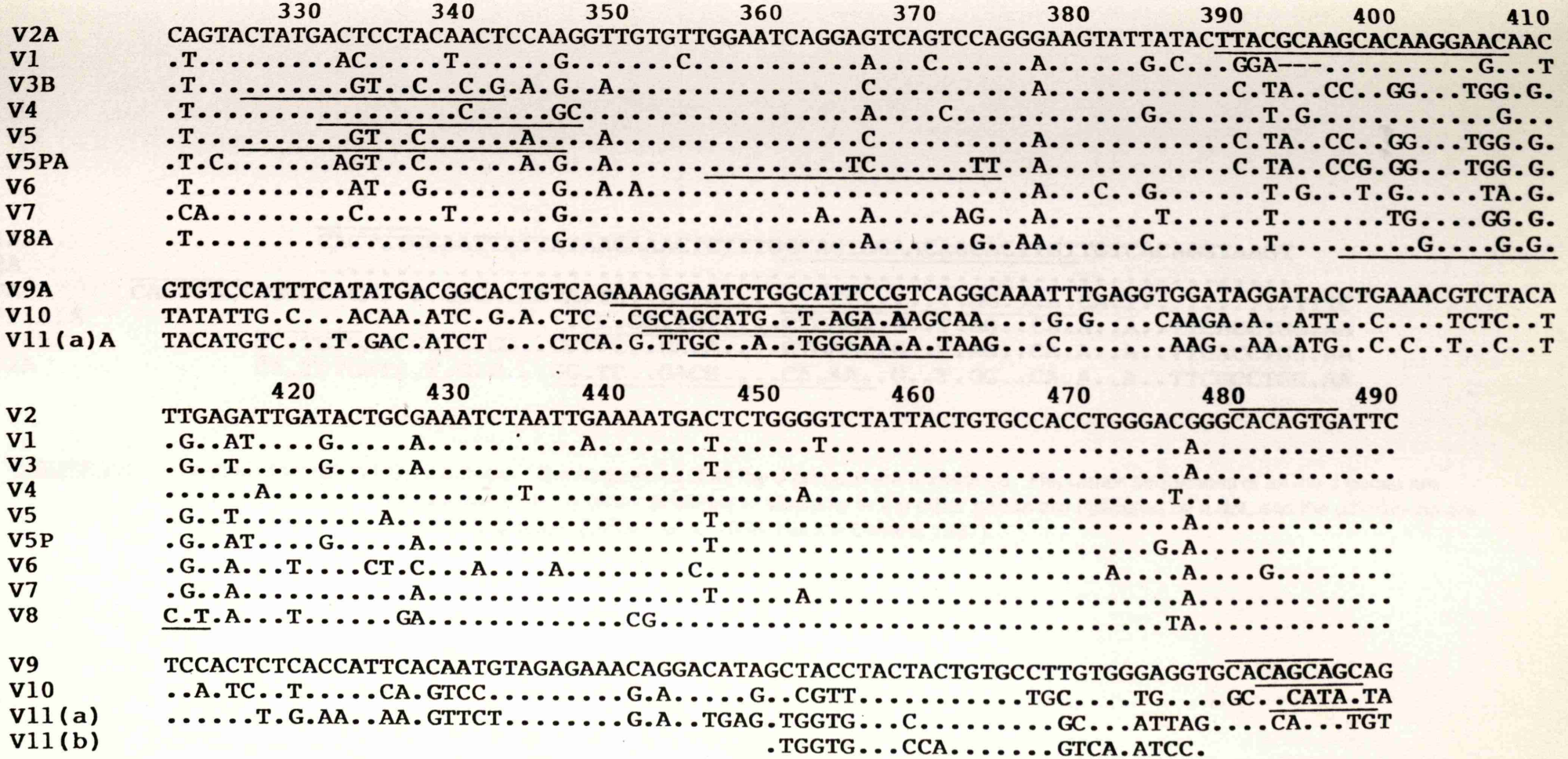


FIGURE 2.3 Location of modified PCR V primers. The sequences of all the V primers are underlined. Heptamer sequences of all the V genes are overlined. Only the full sequence of Vγ2 and Vγ9 are given; positions of similarity in the other genes are indicated by a dot, and the differences are shown by the relevant nucleotide change (Lefrance et al. 1986, Font et al. 1988, Forster et al. 1987, Quertermous et al. 1986, Dariavach & Lefranc 1988).

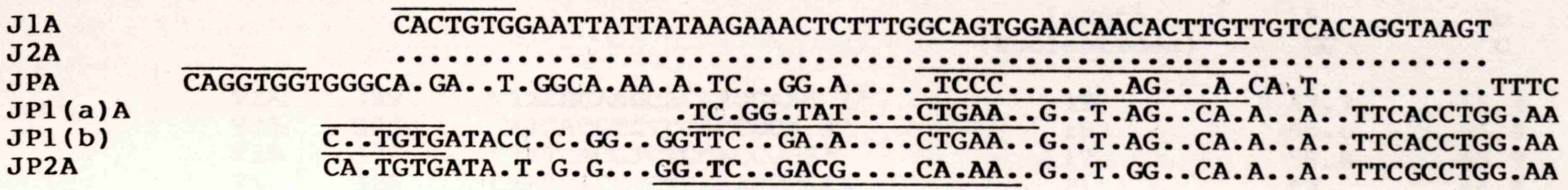


FIGURE 2.4 Location of modified PCR J primers. The sequences of all the J primers are underlined. Heptamer sequences of all the J genes are overlined. Only the full sequence of Jγ1 is given; positions of similarity in the other genes are indicated by a dot, and the differences are shown by the relevant nucleotide change. (Lefranc et al. 1986, Huck & Lefranc 1987).

TABLE 2.3 Details of modified PCR primers

			Length (Nucleotides)	% GC	Tm °C
V2A	389	TTACGCAAGCACAAGGAAC 3'	19	47.7	65.4
V3A	327	TATGACGTCTCCACCGCA 3'	18	55.6	66.8
V3B	326	CTATGACGTCTCCACCG 3'	17	58.8	65.9
V5	326	CTATGACGTCTCCAACTCAAA	21	43	66.9
V5PA	356	GGAATCAGGTCTCAGTCTT 3'	19	47.3	65.4
V8A	397	GCACAGGGAAGAGCCTT 3'	17	58.8	65.9
V8B	357	GAATCAGGAATCAGTCGAGA 3'	20	45.0	66.2
V9A	349	AAAGGAATCTGGCATTCCG 3'	19	47.3	65.4
V10A	352	GCAGCATGGGTAAGACA 3'	17	52.9	63.5
V11A	355	GCTCAGGTGGGAAGACT 3'	17	58.8	65.9
J1/2A		5' ACAAGTGTTGTTCCACTGC	19	47.3	65.4
JPA		5' ATAAGCTTTGTTCCGGGAC	19	47.3	65.4
JP1A		5'*GCTTCAGCAAAATATCCCGA	20	45	66.2
JP2A		5' CTTTTGCAAACGTCTTGATCC	21	42.9	66.9

* The G at the 5' end of this primer has mistakenly been used instead of C.

TABLE 2.4 Estimated sizes of PCR products (base pairs) with modified primers assuming no deletions and no N region

	J1/2A	JPA	JP1A*	JP2A	JP1/2
V2A	133	145	125	124	141
V3A	195	207	187	186	203
V3B	196	208	188	187	204
V5	196	208	188	187	204
V5PA	166	178	158	157	174
V8A	125	137	117	116	133
V8B	165	177	157	156	173
V9A	173	185	165	164	181
V10A	175	187	167	166	183
V11A	171	183	163	162	179

* Estimate based on rearrangements lacking heptamer sequences.

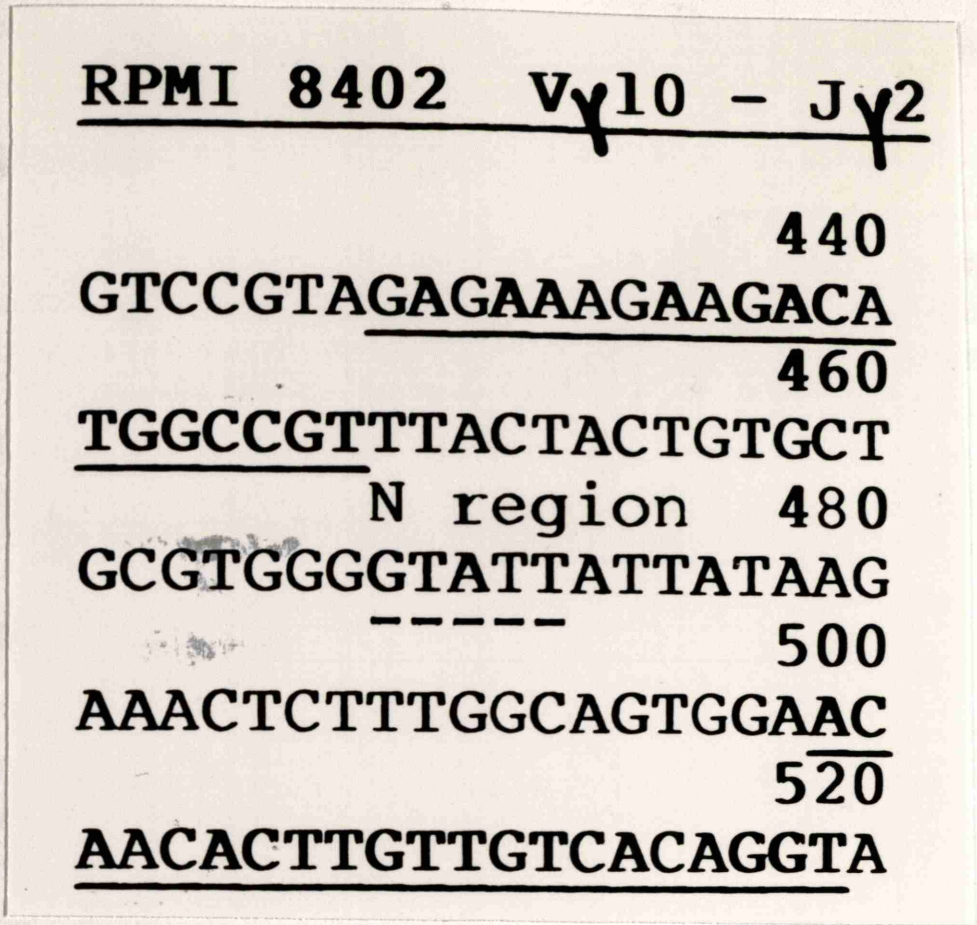


FIGURE 2.5 Nucleotide sequence of RPMI 8402 V10-J2 rearrangement. There are 5 N region insertions (dotted line). The position of the primers for V10 and J2 (original) are underlined. These should give a PCR product of 92 base pairs.

phenol-chloroform extraction and ethanol precipitation shown in Appendix 2.2.

PCR Protocol

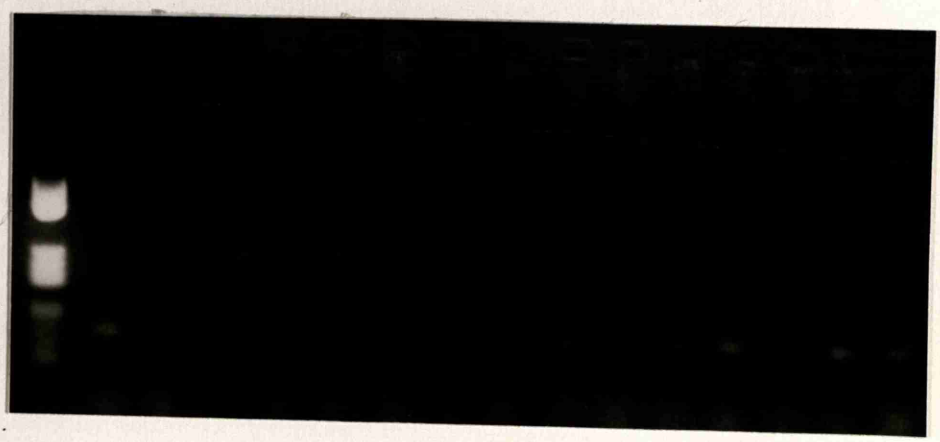
Reaction mixtures for PCR were prepared according to the instructions of the manufacturers of the Taq polymerase used (Appendix 2.3) except that the total volume of the reaction mixture was reduced to 50µl. Experiments were carried out varying the temperature of hybridization, the duration of primer extension in the PCR cycle (see Appendix 2.4 for details of the programmes used) and the temperature at which Taq polymerase was added to the reaction mixture (Appendix 2.3).

Analysis of Results

This was done by ethidium bromide staining of 2.5% agarose gel electrophoresis (Appendix 2.5). DNA molecular weight marker V was routinely included in lane I. This is a HaeIII digest of PBR 322 and the fragment sizes are as follows:

584, 540, 504, 458, 434, 267, 234, 213, 192, 184,124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11, 8 base pairs.

Experiment 1 Effects of different annealing temperatures on amplification of V10-J2 rearrangements in RPMI 8402 by PCR (5 tests at each temperature).

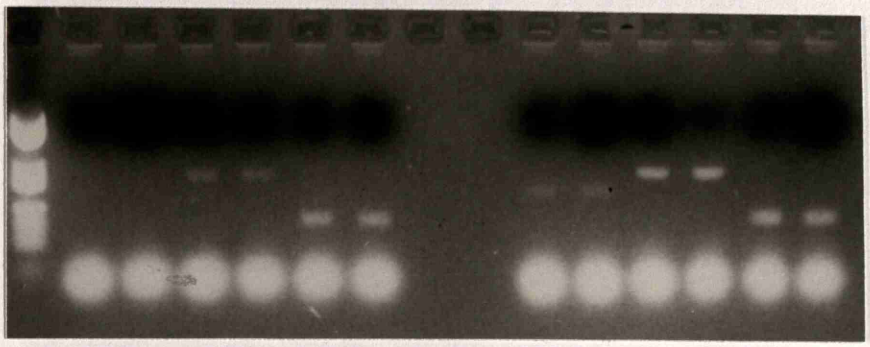


Lane	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Annealing Temp	64°C (Program 1)					62°C (Program 2)					60°C (Program 3)				
Result	+	w	-	-	w	+	+	+	+	+	+	+	+	+	+

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

A single band of appropriate molecular weight is obtained in most tests. Note inconsistent results at 64ºC.

Experiment 2 Effects of extension time on amplification of V4-J2 and V10-J2 rearrangements in RPMI 8402 by PCR, with V2-J1/2 as negative control. Annealing temperature 62ºC.

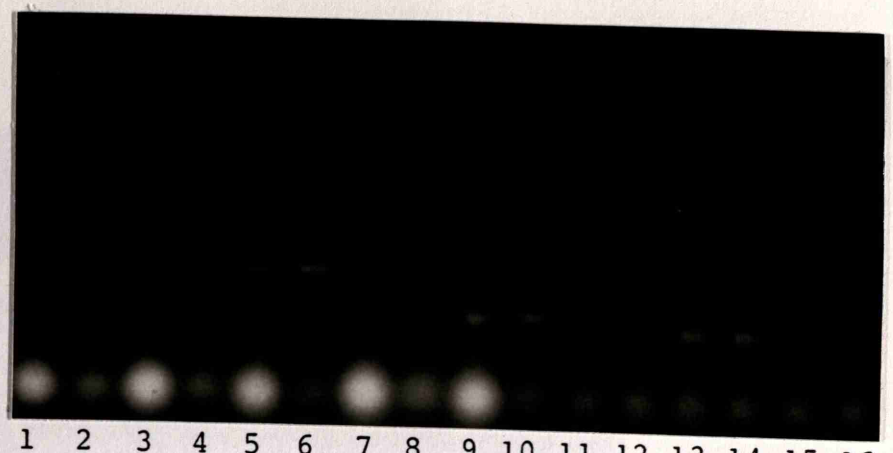


Lane	2	3	4	5	6	7	10	11	12	13	14	15
Extension time	12 seconds (Program 5)						1 minute (Program 6)					
V Primer	2	2	4	4	10	10	2	2	4	4	10	10
Result	-	-	+	+	+	+	+	+	+	+	+	+

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

A specific result is obtained with V4 and V10 primers with shorter extension time of 12 seconds. Longer extension time of 1 minute has given non-specific positive results with V2 - J1/2.

Experiment 3 PCR on RPMI 8402 DNA with all original V and J1/2 primers using programme 5 (annealing temperature 62ºC, extension time 12 seconds).



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
V Primer	2	2	3	3	4	4	5	5	8	8	9	9	10	10	11	11
Results	w	-	-	-	+	+	-	-	+	+	-	-	+	+	-	-

Specific reaction products are obtained with V4 and V10 but an unwanted cross-reaction can also be seen with V8 and a weak reaction in lane 1 with V2.

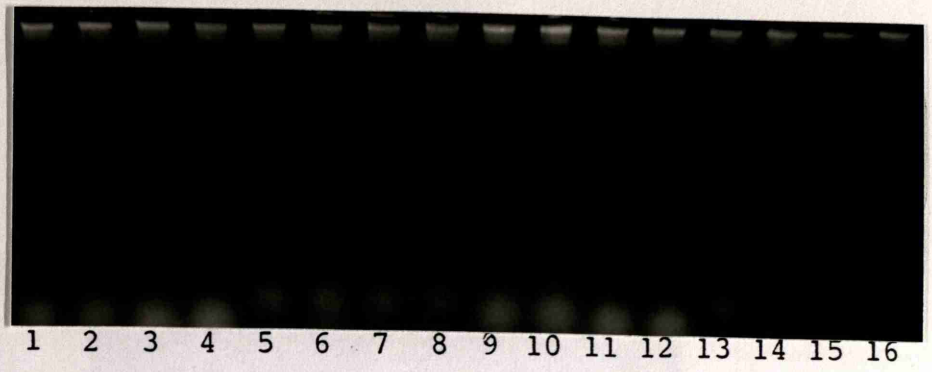
Experiment 4 Attempt to abolish non-specific amplification of RPMI 8402 DNA with V8-J1/2 primers with annealing temperature of 64ºC (Programme 7)

Lane	2	3	4	5	6	7	8	9
V Primer	2	2	4	4	8	8	10	10
Result	-	-	+	+	+	+	+	+

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

Note that V2 has given a negative reaction but V8 still gives a strongly positive cross-reaction.

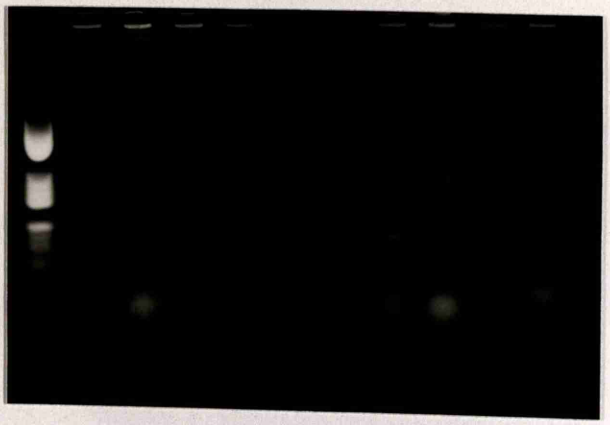
Experiment 5 RPMI 8402 DNA tested with all original J(J1/2, JP, JP1, JP2) and V4 and V10 gene primers (Programme 7).



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
V Primer	———			V4				———	———			V10				———
J Primer	1/2	1/2	P	P	P1	P1	P2	P2	1/2	1/2	P	P	P1	P1	P2	P2
Result	w	w	MB	MB	MB	MB	MB	MB	+	+	MB	MB	MB	MB	MB	MB

A positive (specific) weak (w) reaction is seen with V4 J1/2 and V10-J1/2 primers. Note non-specific (unwanted) multiple bands (MB) with other J primers (JP, JP1, JP2).

Experiment 6 Effects of "hot start" (addition of Taq polymerase at 69ºC) on non-specific PCR amplification of RPMI 8402 DNA with V10-JP, JP1, JP2 primer combinations (Programme 9).



Lane	2	3	4	5		8	9	10	11
	Hot start					Cold start			
J Primer	1/2	P	P1	P2		1/2	P	P1	P2
Result	+	-	-	-		+	MB	MB	MB

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

In lanes 2-5 all reactants with the exception of Taq polymerase were added to the PCR reaction on ice. After laying liquid paraffin over the mixture the tubes were heated at 99ºC for 5 minutes in the thermocycler then cooled to 69ºC and held there. Enzyme preheated to 60ºC was then added while tubes were still in the cycler, the tubes were then heated to 94ºC for 1 minute before entering the normal PCR cycle shown in programme 9.

After initial denaturation of tubes 8-11, Taq polymerase was added and the tubes centrifuged at room temperature (cold start) before entering programme 9. Note that non-specific multiple bands (MB) in lanes 8-11 were abolished with the "hot start" protocol.

"Hot starts" have been used in all subsequent experiments (see Appendix 2.3).

Experiment 7 Amplification of three different preparations of RPMI 8402 DNA (A,B,C) with V4, V8, V10 and J1/2 primers (original) using three different programmes to test for optimal annealing temperature (64ºC, 63ºC, 62ºC) with a "hot start".

Lane	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
DNA preparation	A	A	A	B	B	B	C	C	C	A	A	A	B	B	B
Temperature	64°C (Program 7)									63°C (Program 8)					
V Primer	4	8	10	4	8	10	4	8	10	4	8	10	4	8	10
Result	-	+	+	-	-	-	w	-	w	+	+	+	w	-	-

Lane	17	18	19	20	21	22	23	24	25	26	27	29	30
DNA preparation	C	C	C	MW	A	A	A	B	B	B	C	C	C
Temperature					62°C (Program 9)								
V Primer	4	8	10		4	8	10	4	8	10	4	8	10
Result	-	-	-		+	+	+	w	-	-	w	-	w

Left MW (nucleotides). Lane 1,20: MW marker PBR 322-HaeIII.

Programme 9 with annealing temperature of 62ºC has given the strongest reactions. Note that the different preparations of the same monoclonal DNA vary greatly in the intensity of the reaction. A non-specific reaction is again seen with V8-J1/2 primer combination.

Programme 9 has been used in all subsequent experiments in this chapter.

COMMENT

The importance of suitable annealing temperature and extension time in obtaining adequate PCR amplification of V4-J2 and V10-J2 gene rearrangements in the model system RPMI 8402 is shown in experiments 1-3. Unwanted results were sometimes obtained with V2 and V8 primers. The nucleotide sequence of V2 and V4 are closely similar (Fig 2.1) V4 being thought to have arisen by duplication of V2 (Huck, Dariavach & Lefranc 1989) and the unwanted amplification of RPMI 8402 DNA with V2 primer is probably the result of cross hybridisation of the V2 primer with the corresponding sequence on V4 which differs only at positions 1,3 and 12 from the 3'end of the primer. The V2 cross-reaction was abolished by shortening the extension time at 72ºC from 1 minute to 12 seconds (Experiment 2). It is likely that the unwanted PCR result with V8 primer is also a cross-reaction with the V4 rearrangement of RPMI 8402, the original V8 primer being identical to V4 at 19 of the 24 nucleotides including the critical positions 1,2,3 from the 3' end. It was not abolished by changing the conditions of PCR, but was by the use of a more appropriate primer (V8A, Fig 2.3) which differs from V4 at positions 1,3,5 and 12 from the 3' end (see Experiment 12).

Experiment 6 shows multiple non-specific bands when RPMI 8402 is amplified with V4 and V10 primers paired with primers for JP, JP1 and JP2 instead of J1/2. The products are multiple and mostly of inappropriate size. The three nucleotides at the 3' end of the primer for JP correspond to the J2 segment sequence and a cross-reaction might be expected but strong cross-reactions should not be obtained with the primers for JP1 and JP2 (Fig 2.2). The occurrence of multiple unwanted PCR bands of inappropriate molecular size is well known in other PCR systems (Bourguin et al. 1990). We reasoned that they might reflect mispriming at sub-optimal temperatures when the enzyme Taq polymerase is added to the reaction mixture and centrifuged at room temperature. To avoid this we devised a "hot start" by adding Taq polymerase to the reaction mixture held at 69ºC following the initial denaturation of DNA without mixing of the reagents by centrifugation at room temperature. Experiment 7 shows that the new approach was successful. Since that time "hot start" procedures have been described by others and are now widely used (Faloona et al. 1990, D'Aquila et al. 1991). As a result of the above studies the "hot-start"

protocol (Appendix 2.3) was adopted for PCR amplification of TCRγ gene rearrangements.

ii. Development of a suitable method of detecting dominant clonal rearrangements among PCR products

Having obtained promising results with PCR amplification of the TCR γ gene rearrangements in RPMI 8402 DNA, it was next necessary to find a method to distinguish homogeneous monoclonal or dominant clonal rearrangements from the heterogeneous rearrangements present in polyclonal T-cell populations, for example those found in hyperplastic (reactive) lymph nodes or normal peripheral blood. Since any given pair of V-J primers may give PCR amplification products which vary in size by as many as 40 nucleotides due to additions and deletions at the V-J junction (Chapter 1) it was expected that polyclonal T-cell DNA would give an elongated smear or multiple faint bands on electrophoretic analysis of the PCR products, whereas DNA from the monoclonal T-cell line RPMI 8402 would produce a single, heavy, sharply defined electrophoretic bands with V4-J1/2 and V10-J1/2 primer combinations. Ideally it would be desirable to be able to distinguish between amplification products of rearrangements differing in size by one nucleotide at the hypervariable V-J junction.

The following set of experiments (Exp. 8 and 9) shows that agarose gels stained with ethidium bromide do not give adequate resolution for this purpose but that suitable resolution is obtained with 6% polyacrylamide gel electrophoresis. The staining of polyacrylamide gels with ethidium bromide was unsatisfactory even when double stranded DNA (obtained in non-denaturing conditions) is used to optimise the staining. It was therefore decided to radiolabel the PCR products to obtain maximum sensitivity and to perform the analysis in denaturing gels to avoid possible heteroduplex formation between PCR products of closely similar rearrangements. 32P dCTP was used since it can be incorporated into DNA and emits a very energetic β particle that is easy to detect by autoradiography. Amounts of both radioisotope (32P dCTP) and cold dCTP were optimised using DNA from RPMI 8402. The amount of DNA tested and the total volume of reaction mixture were reduced to 0.5μg and 25μl to minimise expenditure and radiation risks.

METHODS

All experiments in this and subsequent sections were performed using the modified radiolabelled PCR protocol (Appendix 2.3) and with the exception of Experiments 8, 9 and 10 with the modified primers shown in Figs 2.3 and 2.4 and Tables 2.3 and 2.4.

55cm long 0.4mm thick 6% polyacrylamide gels were prepared and high resolution electrophoresis was carried out according to the manufacturer's instructions on a 2010 macrophor sequencing system (Appendix 2.6). Radiolabelled molecular weight marker PBR 322-HaeIII was routinely included in first lane. The method for radiolabelling the marker is given in Appendix 2.8.

Experiment 8 Analysis of PCR amplification products of DNA from reactive lymph nodes (polyclonal T-cells) and cultured monoclonal T-cell line RPMI 8402 by electrophoresis on 2.5% agarose gel (original V4, V8, V10-J1/2 primers).

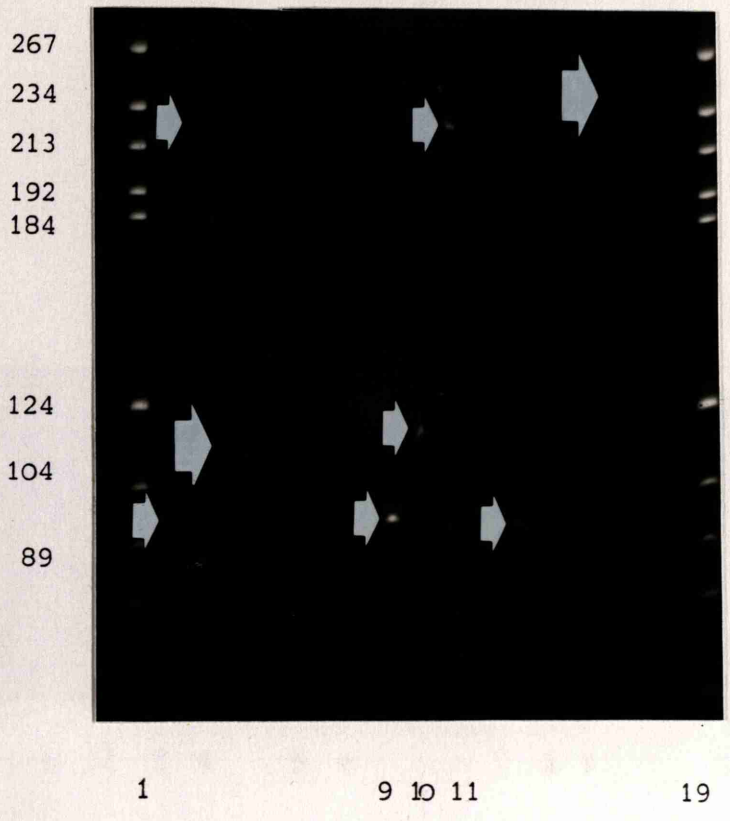
1 2 3 4 5 6 7 8 9 10 11 12 13

Lane	2 3 4 Node	5 6 7 Node	8 9 10 Node + RPMI 8402	11 12 13 RPMI 8402
V Primer	4 8 10	4 8 10	4 8 10	4 8 10
Results	+ + +	+ + +	+ + +	+ + +

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

There is inadequate resolution of bands by agarose gel electrophoresis to distinguish polyclonal DNA (from reactive lymph node) from monoclonal RPMI 8402 or from a 20:1 mixture lymph node and RPMI 8402 DNA.

Experiment 9 Analysis of PCR amplification products of polyclonal T-cell DNA from a reactive lymph node and cultured monoclonal T-cell line RPMI 8402 on 6% non-denaturing polyacrylamide gel (original V4, V8, V10- J1/2 primers). Same reaction products as in Experiment 8.



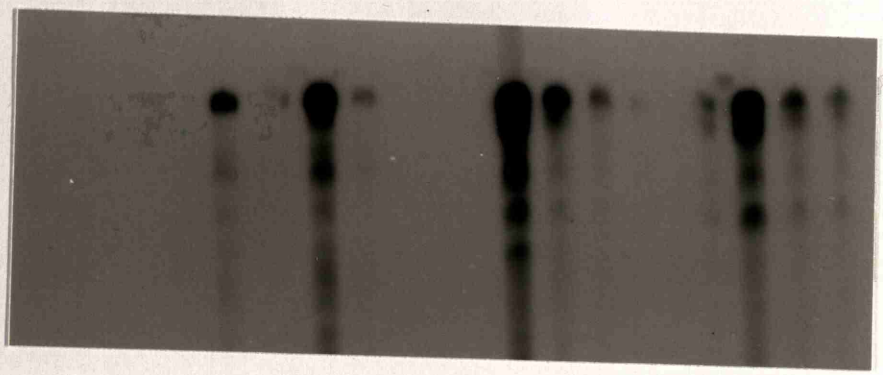
Lane	2	3	4	5	6	7	9	10	11	12	13	14	15
V Primer	10	4	10	4	10	4	10	8	4	10	4	10	4
Result	w	w	s	s	s	s	+	+	+	+	+	s	s

Left MW (nucleotides). Lanes 1,19: MW marker PBR 322-HaeIII.
Lanes 2, 3,12,13: 5% RPMI 8402 DNA in 95% lymph node DNA.
Lanes 4,5,6,7,14,15: reactive lymph node (polyclonal) DNA.
Lane 9,10,11: RPMI 8402 (monoclonal) DNA.

Double stranded PCR products are poorly stained with ethidium bromide on the polyacrylamide gel and difficult to photograph. In contrast to bands of single size given by monoclonal RPMI 8402 (small arrows), PCR products of reactive lymph node DNA have formed smears(s) (examples indicated with large arrows) on high resolution 6% polyacrylamide gel electrophoresis indicating range of product sizes given by polyclonal T-cell DNA. The unwanted cross-reaction with the V8 primer is again seen with RPMI 8402 DNA.

Experiment 10 Amount of radioisotope (32P dCTP) and concentration of cold dCTP for radiolabelling of PCR products for analysis on 6% denaturing polyacrylamide gels.

DNA from RPMI 8402 was subjected to PCR with V10-J1/2 (original) using varying amounts of 32P dCTP and different concentrations of cold dCTP.

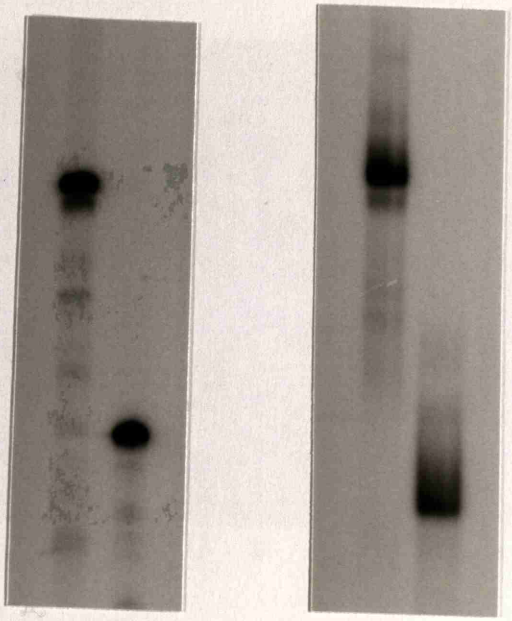


1 2 3 4 5 6 7 8 9 10 11 12

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Final concn. cold dCTP	0.0			0.8µM			4µM			8µM		
32PdCTP added (uCi)	4	0.4	0.04	4	0.4	0.04	4	0.4	0.04	4	0.4	0.04

4µM cold dCTP and 0.4 µCi 32P dCTP give satisfactory results and have been used in all subsequent experiments.

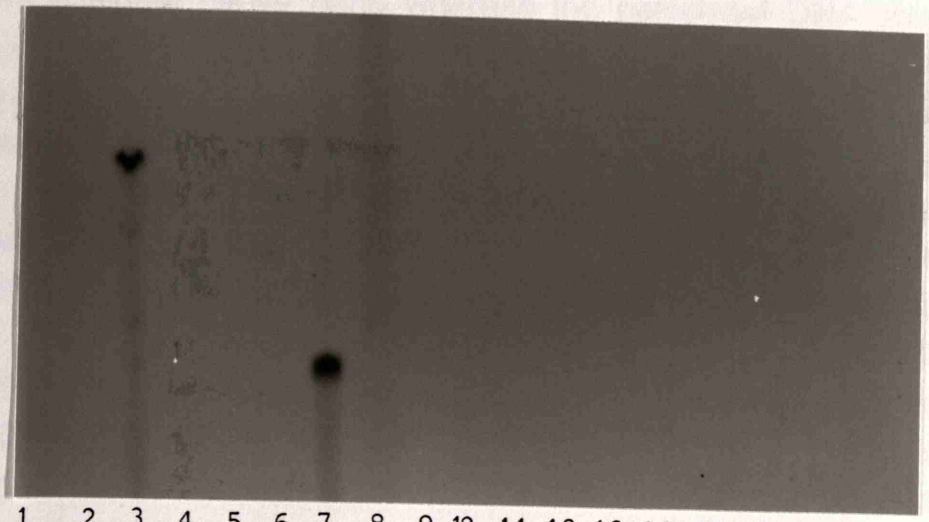
Experiment 11 Analysis of radiolabelled V4-J2 and V10-J2 (modified primers) PCR products of RPMI 8402 T-cell line DNA on denaturing (a) and non-denaturing (b) 6% polyacrylamide gels.



Lane	1	2	1	2
V Primer	4	10	4	10
Type of gel	a		b	
Result	+	+	+	+

A clonal (+) reaction is seen as a dense band of appropriate molecular weight, when PCR is analysed on denaturing polyacrylamide gel (a). In non-denaturing (b) conditions the corresponding bands are much less sharply defined. In all subsequent experiments radiolabelled PCR analysis has been carried out on denaturing 6% polyacrylamide gel electrophoresis.

Experiment 12 Specific amplification of V4-J2 and V10-J2 rearrangements in RPMI 8402 tested with 20 primer combinations (modified primers).



Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
V Primer	2	3	4	P5	8	9	10	11	4	8	10	11	4	8	10	11	4	8	10	11J
J Primer	——J1/2——								——JP——				——JP1——				——JP2——			
Result	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Specific positive results are obtained only with V4-J1/2 and V10-J1/2 primer combinations in keeping with known rearrangements. Note that a negative result is obtained with modified primer V8A.

COMMENT

In Experiment 8 in which PCR products are analysed on 2.5% agarose gel it is not possible to distinguish monoclonal RPMI 8402 DNA from polyclonal lymph node DNA. In theory monoclonal RPMI 8402 should form a single dense band whereas the polyclonal DNA should produce multiple bands or a smear ranging in molecular size over a range of 20-40 nucleotides (Chapter 1). In an attempt to improve on the resolution obtained with 2.5% agarose gels it was decided to analyse the PCR products by polyacrylamide gel electrophoresis with ethidium bromide staining (Experiment 9). Although composed of double stranded DNA, the PCR products stained poorly presumably due to quenching of fluorescence by the polyacrylamide (Sambrook, Fritsch & Maniatis 1989). It was possible however to see the expected single monoclonal bands with RPMI 8402 and a long smear with polyclonal T-cell DNA from reactive lymph node. It was therefore decided to employ a more sensitive technique for detection of PCR amplified products by radiolabelling the reaction mixture with 32P dCTP followed by autoradiography of the polyacrylamide gel. To do this experiments were carried out to obtain optimal radiolabelling with as little 32P radioisotope as possible. It was found that the use of 4μM cold dCTP gave the best results despite a marked imbalance with other dNTPs (dATP, dTTP, dGTP- each 2.5mM) in the PCR (Experiment 10). In non-denaturing polyacrylamide gels this led to greatly increased signals with monoclonal DNA compared to those seen with ethidium bromide staining but the monoclonal bands were not sharply defined (Experiment 11b). This difficulty was overcome by carrying out the polyacrylamide electrophoresis in denaturing conditions (with urea at 50ºC) (Experiment 11a). Accordingly all subsequent experiments were carried out by radiolabelling the PCR with 32P dCTP (Appendix 2.3) and analysing the products by electrophoresis in denaturing polyacrylamide gel (Appendix 2.6).

As shown in Experiments 3,4,7,8 and 9 and discussed above the original V8 primer selected caused non-specific amplification of RPMI 8402 DNA. Replacement with primer V8A abolished the unwanted results (Experiment 12). Other new more appropriate primers were also selected at this time for V2 , V3, V5, V8, V9, V10, V11 γ genes (Figs 2.3 and 2.4 and Tables 2.3 and 2.4) when we became aware of the importance of the last three nucleotides at the 3' end in determining specificity of PCR primers.

Experiment 12 shows that polyacrylamide gel electrophoresis analysis in denaturing conditions with all new primer combinations gives the expected results with RPMI 8402 DNA, there being no unwanted cross-reactions.

iii. Assessment and validation of chosen methods

After defining optimal PCR conditions to amplify V-J gene rearrangements in the RPMI 8402 T-cell line and obtaining satisfactory results by the chosen method, I next undertook to determine whether rearrangements of other TCRγ genes could be detected with appropriate primer combinations. This was done by testing DNA samples of other T-cell lines with known γ gene rearrangements by the method developed in the model system with RPMI 8402. The sensitivity of the procedure was tested by serial dilutions of cell line DNA in polyclonal DNA obtained from reactive lymph nodes and testing with appropriate primers.

METHODS

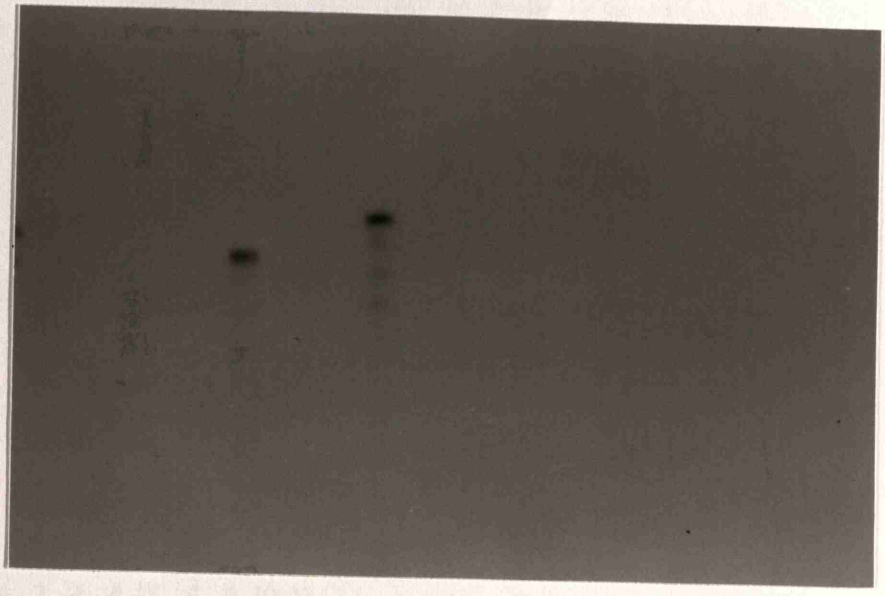
DNA was prepared as shown in Appendix 2.2 from the following cultured monoclonal T-cell lines.

Name of cell line	Source	Rearrangements
Jurkat J6	Dr Ruth Jarrett (Glasgow)	V8-J2, V11-J2
SUPT-1	Dr TH Rabbitts (Cambridge)	V3-J2, V4-J2
MOLT - 16	Dr TH Rabbitts (Cambridge)	V8-J2

Polyclonal DNA was similarly prepared by the methods shown in Appendix 2.2 from frozen reactive lymph nodes from surgical pathology specimens.

The sensitivity of the method for demonstrating γ gene rearrangements was investigated by making dilutions of monoclonal T-cell DNA in polyclonal T-cell DNA and testing 1μg, 1ng, 1pg and 1fg of the mixture by PCR. Samples of DNA from cultured monoclonal T-cell lines RPMI 8402 and Jurkat J6 were used for the purpose. PCR was done according to the modified radiollabelled PCR protocol given in Appendix 2.3.

Experiment 13 Specific amplification of V8-J1/2, V11-J1/2 rearrangements in Jurkat J6 cell line tested with 20 primer combinations (modified primers).

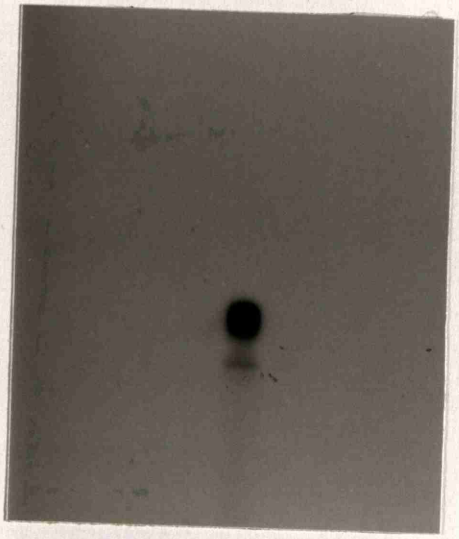


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
V Primer	2	3	4	P5	8	9	10	11	4	8	10	11	4	8	10	11	4	8	10	11
J Primer	——J1/2——								——JP——				——JP1——				——JP2——			
Result	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

Positive results are obtained with V8-J1/2 and V11-J1/2 primer combinations in keeping with the known rearrangements.

Experiment 14 Specific amplification of V8-J1/2 rearrangement in MOLT-16 T-cell line tested with 8V and J1/2 primers (modified primers).

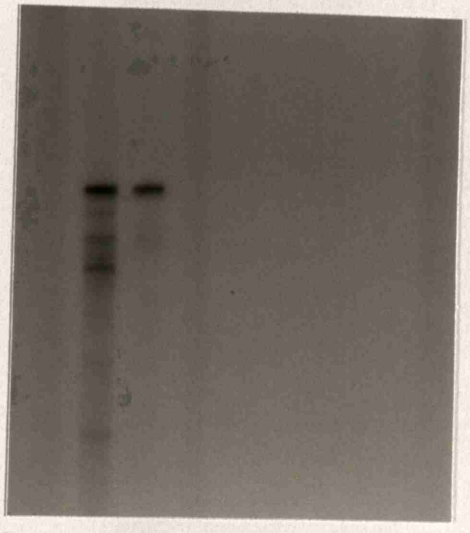


1 2 3 4 5 6 7 8

Lane	1	2	3	4	5	6	7	8
V Primer	2	3	4	P5	8	9	10	11
Result	-	-	-	-	+	-	-	-

Positive result is obtained only with V8-J1/2 in keeping with known rearrangement in MOLT-16 cell-line.

Experiment 15 Specific amplification of V3-J1/2 and V4-J1/2 rearrangements in SUPT-1 T-cell line tested with 8 V and J1/2 primers (modified primers).

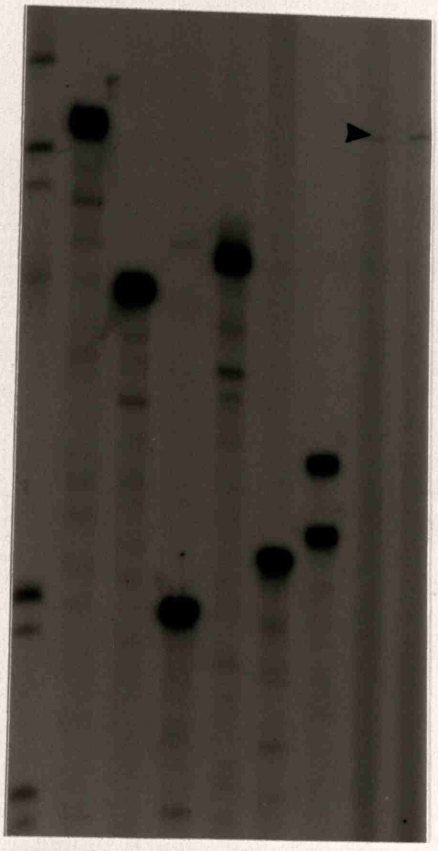


1 2 3 4 5 6 7 8

Lane	1	2	3	4	5	6	7	8
V Primer	2	3	4	P5	8	9	10	11
Result	-	+	+	-	-	-	-	-

Positive results are obtained only with V3-J1/2 and V4-J1/2 in keeping with known rearrangements in SUPT-1.

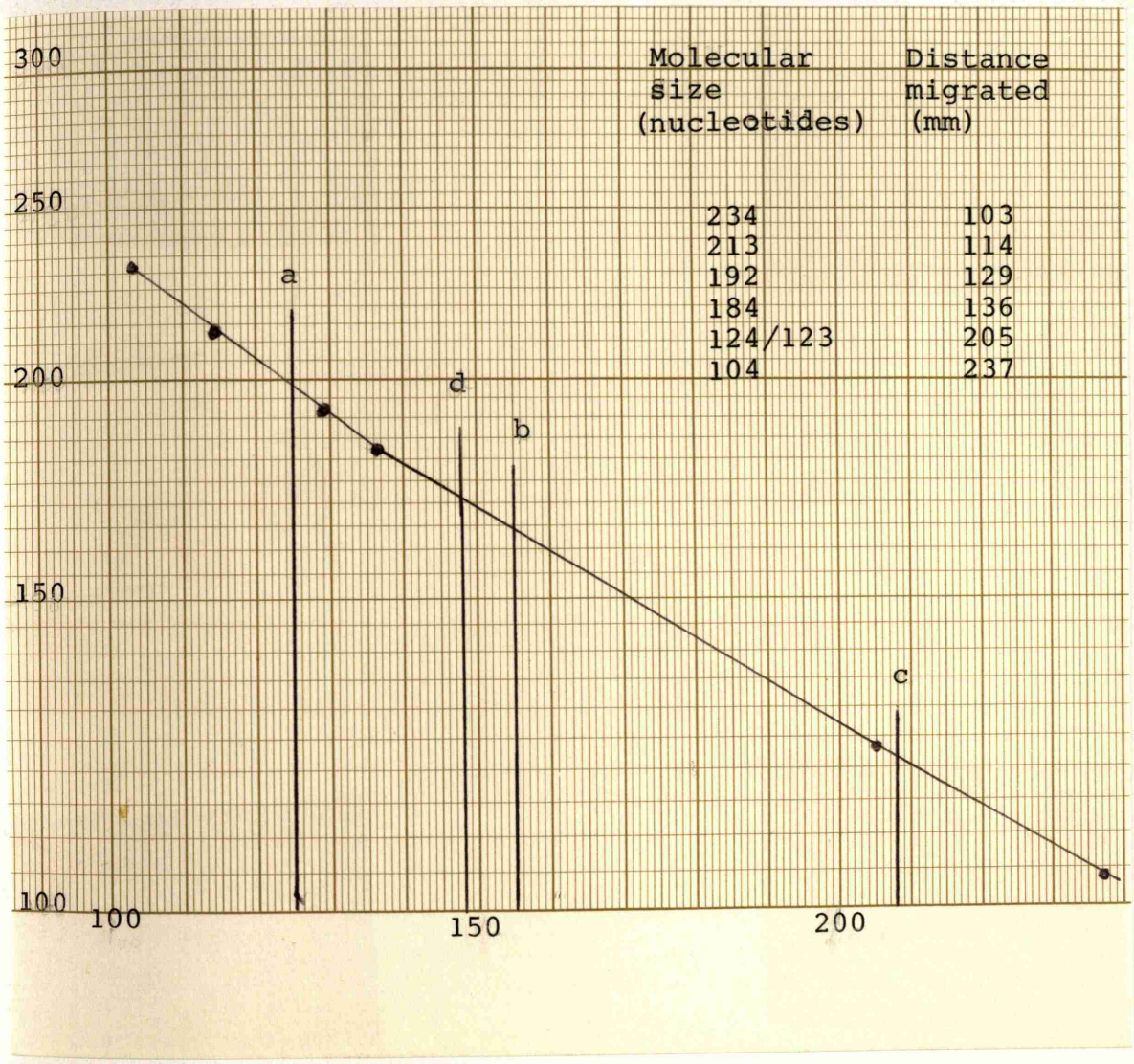
Experiment 16 Molecular weight of PCR products of TCRγ gene rearrangements in DNA from cultured T-cell lines.



Lane	2	3	4	5	6	7	8	9
V Primer	4	10	8	11	8		3	4
J Primer	———		———		J1/2	———		
Cell line	RPMI 8402		Jurkat J6		MOLT -16		SUPT-1	
Result	+	+	+	+	+		w	w

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

Molecular weights were calculated from the graph shown overleaf and are given in Table 2.6. In this experiment the reaction of SUPT-1 was very weak (arrow).



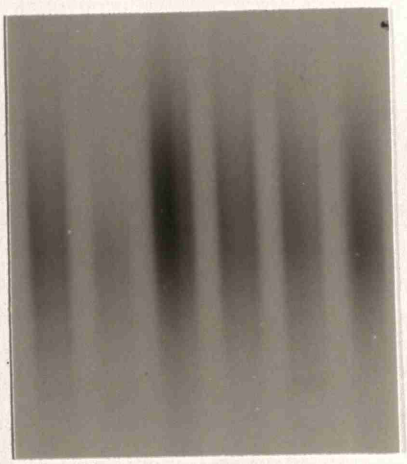
Calibration curve using molecular weight marker PBR 322-HaeIII.
Ordinate: molecular weight in nucleotides (logarithmic scale).
Abscissa: distance migrated in mm.
Findings with PCR amplified rearrangements in cell lines RPMI 8402 ((a) V4-J1/2 (b) V10-J1/2) and Jurkat J6 ((c) V8-J1/2 (d) V11-J1/2) are shown.

TABLE 2.6 Observed and expected sizes of PCR products of rearranged TCRγ genes in four T-cell lines using DNA PBR322-HaeIII marker

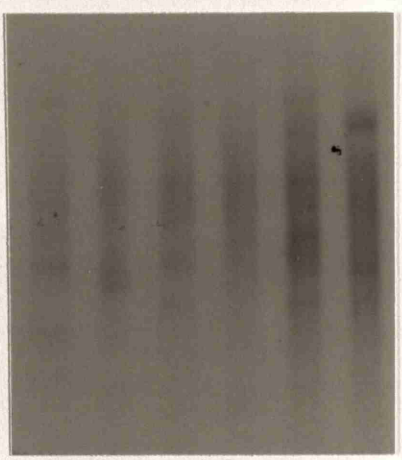
Cell line	V-J rearrangement	Size of PCR products (bp) Observed	Expected
RPMI 8402	V4-J1/2	196	181-221
	V10-J1/2	165	165
Jurkat J6	V8-J1/2	123	105-145
	V11-J1/2	172	151-191
MOLT-16	V8-J1/2	129	105-145
SUPT-1	V3-J1/2	194	175-215
	V4-J1/2	192	181-221

Experiment 17 Amplification of V2-J1/2, V5-J1/2 and V9-J1/2 (modified primers) rearrangements in polyclonal lymph node DNA from six subjects.

(a)



(b)



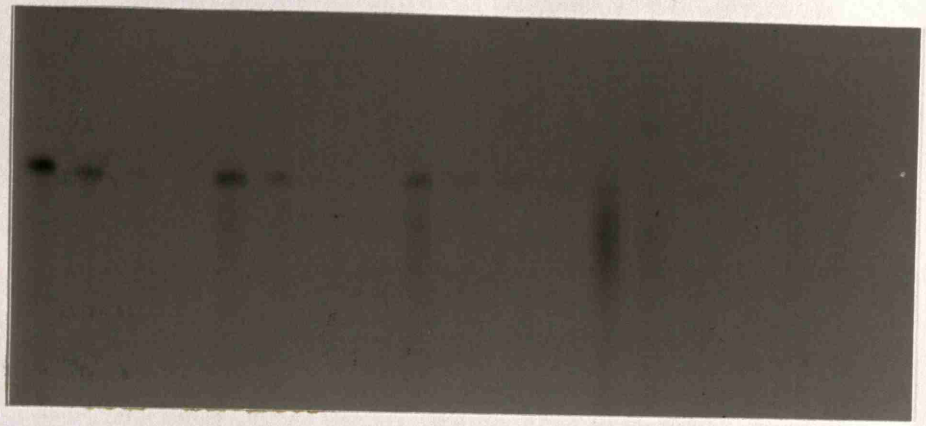
(c)

(a) V2-J1/2
(b) V5-J1/2
(c) V9-J1/2

Polyclonal lymph node DNA formed ill-defined autoradiographic smears (sometimes including one or more light bands) presumably due to variation in length of different V-J junctions.

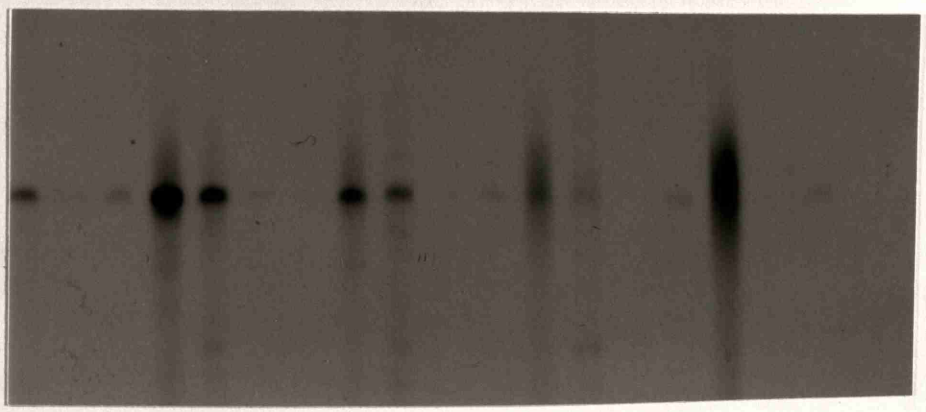
Experiment 18 Sensitivity of PCR method for detection of monoclonal T-cell DNA in presence of polyclonal T-cell DNA from reactive lymph node (1 µg, 1ng, 1pg and 1fg of DNA tested).

(a)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

(b)

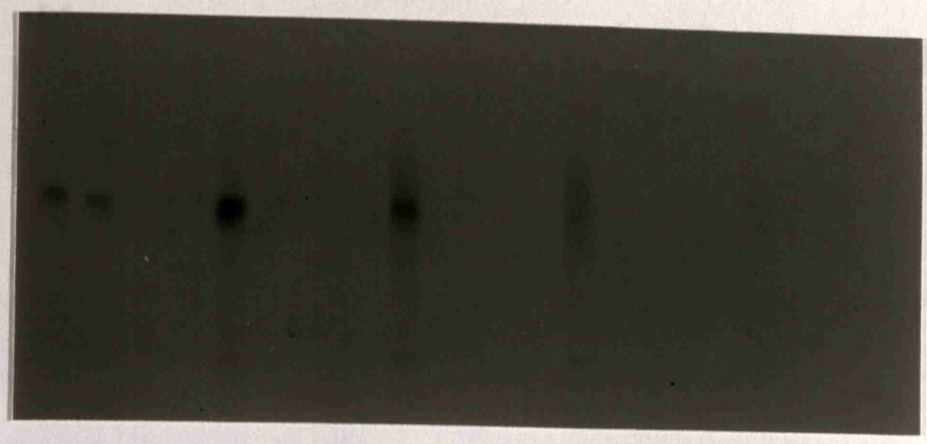


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

The very weak bands seen with fg samples in (b) lanes 4 and 16 are thought to be contamination artefacts.

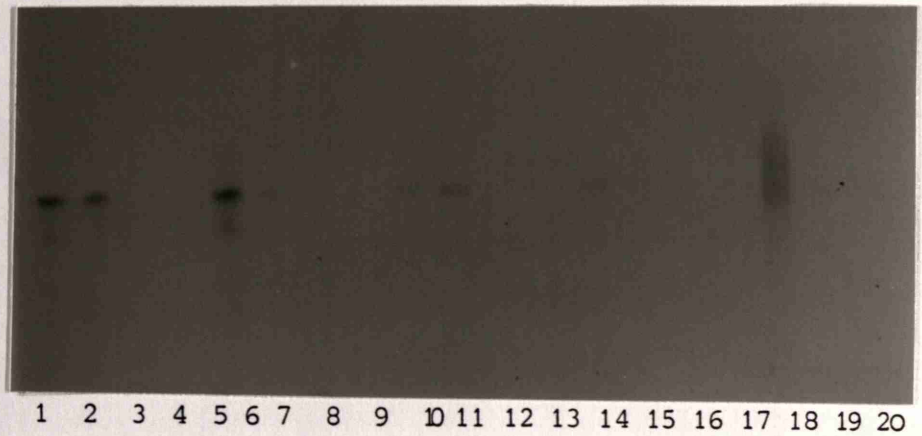
Experiment 18 (continued)

(c)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

(d)



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Lane	1 2 3 4	5 6 7 8	9 10 11 12	13 14 15 16	17 18 19 20
Dilution in lymph node DNA	-Undiluted-	-1 in 20-	—1 in 100—	—1 in 1000—	Lymph node DNA only
Total DNA	ug ng pg fg	ug ng pg fg	ug ng pg fg	ug ng pg fg	ug ng pg fg

(a) V4-J1/2 PCR products of RPMI 8402 DNA.
(b) V10-J1/2 PCR products of RPMI 8402 DNA.
(c) V8-J1/2 PCR products of Jurkat J6 DNA.
(d) V11-J1/2 PCR products of Jurkat J6 DNA.

The results are summarised in Table 2.5. Nanogram samples of RPMI8402 tested with V10-J1/2 and Jurkat DNA tested with V8-J1/2 gave strong reactions diluted 1 in 100 in lymph node DNA but larger amounts were required to demonstrate the other rearrangements in these cell lines.

TABLE 2.5 Sensitivity of PCR for detecting clonal T cell γ gene rearrangements

Cell line (Primers)	Dilution in lymph node DNA	PCR result with DNA sample of 1µg	1ng	1pg	1fg
RPMI 8402 (V4-J1/2)	Undiluted	+	+	-	-
	1:20	+	+	-	-
	1:100	+	-	-	-
	1:1000	-	-	-	-
RPMI 8402 (V10-J1/2)	Undiluted	+	+	-	-
	1:20	+	+	-	-
	1:100	+	+	-	-
	1:1000	-	-	-	-
Jurkat J6 (V8-J1/2)	Undiluted	+	+	-	-
	1:20	+	+	-	-
	1:100	+	+	-	-
	1:1000	+	-	-	-
Jurkat J6 (V11-J1/2)	Undiluted	+	+	-	-
	1:20	+	-	-	-
	1:100	-	-	-	-
	1:1000	-	-	-	-

COMMENT

Experiments 13-15 with DNA from three T-cell lines other than RPMI 8402 gave positive results only with the relevant primers and the molecular sizes of the PCR products were within the expected range (Experiment 16). Taken together, the experiments show specific PCR amplification with primers for V3, V4, V8, V10 and V11 paired with J1/2. Evidence that the primers for V2, V5 and V9 are effective and specific is less direct. They produced smears of appropriate molecular size when tested with lymph node polyclonal DNA (Experiment 17) and gave no reaction with monoclonal DNA containing V3, V4, V8, V10 and V11 γ gene rearrangements. The primer for ΨV5 was used in some of these experiments because we did not know the sequence of the functional V5 gene at the time.

The dilution tests reported in Experiment 18 and Table 2.5 show that the test is somewhat more sensitive with some primers than others. Positive results were obtained with 1ng of 1 in 100 mixture of RPMI 8402 or Jurkat J6 in polyclonal T-cell DNA from a reactive lymph node. Assuming that the majority of T-cells belong to clones in which one of the 8 potentially functional V genes is rearranged with J1 or J2 and that rearrangements of each V gene commonly show a range of sizes of 40 nucleotides at the hypervariable V-J junction, the PCR products of most V-J rearrangements will form approximately 320 (8 x 40) bands distinguishable on high resolution polyacrylamide gel electrophoresis. Each band on average will thus represent a little less than 1 per cent of the total TCRγ gene rearrangements. It is therefore unlikely that a single clone will be recognisable if it consists of less than 1 percent of all the T-cells present.

Compared with Southern blot analysis, the PCR method is much more sensitive since results can be obtained with a few nanograms of DNA, whereas 10μg is required for Southern blotting. Both techniques are similar in their ability to demonstrate monoclonal T-cells against a background of 95-99 per cent polyclonal DNA (Greaves et al. 1986. Arnold et al. 1983).

In some experiments (eg. Exp 11 and Exp 16) it was observed that in addition to bands of expected molecular sizes others of inappropriate molecular weight may be produced by the PCR. This phenomenon is also found with the PCR in other fields of study and presumably represents

mispriming events at an early stage in the reaction and possibly incomplete extension at the end of the procedure. Non-specific PCR products of inappropriate molecular size are easily recognised when one pair of primers is used but would cause difficulties in interpretation if more than one primer combination was tested in a single tube. This is one reason why the original plan to incorporate multiple primers each strategically selected to give PCR products of different sizes (see Fig 2.2) had to be abandoned. A second reason is the difficulty of finding specific sites for V3, V4, V5 and V8 other than at the variable regions around nucleotides 330-350 and 390-420 (Fig 2.1).

iv. Extraction and processing of DNA for amplification of TCRγ gene rearrangements by PCR

METHODS

To examine whether purification of extracted DNA is necessary prior to PCR, PCR was performed on 2000 cells of the RPMI 8402 cultured T-cell line in four different ways.

A. Directly on the cells. Aliquots of 2000 cells which had been frozen in 10µl PBS at -20ºC overnight were used as the target DNA.

B. After boiling. Aliquots of 2000 cells in 10µl PBS which had been frozen at -20ºC overnight were pelleted, resuspended in 10µl water and boiled for 5 minutes before testing.

C. Following proteinase K digestion. Aliquots of 200 cells were suspended in 10µl non-ionic detergent buffer (NIB) (Appendix 2.9) containing proteinase K (3µl/500µl NIB) incubated at 60-65ºC for 1-2 hours then at 95ºC for 10 minutes to inactivate proteinase K.

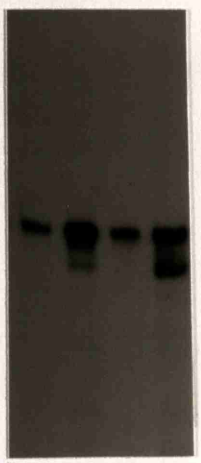
D. On DNA prepared by extraction with phenol/chloroform as given in Appendix 2.2.

Method A was also tested on 10 cells, 100 cells, 1000 cells and 2000 RPMI 8402 cells. Its sensitivity was also tested against a background of polyclonal T-cells by mixing RPMI 8402 cells with PBMC from a healthy individual in concentrations of 0.5%, 1%, 5% and 10% and subjecting directly to PCR.

Experiment 19 Comparison of 4 methods of preparing DNA for PCR amplification of V10-J2 rearrangement in T-cell line RPMI 8402.

PCR was performed on 2000 cultured cells as follows:

A. Directly on cells after freezing and thawing.
B. After boiling cells with 10ul H_20 for 5 minutes.
C. After incubating at 60-65ºC with 10ul NIB + Proteinase-K for 1 hour and inactivating Proteinase-K by incubating at 95ºC for 10 minutes.
D. On DNA purified by phenol/chloroform extraction and precipitation with ethanol at 4ºC.

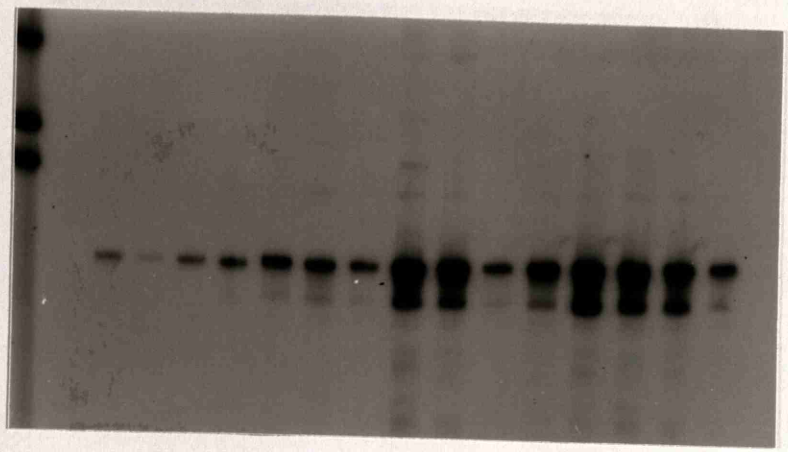


1 2 3 4

Lane	1	2	3	4
Preparation	A	B	C	D
Result	+	+	+	+

Equally satisfactory results were obtained with all four preparations.

Experiment 20 Sensitivity of detection of V10-J2 rearrangement by PCR performed directly on cells after freezing and thawing of RPMI 8402 T-cell line.



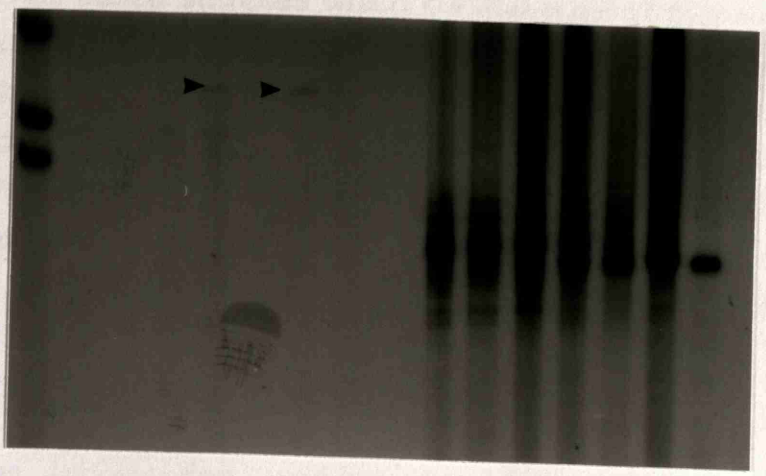
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Lane	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Number of cells tested	—10—				—100—				—1000—				—2000—			
Result	-	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

Positive clonal reaction was seen in samples containing as few as 10 target molecules.

Experiment 21 Sensitivity of detection of V4-J2 and V10-J2 rearrangements in RPMI 8402 cells diluted in polyclonal peripheral blood mononuclear cells by direct PCR on 2000 cells after freezing and thawing.



Lane	2	3	4	5	6	7	8	10	11	12	13	14	15	16
Percentage of clonal cells	0	0.05	0.1	0.5	1	5	100	0	0.05	0.1	0.5	1	5	100
Primers	——V4-J1/2——							——V10-J1/2——						
Result	-	-	w	w	-	w	-	-	-	-	-	+	+	+

Left MW (nucleotides). Lane 1 MW marker PBR 322-HaeIII.

A very weak reaction is seen with V4-J1/2 primers (arrows) while clonal reaction is visible with V10-J1/2 primer combination with as few as 1% RPMI 8402 cells.

COMMENT

In addition to establishing the conditions for PCR and obtaining a suitable method of analysing the PCR products an important problem in studying small tissue samples which contain a minor population for T-cells with different γ gene rearrangements is the efficient recovery from the tissue of effective template for PCR amplification. Proteolytic digestion followed by phenol/chloroform extraction and ethanol precipitation is generally recommended for the preparation of DNA for PCR. As shown in Experiment 7, different samples of DNA prepared in this way from RPMI 8402 cells may give positive or negative results in PCR with appropriate primers. The reason for this is unknown but it is possible that contamination with nuclease or polymerase inhibitors is involved.

Other laboratories working on other systems have obtained satisfactory PCR results on samples prepared in various other ways. We have experimented with different methods of preparing PCR targets for amplification. In Experiment 19 equally satisfactory results were obtained by direct testing of aliquots of RPMI 8402 cells which had been frozen and thawed; frozen, thawed and boiled; digested in proteinase-K and boiled to inactivate the enzyme; and purified by standard proteinase-K digestion, phenol/chloroform extraction and ethanol precipitation. Following freezing and thawing, positive results were obtained with as few as 10 cells (Experiment 20). Good bands were also obtained with 1% of RPMI 8402 cells in 99% mononuclear cells from normal peripheral blood with the V10-J1/2 primer combination but the results were much weaker with V4-J1/2, the other primer combination tested (Experiment 21).

While these findings are of interest they only give an indication of how specimens might be processed for the analysis of T-cell clones in various human tissues for routine diagnosis or research purposes.

Discussion

The pilot studies in this chapter have identified a set of suitable primers, incubation times and temperatures for PCR amplification of the TCRγ gene rearrangements known to be present in four cultured monoclonal T-cell lines. Autoradiography of the radiolabelled PCR products on high resolution 6% denaturing polyacrylamide gels

demonstrates sharply defined dense monoclonal bands while smears which reflect the range of sizes of the amplified V-J junctions are obtained with polyclonal DNA.

The method is extremely sensitive. The V10-J2 rearrangement present in RPMI 8402 has been shown in as few as 10 cells. Monoclonal rearrangements can be detected in nanogram quantities of DNA of cultured T-cell lines even in the presence of 20-100 parts of polyclonal lymph node DNA. It is unlikely that significant improvement in results would be obtained by further refinement in technique. Unfortunately if primers specific for the various V and J genes are to be used, each DNA sample requires at least 32 successful PCR tests in order to amplify all the rearrangements likely to be present.

PCR amplification of V10-J2 rearrangement in RPM1 8402 was equally successful in cell preparations which had been frozen and thawed, boiled or digested with proteinase-K or on purified DNA obtained by phenol/chloroform extraction.

In the following chapters purified DNA was used in order to minimise possible effects of nucleases or PCR inhibitors which might be present in normal or pathological tissue samples. It was decided to use 0.5 µg rather than nanogram quantities of DNA in each test to increase the likelihood of detecting clones forming less than 5% of the total T-cell proliferation. This made it necessary to restrict the primer combinations tested to those for J1/2 and the eight commonly rearranged V genes with the result that rearrangements involving JP, JP1 and JP2 would not be detected.

CHAPTER 3

Polymerase Chain Reaction Amplification of T-cell Receptor γ Gene Rearrangements in T-cell Neoplasia

Introduction

In Chapter 2 it was shown that the appropriate TCRγ gene rearrangements can readily be demonstrated by PCR in cultured monoclonal T-cell lines whose γ gene rearrangements are already known and that the dense monoclonal bands obtained are easily distinguished from the "polyclonal" smears obtained with DNA extracted from reactive lymph nodes or normal peripheral blood mononuclear cells.

Dominant TCR gene rearrangements have been demonstrated in T-cell lymphomas and leukaemias by various workers using Southern blot analysis and more recently PCR (Table 1.1).

In order to assess the ability of our method to distinguish neoplastic from non-neoplastic T-cell clones in clinical samples, electrophoretic band patterns obtained with biopsies infiltrated by T-cell lymphoma were compared with those in reactive (non-neoplastic) lymph nodes. PCR results were compared with Southern blot analysis of TCRγ gene rearrangements in some cases. Since the actual density of the auto-radiographic bands must depend not only on the relative amounts of the various clonotypes amplified but also on such variables as the quality of the DNA sample, the number of T-cells present in the original specimen, the efficacy of the particular primer combination, the batch of Taq polymerase and the specific activity of 32P dCTP, a dominant clonotype was defined as one which gives rise to a sharp discrete band which is disproportionately dense when compared with other PCR products (bands or smears) in the same lane. Bands which were conspicuously abnormal were categorised as ++ and those which were considered borderline were scored +.

Materials and Methods

Clinical Specimens

Unfixed tissue samples from routine diagnostic biopsies of 6 T-cell lymphomas were obtained from the -70ºC store in the Department of Pathology, Glasgow Royal Infirmary.

High molecular weight DNA extracted from 5 routine diagnostic biopsies from 5 patients with T-cell lymphoma was provided by Sarah Gledhill, Leukaemia Research Virus Centre, Department of Veterinary Pathology, University of Glasgow, who had carried out restriction enzyme analysis (Southern blotting) of TCRγ gene rearrangements on each of these samples (Gledhill et al. 1990).

High molecular weight DNA extracted from 22 routine diagnostic biopsies from 21 cases of T-cell lymphoma was provided by Dr. Tim Diss, University College of London Medical School, who had carried out restriction enzyme analysis (Southern blotting) of rearrangements of TCRγ genes on each of these samples.

In all cases studied the diagnosis was established by histological and immunohistochemical examination of diseased tissue.

Unfixed reactive lymph nodes were obtained as non-neoplastic controls from 12 patients undergoing surgical operations at Glasgow Royal Infirmary.

Clinical details of cases and controls are given in Table 3.1 and Appendices 3.1 and 3.2.

Nucleotide sequencing studies were performed on four T-cell lymphoma cases by F.R. Imrie (Imrie, Karim & Goudie 1992).

DNA Extraction

High molecular weight DNA was extracted from several hundred 5 micron cryostat sections from each frozen lymph node using standard proteolytic digestion, phenol/chloroform extraction and ethanol precipitation and DNA content was determined by UV spectrophotometry (Appendix 2.2).

PCR Amplification

PCR amplification of 0.5μg of DNA was performed for 30 cycles using the modified radiolabelled PCR protocol as described in Appendix 2.3 and the primers specific for 8 Vγ and Jγ1/2 genes shown in Tables 2.3 and 2.4. PCR products were analysed on autoradiographs of 6% polyacrylamide gels run under denaturing conditions according to protocols given in Appendix 2.6.

Results

T-cell lymphoma

The results of PCR obtained with 8 Vγ and Jγ1/2 primer combinations in DNA from 33 routine diagnostic biopsies from 32 T-cell lymphomas are given in detail in Appendix 3.1 and summarised in Tables 3.1 and 3.2. Illustrative examples are shown in Figs 3.1 - 3.3.

Two hundred and sixty one PCRs were carried out on the 33 DNA samples. Successful reactions were obtained in 234 and 27 PCRs failed to give any reaction. The first five cases studied (cases L1-L5) gave similar sized dominant V8-J1/2 rearrangements (Fig 3.4) (Appendix 3.1). In four of these cases (cases L1,L3,L4 and L5) sequencing was performed and the dominant bands were found to have identical nucleotide sequences across the V-J junction (Fig 3.5) (Imrie, Karim & Goudie 1992). These identical bands were concluded to be due to contamination with V8 amplification products from a PCR on another case when the technique was being developed. These dominant bands with V8-J1/2 are marked with an asterisk in Appendix 3.1 and have been excluded from the analysis of results. The DNA from case L10 was unreactive in PCR and is also not included in the analysis of results.

Dominant bands like those seen with cultured T-cell lines (Chapter 2) were detected in twenty-one of thirty-one cases. In ten of these cases the strongest reaction was + and in eleven ++. One dominant band was found in nine cases (cases L2,3,5,6,11,13,16,26 and L27) two in eleven (cases L4,7,12,15,19,20,21,22, 24,25 and L29) and three in one case (case L9).

TABLE 3.1 Dominant TCRγ gene rearrangements in routine diagnostic biopsies of T-cell lymphoma

Case No.	Age/Sex	Diagnosis	Dominant clonal rearrangements PCR	Southern blot
L1	F	T-NHL	-	ND
L2	34/F	T-cell lymphoma	V3 +	ND
L3	NK	CTCL	ΨV5 +	ND
L4	75/F	Pleomorphic T-cell lymphoma	V3 + V9 +	ND
L5	20/F	Lymphocytic T-cell lymphoma	V2 ++	ND
L6	16/M	T-cell lymphoma	V8 +	ND
L7	62/M	AIL	V3 ++ V9 +	Clonal rearrangement
L8	43/M	T-NHL	-	No clonal rearrangement
L9	35/F	T-NHL	V3 + V8 + V11+	Clonal rearrangement
L10	70/M	T-NHL	NR	Clonal rearrangement
L11	15/F	T-NHL	V2 ++	Clonal rearrangement
L12	46/M	Large T-cell lymphoma	V9 + V10 ++	V10
L13	58/F	CTCL	V9 ++	V2 or 4, V9
L14	7/F	Cutaneous lympho-blastic T-cell lymphoma	-	-
L15	59/M	Pleomorphic T-cell lymphoma	V9 ++ V10 +	-
L16	57/M	CTCL	V9 ++	V9

Table 3.1 (continued)

Case No.	Age/Sex	Diagnosis	Dominant clonal rearrangements PCR	Southern blot
L17(a)	58/M	Cutaneous pleomorphic T-cell lymphoma	-	-
L17(b)		Cutaneous pleomorphic T-cell lymphoma	-	V3
L18	62/F	Pleomorphic T-cell lymphoma	-	V9 or V11
L19	64/M	Pleomorphic T-cell lymphoma	V3 + V11 +	V2 or V4
L20	NK	EATL	V2 ++ V8 ++	V2, V8
L21	50/M	Cerebriform T-cell lymphoma	V2 ++ V11 +	V2 or 4, V11
L22	40/M	Pleomorphic T-cell lymphoma	V4 + V11 +	V4
L23	40/M	Pleomorphic T-cell lymphoma	-	V3, V2 or 11
L24	20/M	T-ALL	V5 ++ V10 +	V5, V10
L25	54/M	Pleomorphic T-cell lymphoma	V3 + V5 +	Not clear
L26	NK	T-cell lymphoma	V5 +	-
L27	59/M	T-zone lymphoma	V2 ++	V2 or 4
L28	/M	EATL	-	V3, V5
L29	NK	EATL	V5 + V9 ++	V8, V9

Table 3.1 (continued)

Case No.	Age/Sex	Diagnosis	Dominant clonal rearrangements PCR	Southern blot
L30	29/M	Cutaneous large T-cell lymphoma	-	-
L31	NK	EATL	-	-
L32	NK	Pleomorphic T-cell lymphoma	-	-

-	=	No dominant band
+	=	Borderline dominant band
++	=	Unequivocally dominant band
AIL	=	Angioimmunoblastic lymphadenopathy
CTCL	=	Cutaneous T-cell lymphoma
EATL	=	Enteropathy associated T-cell lymphoma
T-NHL	=	T-Non-Hodgkin's lymphoma
ND	=	Not done
NK	=	Not known
NR	=	No reaction

TABLE 3.2 PCR amplified dominant TCRγ gene rearrangements in biopsies of T-cell lymphoma

	No. of cases		
	-	+	++
Nodal	4	9	7
Cutaneous	4	1	2
Enteropathy associated	2	0	2
Total	10	10	11
Lymph node controls	11	1	0

- = No dominant band
+ = Borderline dominant band
++ = Unequivocally dominant band

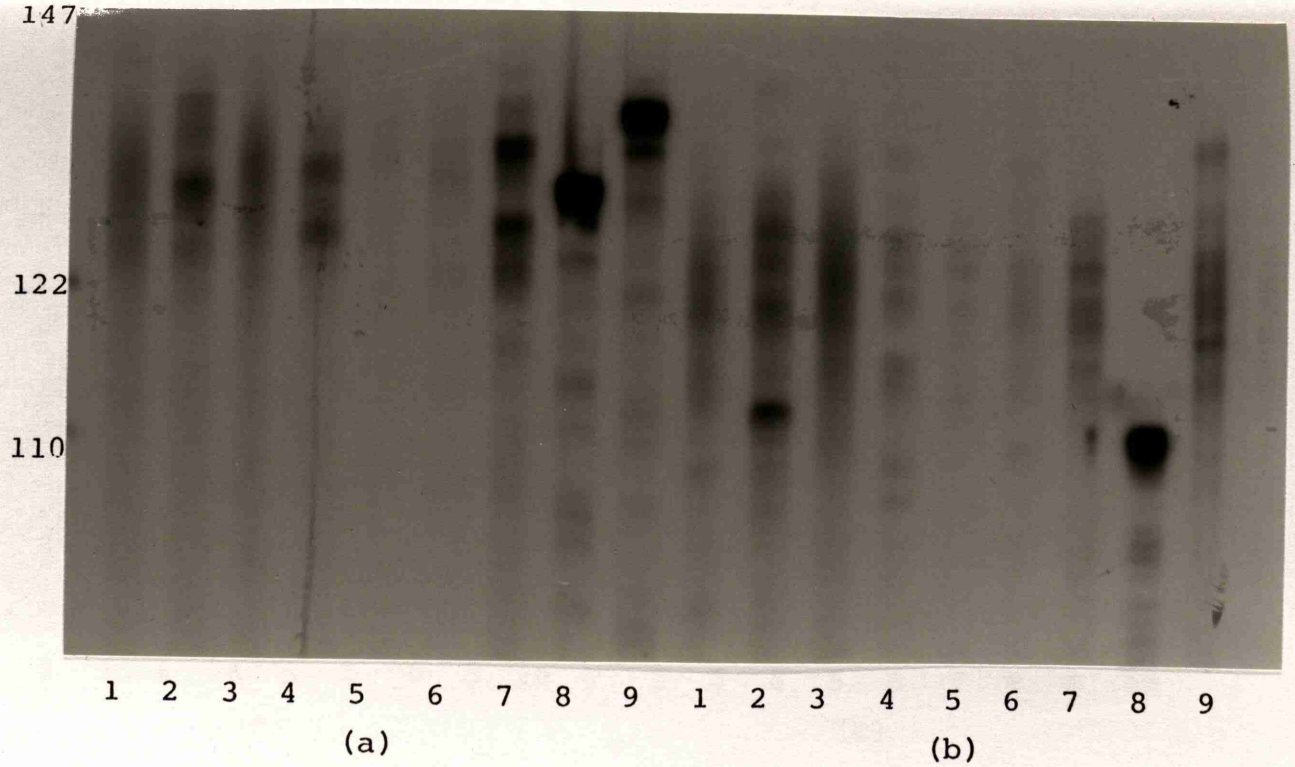


Figure 3.1 PCR analysis of TCRγ gene rearrangements in malignant T-cell lymphoma.
(a) V2-J1/2 (b) V8-J1/2 rearrangements.
Lanes 1-9: cases L12,L13,L14,L15,L17,L18,L19,L20 and L21.
Left MW in nucleotides.
With V2-J1/2 a dense dominant band (++) is present in case L20 and in case L21. With V8-J1/2 a second (++) band is present in case L20.

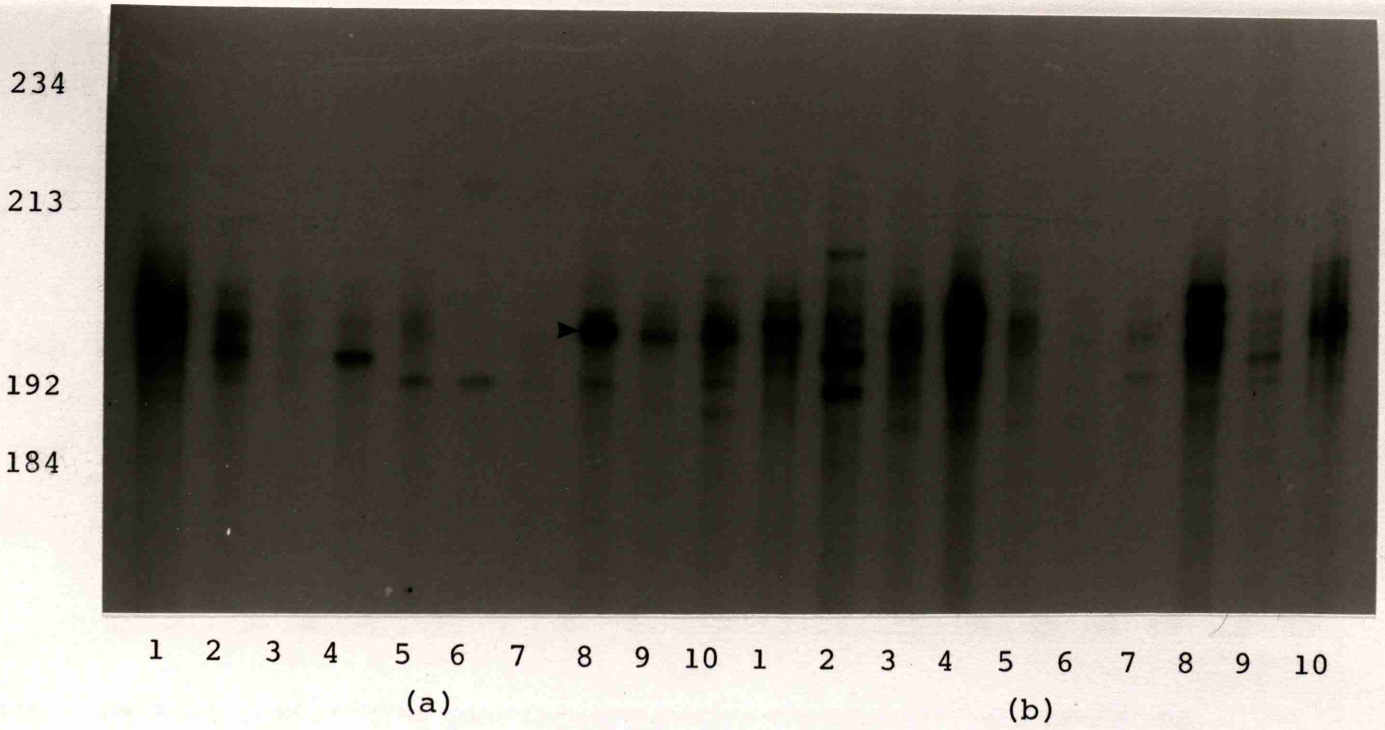


Figure 3.2 PCR analysis of TCRγ gene rearrangements in malignant T-cell lymphoma.
(a) V3-J1/2 (b) V5-J1/2 rearrangements.
Lanes 1-10: Cases L12,L13,L14,L15,L16,L17,L18,L19,L20 and L21.
Left MW in nucleotides.
With V3-J1/2 a dominant + band (arrow) is present in case L19.

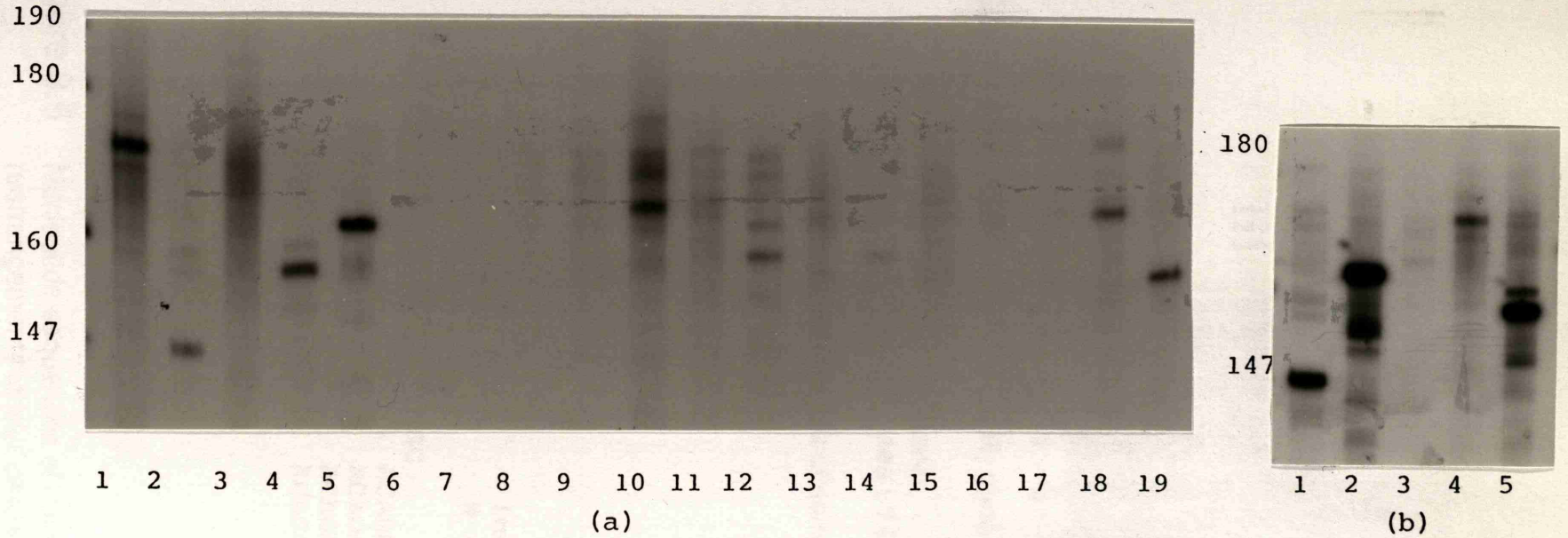


Figure 3.3 PCR analysis of TCRγ gene rearrangements in malignant T-cell lymphoma.
(a) V9-J1/2 and V11-J1/2 rearrangements.
Lanes 1-10: cases L12,L13,L14,L15,L16,L17,L18,L19,L20 and L21 with V9-J1/2. Lanes 11-19: same cases (except case L20) with V11-J1/2. Left MW in nucleotides.
With V9-J1/2 a sharp dominant (+) band is present in case L12, case L13, case L15 and case L16. With V11-J1/2 a dominant (+) band is present in case L21.
(b) Repeat PCR with V9-J1/2.
Lanes 1-5: cases L13,L16,L21,L12 and L15. Left MW in nucleotides.
A dominant (+) band is present in case L12. Dominant (++) band is present in case L13, case L15 and case L16.

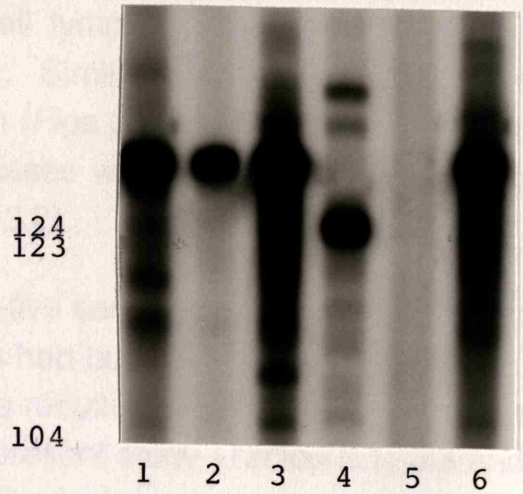


Figure 3.4 PCR analysis of TCRγ gene rearrangements in malignant T-cell lymphoma.
V8-J1/2 rearrangements.
Lanes 1-3 and 6: cases L1,L3,L4 and L5. Left MW in nucleotides.
Dominant ++ band is of same electrophoretic mobility in cases L1,L3,L4 and L5.

Case No.	V8 (germline)	Inserted N region	J1/2 (germline)
	TGCCACCTGGGATAGG		GAATTATTATAAGAAACT
1.	....	ACAGATAGGATC	....
2.	....	ACAGATAGGGTC	....
3.	....	ACAGATAGGATC	....
4.	....	ACAGATAGGATC	....

Figure 3.5 Nucleotide sequences of dominant V8-J1/2 γ gene rearrangements in four cases of T-cell lymphoma illustrated in Fig. 3.4, lanes 1,2,3, and 6. Each has deletions of six nucleotides at the 3' end of the V8 germline gene and four nucleotides at the 5' end of germline J1/2 plus an insertion of the same 12 uncoded (N region) nucleotides at the V-J junction.

Dominant bands were found in nodal, cutaneous and enteropathy-associated T-cell lymphoma cases (Table 3.2). Examples are shown in Figs 3.1 - 3.3). Similar results were obtained on repeated testing of the same specimen (Figs 3.3a and b). No dominant bands were found in the remaining ten cases which produced smears often accompanied by minor bands (eg. Fig 3.2).

In twenty-five samples from 24 cases of T-cell lymphoma TCRγ gene rearrangements had been examined in other laboratories by Southern blot analysis and the results were available for comparison with those obtained by PCR in the present study (Tables 3.1, 3.3 and 3.4). In thirteen samples both tests showed dominant rearrangements and in six both lacked dominant rearrangements. (In case L8 no dominant γ gene rearrangements were found by PCR or Southern blot analysis and Southern blot for β gene rearrangements was also negative (Gledhill et al. 1990)). In two samples dominant rearrangements were found only by PCR and in four only by Southern blot (in three of these the Southern blot rearrangement had not been confirmed by digestion with a second restriction enzyme).

In twenty-one cases γ gene rearrangements were studied by Southern blot analysis by Dr. Tim Diss who attempted to identify which V genes were rearranged from the size of the restriction fragments (Table 3.1). In fourteen samples the specific dominant V gene rearrangements had been identified by PCR and Southern blot analysis. The findings are compared in Table 3.4. The results were concordant in twelve rearrangements. PCR was positive and Southern blot negative in eight and PCR negative and Southern blot positive in nine.

Reactive lymph nodes

Detailed results obtained with 8 Vγ and Jγ1/2 primer combinations in DNA from reactive lymph nodes from 12 patients are shown in Appendix 3.2 and examples illustrated in Figs 3.6 - 3.8.

Ninety-six PCRs were performed. Seventy-six PCRs gave successful reactions and in 20 the reaction failed. Polyclonal smears were produced in all samples, sometimes accompanied by one or more faint minor bands. Minor bands were much less numerous than in lymphoma cases (Figs 3.1 -

TABLE 3.3 Summary of PCR and Southern blot results in 26 lesions from 25 cases diagnosed histologically as T-cell lymphoma.

PCR + SB +	13
PCR - SB -	6
PCR + SB -	2
PCR - SB +	5

SB = Southern blot
\- = Negative
\+ = Positive

TABLE 3.4 Correlation of specificity of dominant rearrangements Vγ gene rearrangement obtained by PCR and Southern blot in T-cell lymphomas.

	V2	V3	V4	V5	V8	V9	V10	V11
PCR + SB +	3		1	1	1	3	2	1
PCR + SB -		1		2		2	1	2
PCR - SB +	3	3		1	1	1		

SB = Southern blot
\- = Negative
\+ = Positive

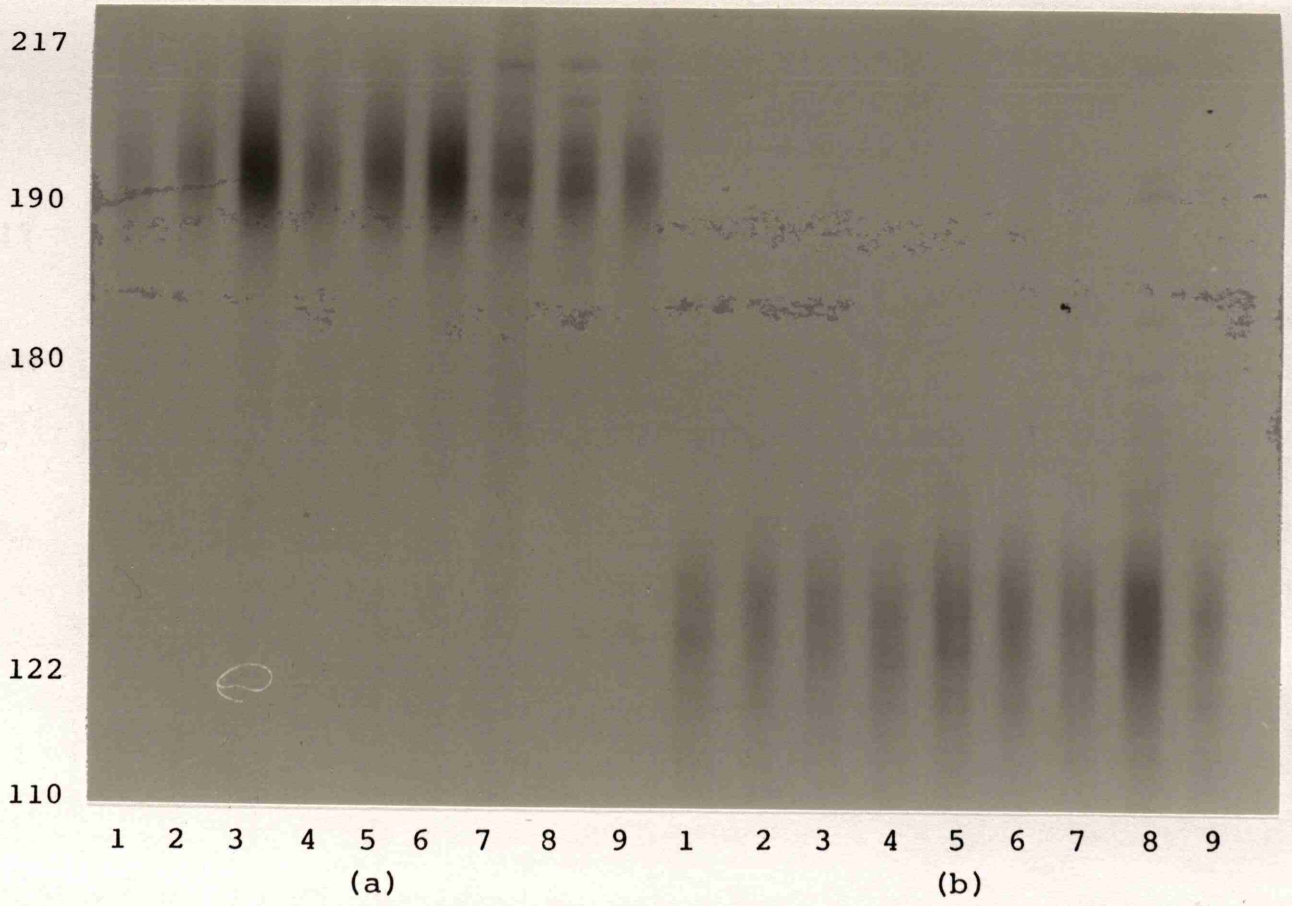


Figure 3.6 PCR analysis of TCRγ gene rearrangements in non-neoplastic lymphoid tissue.
(a) V4-J1/2 (b) V8-J1/2 rearrangements.
Lanes 1-9: reactive lymph nodes from control cases RN4-RN12. Left MW in nucleotides.
No dominant band is present.

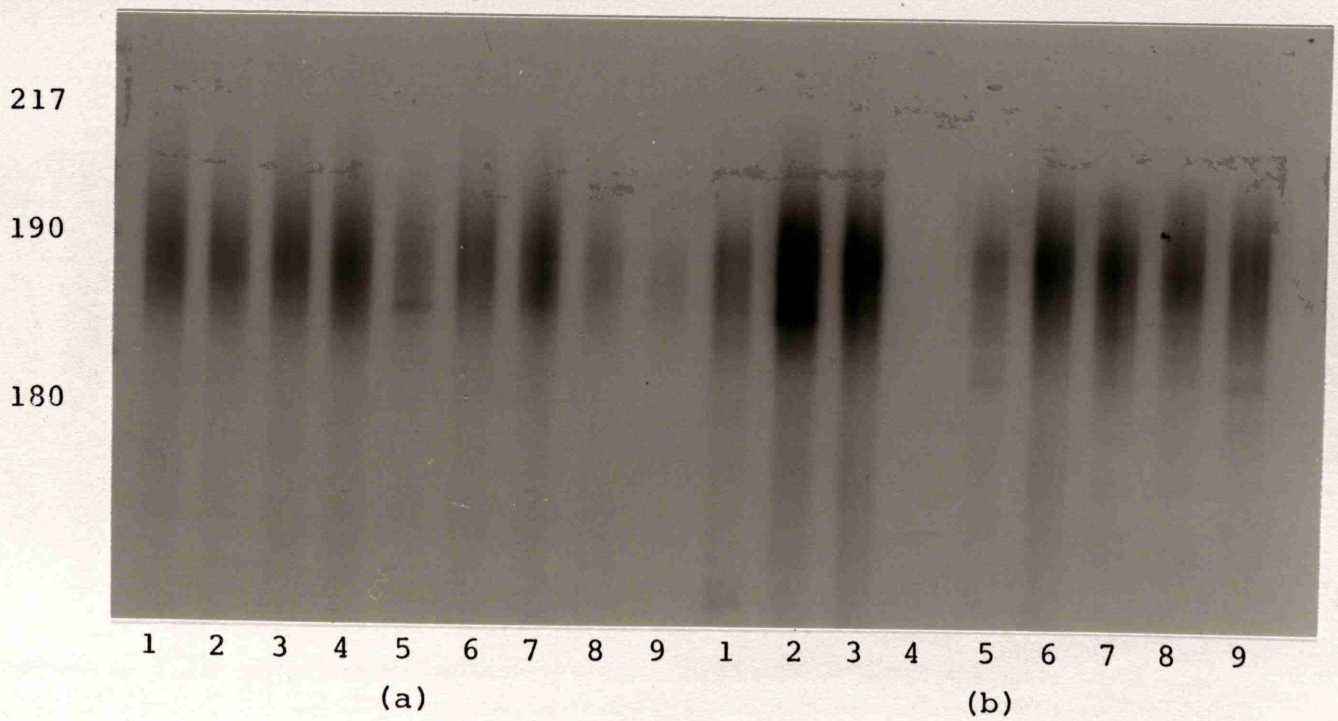
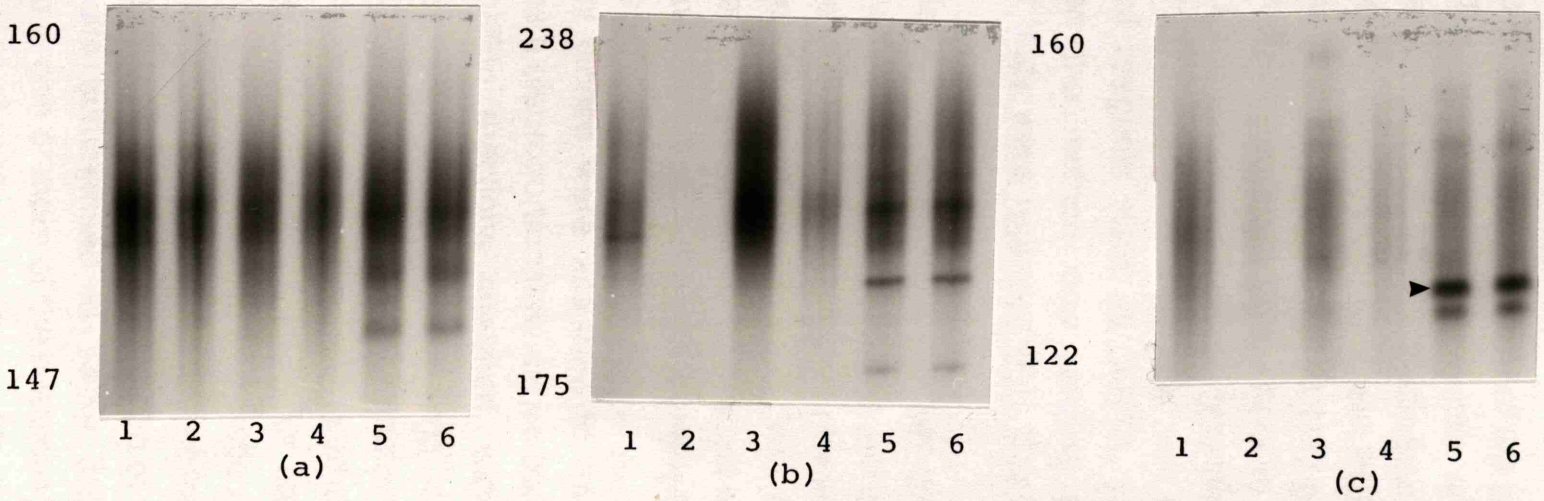


Figure 3.7 PCR analysis of TCRγ gene rearrangements in non-neoplastic lymphoid tissue.
(a) V10-J1/2 (b) V11-J1/2 rearrangments.
Lanes 1-9: reactive lymph nodes from control cases RN4-RN12. Left MW in nucleotides.
No dominant band is present. No reaction is obtained in Lane 4(b)



Figures 3.8 PCR analysis of TCRγ gene rearrangements in non-neoplastic lymphoid tissue.
a) V2-J1/2 (b) V3-J1/2 (c) V8-J1/2 rearrangements in three control cases tested in duplicate.
Lanes 1-2: control case RN1. Lanes 3-4: control case RN2.
Lanes 5-6: control case RN3. Left MW in nucleotides.
With V8-J1/2 a dominant + band (arrow) is present in case RN3. No reaction in lane 2 (b) probably due to technical error. Note reproducibility of the results in duplicate tests.

3.3). A dominant + reaction was found in only one case (Fig 3.8c). Duplicate tests demonstrated the reproducibility of the results (Fig 3.8).

Discussion

The quality of the DNA, efficacy of the particular primer combination, the batch of Taq polymerase, the specific activity of the 32P dCTP and duration of the exposure of the autoradiogram are factors which influence the results obtained by PCR. Consistent PCR non-reactivity of individual samples (as with case L10 Appendix 3.1) is probably due to the presence of inhibitors in the DNA. Technical errors in the performance of the test may account for the failure to obtain amplification in individual tests for example in Fig 3.8b. Failure of whole test batches was usually due to deterioration of reagents such as dNTPs, primers or Taq polymerase. However when the method was working the results were generally reproducible if tests were repeated (Fig 3.3a and b) or done in duplicate (Fig 3.8).

The molecular sizes of the individual dominant bands (or polyclonal smear) were within the expected range as deduced from the published sequences (Tables 2.3, 2.4). Cross contamination as evidenced by the presence of identical rearrangements in DNA from different patients during initial experiments (Figs 3.4 and 3.5) was not encountered again in the rest of the study, no two of the reported dominant bands being of the same electrophoretic mobility.

Dominant clones were assumed to be present in those cases in which there was a disproportionately dense band in at least one of the eight primer combination reactions analysed, with or without a background smear due to the presence of polyclonal reactive lymphocytes. The distinction between a borderline + reaction and some of the more prominent minor bands considered to be part of the polyclonal background is arbitrary and subjective. Discrete minor bands were more frequent in lymphomatous lesions than in non-neoplastic lymph nodes (compare Figs 3.1 - 3.3 with Figs 3.6 - 3.8) suggesting infiltration of the lymphoma by an oligoclonal rather than polyclonal population of reactive lymphocytes.

The results summarised in Table 3.2 demonstrate a dominant clonotypic band in one or more of the reactions in 68% of T-cell lymphomas and in 8% (one out of twelve) of non-neoplastic reactive lymph nodes. By

far the most likely explanation of the absence of rearrangements in 32% of lymphoma cases is the use of an incomplete set of primers. It is known that J1 or J2 are rearranged in the majority of T-cell clones (Lefranc et al. 1986, Chen et al. 1988). Tests were not performed with primers for JP, JP1 and JP2 because these are rearranged in only a small proportion of T-cells (Chen et al. 1988) and there was insufficient material to test 0.5μg of DNA with all 32 primer combinations. Experiment 18 in Chapter 2 has shown that further dilution of DNA decreases the density of the electrophoretic band given by dominant rearrangements. The occurrence of lymphoma cases where no clonal rearrangements were detected could also be due to diagnostic errors or the occurrence of malignant transformation in T-cells before rearrangement has taken place in the cells or the deletion of the rearranged locus in the malignant clone. The finding of concordance of PCR and Southern blot analysis in only 12 of 29 rearrangements studied by both methods (Table 3.4) is difficult to interpret. The 9 PCR negative Southern blot positive rearrangements are to be expected with the use of J1/2 primers only. The 8 PCR positive Southern blot negative rearrangements may reflect greater sensitivity of PCR when appropriate rearrangements are present.

The frequency of dominant clones cannot be analysed in the various histological categories of T-cell lymphoma in the present study due to the small number of cases in each category and lack of a standard classification of lymphoma subtypes of the specimens submitted from other laboratories.

The dominant band (+) found in one of the reactive lymph nodes may represent a restricted immune response to some particular antigen or it may be due to the presence of an otherwise latent benign neoplastic clone. The multiple minor bands demonstrated in reactive lymph nodes are the first indication of the relative amounts of different clones of lymphocytes in samples of non-malignant lymphoid tissue. The ability of the technique used to distinguish malignant from non-malignant T-cell proliferation in 68% of cases suggest that amplification of TCRγ gene rearrangements by PCR may be a suitable method for detecting benign T-cell neoplasia in lymphocytes infiltrating the lesions of autoimmune and other immunopathological disorders.

CHAPTER 4

Polymerase Chain Reaction Amplification of T-cell Receptor γ Gene Rearrangements in Normal Skin and Cutaneous T-cell Lymphoma

Introduction

Infiltration of the skin by lymphocytes is a prominent feature in many dermatological disease states ranging from malignant cutaneous lymphoma to common benign inflammatory skin diseases like psoriasis. The infiltrating cells are predominantly memory type (CD4+/CD45RA-) lymphocytes (Markey et al. 1990). In normal human skin lymphocytes are predominantly of T-cell type and are activated and evenly distributed over the CD4+ and CD8+ subsets(Bos et al. 1987). They have a characteristic anatomical distribution being mainly localised around the postcapillary venules. The distinct location of T-cells suggests that the skin represents a specific site for T-lymphocyte homing. Parallels of lymphocyte recruitment exist in other organs like Peyer's patches in the gut(Butcher, Scolley & Weissmann 1980). The suggested mechanism is that subpopulations of memory T-cells exhibit non-random recirculation patterns and that particular subsets home to specific tissues, a phenomenon mediated by interaction of tissue-specific vascular adhesion molecules and ligands expressed on specialised sets of lymphocytes (Picker et al. 1991).

The molecular basis for affinity of T-cells for skin remained obscure until it became clear that there is a subset of skin-seeking memory T-cells which are identified by a cutaneous lymphocyte-associated antigen recognised by the monoclonal antibody HECA-452(Picker et al. 1991, MacKay 1991). They constitute about 10% of T-cells in peripheral blood and 80% of those in normal and chronically inflamed skin(Picker et al. 1990). It is thought that these cells home to skin because they express a ligand which selectively adheres to the endothelial adhesion molecule ICAM-I. The ligand has not been identified but may be the cutaneous lymphocyte-associated antigen itself (Picker et al. 1991). Excessive accumulation of cutaneous lymphocytes in chronic inflammation is thought to occur due in part to increased expression of ELAM-I (which can be activated by macrophages, T-cells or bacterial products) and other

adhesion molecules and in part to proliferation of T-cells in response to local antigenic stimulation. These findings suggest the existence of corresponding subsets of skin-homing T-cell clones.

Apart from cutaneous T-cell lymphomas very little is known about individual T-cell clones in normal and diseased human skin.

Cutaneous T-cell lymphoma (CTCL) is a neoplasm of T-cells which initially presents with widespread cutaneous involvement and subsequently progresses to involve lymph nodes, peripheral blood and other viscera and frequently results in death. Mycosis fungoides first described in 1806 by Alibert to characterise a tumour resembling mushrooms. Sezary syndrome was originally described in 1938 by Sezary and Bouvrain as a triad of erythroderma, leukaemia composed of large mononuclear cells with convoluted nuclei and enlarged lymph nodes infiltrated by the same abnormal cells as were found in the blood. These two conditions were long considered to be two separate disease entities. It was not until 1975 that they were both recognised as part of the spectrum of CTCL (Edelson et al. 1975) characterised by infiltration of the epidermis (epidermotropism) and adjacent dermis by abnormal lymphocytes. The disease initially presents with skin lesions which tend to evolve from macules and flat patches to papules and slightly elevated plaques and then to nodules or tumours (large dome shaped lesions). Histologically the patch stage of the disease is characterised by increased numbers of mononuclear cells which may or may not be atypical in an otherwise normal skin. In the plaque stage many of the mononuclear cells are atypical and they often infiltrate the epidermis which may be normal, atrophic or psoriasiform. Often there is mixed inflammatory infiltrate composed of plasma cells and eosinophils interspersed among atypical mononuclear cells. As the disease progresses the mononuclear cells become more atypical and lose their affinity for epidermis as is reflected by non-epidermotropism in the tumour stage (Paterson & Edelson 1987).

It has been shown that the neoplastic cells found in the blood in Sezary syndrome proliferate in the presence of the T-cell mitogen phytohaemagglutinin (Crossen et al. 1971) and have the unique membrane property of thymus derived lymphocytes of forming rosettes with sheep erythrocytes (Broome et al. 1973, Brouets, Flandrin & Seligmann 1973) demonstrating that the condition is a neoplasm of T-lymphocytes and not of

monocytes as initially thought. Immunohistochemical studies have also shown that the neoplastic cells of CTCL are helper T-cells since they react with monoclonal antibodies specifically reactive with differentiation antigens CD3 and CD4 found on helper T-cells(Kung et al. 1981). Cells of mycosis fungoides (patch/plaque stage) have been shown to react with the monoclonal antibody HECA-452. In the tumour stage of the disease associated with loss of epidermotropism and widespread dissemination, neoplastic cells lack HECA-452 expression (Picker et al. 1990). These findings provide evidence that CTCL is derived from normal cutaneous T-cells. The suggested mechanism is neoplastic amplification of skin-seeking T-cells targeting the skin and affecting other areas only on the formation of non-skin associated sub-clones(Kung et al. 1981, Picker et al. 1991).

There is substantial evidence that CTCL is a monoclonal process. Edelson et al. (1979) found karyotypic abnormalities in patients with CTCL. Though karyotypes were different for each individual, monoclonality was demonstrated (homogeneous population of abnormal T-cells) in all three patients studied. Whang-Peng et al. (1982) studied 41 patients with CTCL, four with limited plaques, thirteen with generalised plaques, eight with cutaneous tumours and sixteen with generalised erythroderma. The cytogenetic findings were parallel to clinical findings, the patients with limited plaques showing few if any abnormalities, the patients with generalised plaques and tumours showing frequent abnormalities and patients with erythroderma demonstrating the most extreme chromosomal abnormalities. The existence of three or more cells with the same chromosomal abnormality was considered evidence of clone formation and was demonstrated in only eight patients, all in the terminal phase of the disease.

Phenotypic studies of the malignant cells in CTCL by monoclonal antibodies are consistent with the findings that disease arises from clonal expansion of a single T-cell. Kung et al. (1981) demonstrated that neoplastic T-cells from lymph node suspensions and peripheral blood of patient with CTCL are predominantly CD4 and not a mixture of CD4 and CD8 T-cells as found in normal tissues. These findings were also demonstrated in skin infiltrates.

Using probes for TCR genes clonal rearrangements have been documented in skin, lymph nodes and peripheral blood from patients with

mycosis fungoides and Sezary syndrome. Weiss et al. (1985) studied clonal β gene rearrangements in tissue samples of CTCL, (blood, lymph nodes and skin lesions). The presence of clonal TCR rearrangements correlated with the histological evidence of disease, patients with mycosis fungoides presenting as skin lesions and with lymph node involvement showed clonal TCR rearrangements in tissue obtained from the lesion and the lymph node but not peripheral blood. Furthermore 25-90% of lymph nodes not clearly involved histologically contained clonal β chain rearrangements identical to those detected in skin lesions from the same patient. Rafalkier et al. (1987) found clonal TCR β rearrangements in five cases of advanced mycosis fungoides lesions and in four examples of Sezary syndrome. In 12 cases of early CTCL (early plaque lesions) no rearrangements were detected. In a separate study performed by Weiss et al. (1989) on peripheral blood lymphocytes from 26 patients with mycosis fungoides and Sezary syndrome TCR rearrangements were detected in seven, three of whom had no morphologically detectable atypical lymphocytes in their blood. Dosaka et al. (1989) studied 22 plaques from seven patients and found a dominant clone in two cases. In one case the dominant clone was the same in two lesions. In a second case the same dominant clone was detected after a year in two other lesions.

Although these (and similar) studies have confirmed the monoclonal nature of malignant T-cells in mycosis fungoides and Sezary syndrome, evidence of a large (neoplastic) T-cell clone has seldom been found in the plaque stage of mycosis fungoides, which may persist for many years before progressing to the tumour stage of the disease.

In the present chapter PCR studies are performed on peripheral blood, punch biopsies from two areas of clinically unaffected skin and two separate lesions from three patients with plaque stage mycosis fungoides who have never received chemotherapy. PCR results are compared in skin and blood from the same patient to determine whether the method can detect dominant clonal rearrangements selectively localised to the lesions before an obvious tumour mass develops. In one case of Sezary syndrome PCR results are compared in skin, blood and T-cell lines derived from the patient's skin and blood.

Skin biopsies and venous blood from five healthy individuals are studied as controls.

Cases Studied

Three patients with histologically confirmed plaque stage mycosis fungoides each agreed to provide a sample of venous blood and two biopsies from separate lesions and two areas of clinically unaffected skin. All three patients had been treated with topical steroids but none had received PUVA therapy or other forms of chemotherapy. Details are summarised in Tables 4.1 and 4.2.

Case CL1

A female aged 70 years developed erythematous plaques on trunk and limbs at the age of 47 years and was diagnosed clinically and histologically as parapsoriasis. Thirteen years later mycosis fungoides was diagnosed (Fig 4.1) There was no lymph node enlargement. Peripheral blood was normal.

Case CL2

A female aged 80 years presented three years previously with indurated lichenified plaques affecting the back and right axilla (Fig. 4.2). There was no lymph node enlargement. Peripheral blood was normal.

Case CL3

A male aged 40 years had a generalised psoriatic rash for many years. Mycosis fungoides had been diagnosed histologically a few months previously when he developed tumid lesions on the back.

20 mls of venous blood and 6mm punch biopsies from anaesthetised skin were obtained from each of the three patients. Sites from which biopsies were taken in each case are given in Table 4.2.

Case CL4

At the Department of Veterinary Pathology, University of Glasgow, Dr. Ruth Jarrett has established T-cell lines from skin and peripheral blood of a patient with an aggressive form of Sezary syndrome including exfoliative erythroderma, generalised lymphadenopathy and circulating neoplastic cells characterised by atypical cerebriform nuclei. In immunocytochemical studies the cells reacted with CD2 (pan T-cell) CD4

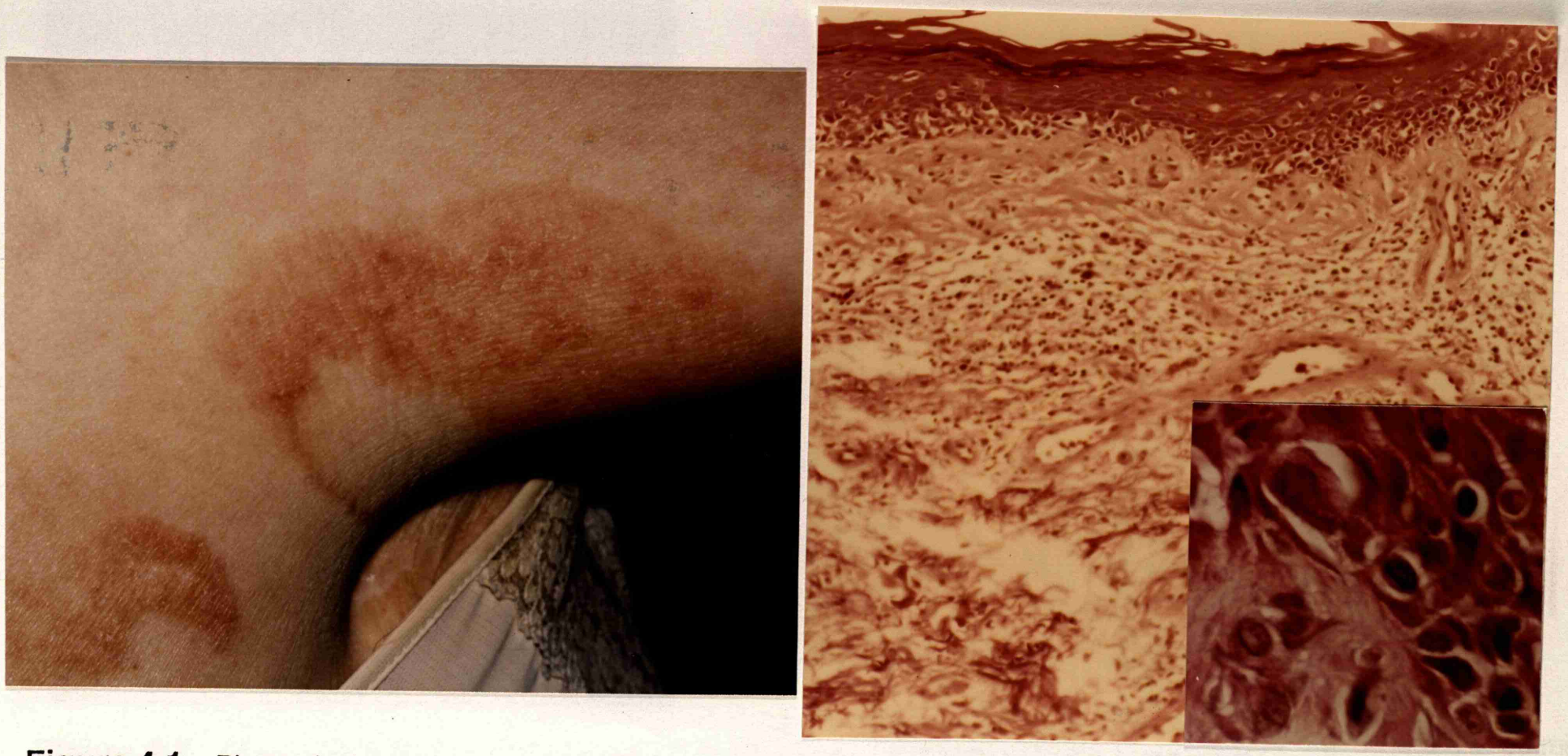


Figure 4.1 Plaque lesions of mycosis fungoides in case CL1. Lymphoid infiltrate (CD3+, CD4+, HECA-452+) in dermis and epidermis. Note the presence of epidermotropism.

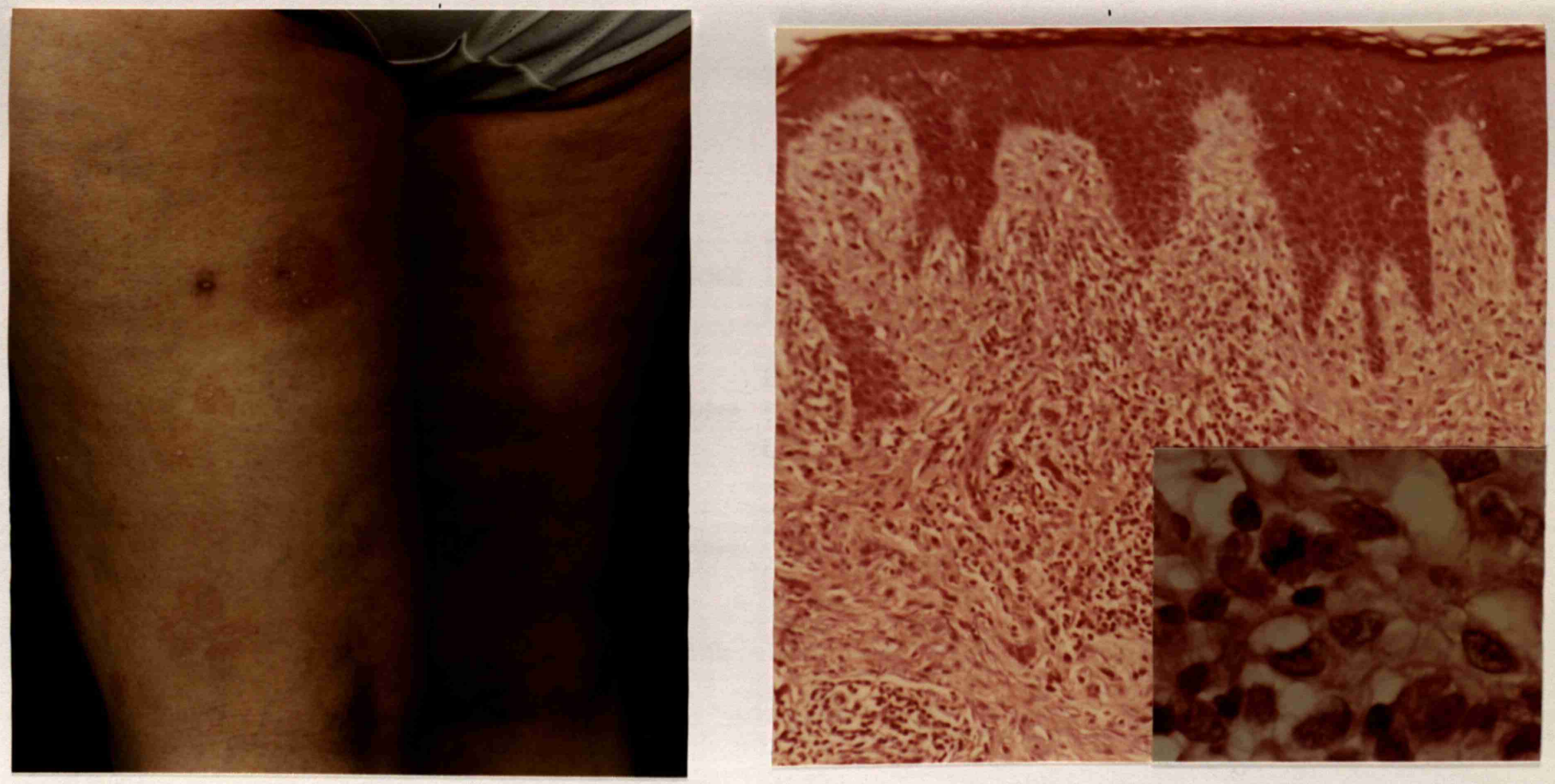


Figure 4.2 Plaque lesions of mycosis fungoides in case CL2. Lymphoid infiltrate (CD3+, CD4+, HECA- 452-) in dermis. Note the absence of epidermotropism.

TABLE 4.1 Dominant TCRγ gene rearrangements in CTCL and normal healthy controls

Case No.	Age/Sex	Diagnosis	Sample	Dominant rearrangement
CL1	70/F	Plaque stage mycosis fungoides	Blood	-
			Lesion	V2+⊗ V10 ++⊗
			Unaffected skin	-
CL2	80/F	Plaque stage mycosis fungoides	Blood	V9 ++
			Lesion	V3 ++⊗ V4 ++⊗
			Unaffected skin	V3 ++⊗ V4 +⊗ V9 ++
CL3	40/M	Plaque stage mycosis fungoides	Blood	-
			Lesion	-
			Unaffected skin	-
CL4	60/F	Sezary syndrome	Blood	V2 ++ V2 ++
			Lesion	V2 ++ V2 ++
			Cell line (blood)	V2 ++ V2 ++
			Cell line (skin)	V2 ++ V2 ++

Table 4.1 (continued)

Control Case	Age/Sex	Diagnosis	Sample	Dominant rearrangement
NC1	60/M	--	Blood	V2 ++
			Skin	-
NC2	55/M	--	Blood	-
			Skin	-
NC3	16 M	--	Blood	-
			Skin	-
NC4	30 F	--	Blood	-
			Skin	-
			Gingiva	-
NC5	45 M	--	Blood	V2 +
			Skin	-
			Gingiva	-

⊗ = Detected in both biopsies
\- = No dominant band
\+ = borderline dominant band
++ = unequivocally dominant band

TABLE 4.2 Sites of skin biopsies taken in CTCL cases

Case	Site of biopsy	DNA No.
CL1	Right leg normal	293
	Left leg normal	294
	Right leg lesion	295
	Left leg lesion	296
CL2	Right medial thigh normal	248
	Left medial thigh normal	249
	Right medial thigh lesion	250
	Left medial thigh normal	251
CL3	Right upper back normal	324
	Left upper back normal	325
	Right upper back lesion	326
	Left upper back lesion	327

(helper T-cell) but lacked expression of CD3 (pan T-cell surface). The cells were positive with lymphocyte acid phosphatase, lymphocyte beta glucuronidase and with lymphocyte alpha-napthylbutyrate esterase. Southern blot analysis showed that the TCR β gene was in germline configuration while TCRγ V2 gene was shown to be rearranged when probed with pH60 for the Jγ1 segment. It was desirable to establish if the same rearrangements of the TCRγ gene were present in the cell lines and in the blood and skin. Because of the small amount (<10μg) of fragmented DNA available from the skin biopsy it was not possible to carry out the investigation by Southern blotting. PCR studies were therefore carried out on DNA from peripheral blood mononuclear cells, from the skin biopsy and from both cell lines.

Healthy controls

Four healthy adults (3 males, 1 female) volunteered to supply skin biopsies for this study. Samples of venous blood and 6mm punch biopsies of anaesthetised skin were obtained from all four. Two of the subjects each supplied two $4mm^2$ biopsies of normal gingival mucosa. Paired earlobe skin from otoplasty performed on a healthy male for cosmetic reason was obtained in another case.

Age and sex of the control subjects are given in Table 4.1 and sites from which biopsies were taken are given in Table 4.3.

Methods

DNA extraction

From Skin biopsies and gingival mucosa

After punch biopsy of the skin and gingival mucosa the tissue was rapidly frozen in liquid nitrogen, wrapped in foil and stored at -70°C until used for DNA extraction. DNA was extracted from 5 micron cryostat sections of the whole biopsy specimens using a modification of the standard method for small amounts of tissue as given in Appendix 2.7.

From peripheral blood

PBMC were separated and DNA extracted and purified according to the standard protocol given in Appendix 2.2.

TABLE 4.3 Sites of biopsies taken in normal healthy controls

Case	Site of biopsy	DNA No.
NC1	Right forearm (Posterior)	240
	Left forearm (Posterior)	241
	Right thorax	242
	Left thorax	243
NC2	Right forearm	244
	Left forearm	245
	Right elbow	246
	Left elbow	247
NC3	Right ear	216A
	Left ear	216B
NC4	Right arm	230
	Right gingiva (midpoint upper right molar)	231
	Left gingiva (midpoint upper left molar)	232
NC5	Right arm	235
	Right gingiva (midpoint upper right molar)	237
	Left gingiva (midpoint upper left molar)	238

In Case CL4 DNA extracted from PBMC, both cell lines and skin was supplied by Dr. Ruth Jarrett.

PCR amplification

PCR amplification on 5µl aliquots of DNA was performed for 35 cycles using the modified radiolabelled PCR protocol as given in Appendix 2.3 with the primers specific for 8 Vγ genes and Jγ1/2 given in Tables 2.3 and 2.4. PCR products were analysed on 6% polyacrylamide gel run under denaturing conditions and autoradiographs prepared as shown in Appendix 2.6.

In two experiments on case CL1 electrophoresis was repeated on PCR products which had been diluted by further addition of formamide dye mix (Appendix 2.9) in order to improve their resolution.

Results

Detailed results of tests obtained with 8 Vγ and the Jγ1/2 primer combinations in four cases of CTCL are given in Appendix 4.1. The results are summarised in Tables 4.1 and 4.4.

Case CL1

The results are illustrated in Fig 4.3. With Vγ2 a dominant + band was present in both lesions and a corresponding weak band was present in the one sample of clinically unaffected skin which produced a visible reaction (Fig 4.3(a)). With Vγ10 a dominant ++ band was present in both lesions and corresponding weak bands were present in both samples of clinically unaffected skin but not in peripheral blood (Fig 4.3(d)). The same results were obtained in two separate tests. V5-J1/2 gave no results when tested and V11-J1/2 was not tested as no DNA was left.

The remaining 21 satisfactory tests with other Vγ and Jγ1/2 primer combinations gave multiple minor bands and/or polyclonal smears (Figs 4.3(b) and (c).

Case CL2

The results are illustrated in Figs 4.4 - 4.8. With Vγ3 a dominant ++ band was present in each of the skin samples but not in peripheral blood

TABLE 4.4 Number of cases, samples and successful PCRs in patients with CTCL and normal healthy controls

	No. of Cases	No. of samples (PCR tests)			
		Blood	Skin lesion	Clinically unaffected skin	Gingiva mucosa
CTCL	4	4 (27)	7 (33)	6 (37)	---
Normal control	5	5 (30)	---	12 (67)	4 (23)

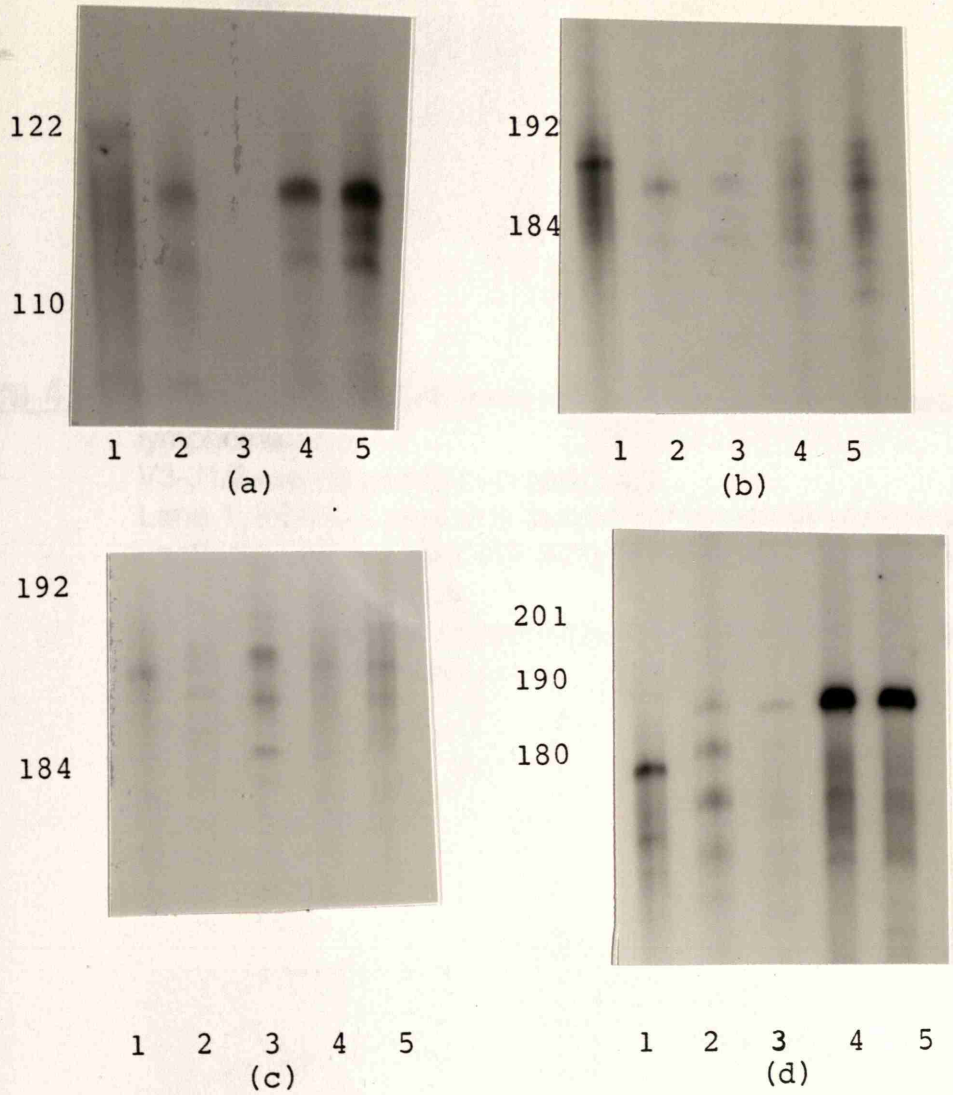


Figure 4.3 PCR analysis of TCRγ gene rearrangements in cutaneous T-cell lymphoma.
a) V2-J1/2 (b) V3-J1/2 (c) V4-J1/2 (d) V10-J1/2 rearrangements in case CL1.
Lane 1: PBMC. Lanes 2-3: samples of two areas of clinically unaffected skin. Lanes 4-5: samples of two plaques lesions. Left MW in nucleotides.
With V2-J1/2 a dominant + band is present in both plaque lesions and a corresponding weak band is present in the one sample of clinically unaffected skin in which a reaction is obtained. With V10-J1/2 a dominant ++ band is present in two plaque lesions and corresponding minor bands in both samples of clinically unaffected skin.

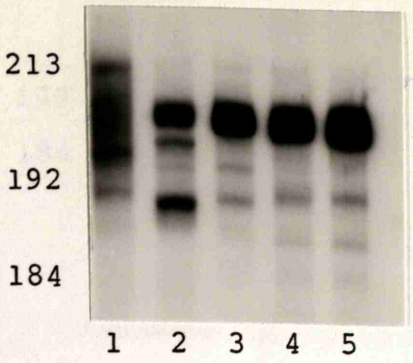


Figure 4.4 PCR analysis of TCRγ gene rearrangements in cutaneous T-cell lymphoma.
V3-J1/2 rearrangements in case CL2.
Lane 1: PBMC. Lanes 2-3: samples of two areas of clinically unaffected skin. Lanes 4-5: samples of two plaque lesions.
Left MW in nucleotides.
A dominant ++ band is present in each of the skin samples but not in peripheral blood.

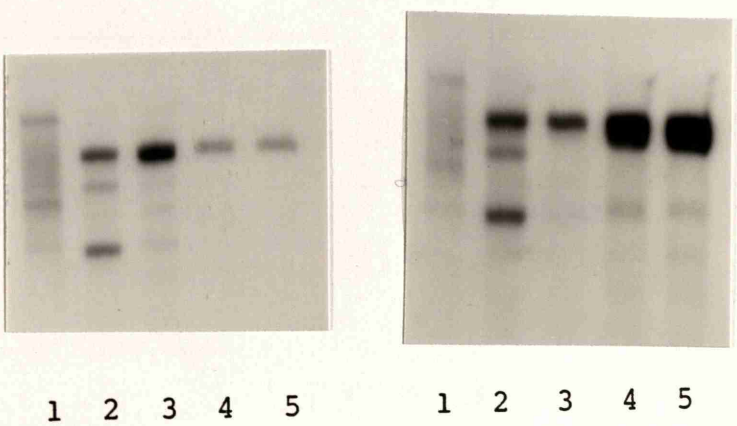


Figure 4.5 Dilution experiment on V3-J1/2 PCR products shown in Fig 4.4. (Left) PCR products in lanes 1-3 diluted 1/5 and in lanes 4-5 diluted 1/50 showing that dominant rearrangements in all skin samples is of the same molecular size. (Right) PCR products in lanes 1-3 diluted 1/2 and in lanes 4-5 diluted 1/10. Much more of the dominant rearrangement is present in the lesions than in clinically unaffected skin.

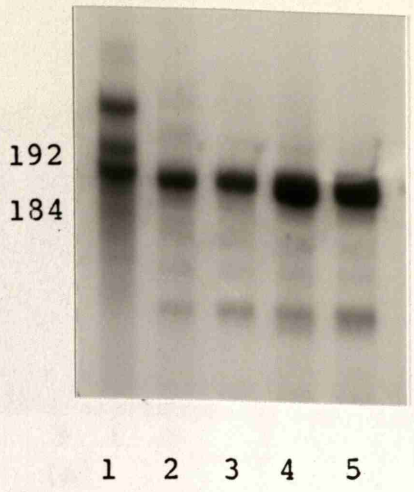


Figure 4.6 PCR analysis of TCRγ gene rearrangements in cutaneous T-cell lymphoma.
V4-J1/2 rearrangements in case CL2.
Lane 1: PBMC. Lanes 2-3: samples of two areas of clinically unaffected skin. Lanes 4-5: samples of two plaque lesions.
Left MW in nucleotides.
A dominant ++ band is present in both plaque lesions and a corresponding dominant + band is present in both samples of clinically unaffected skin.

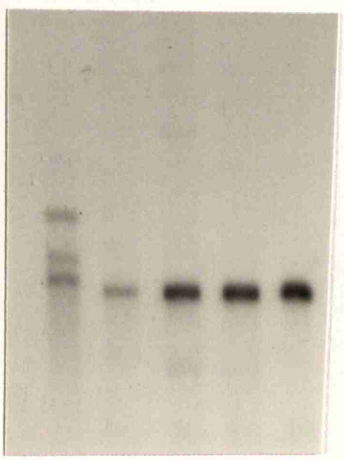


Figure 4.7 Dilution experiment on V4-J1/2 PCR products shown in Fig 4.6. PCR products in lanes 1-3 diluted 1/5 and in lanes 4-5 diluted 1/50. The dominant rearrangements in the lesions is present in clinically unaffected skin as well, but not in peripheral blood.

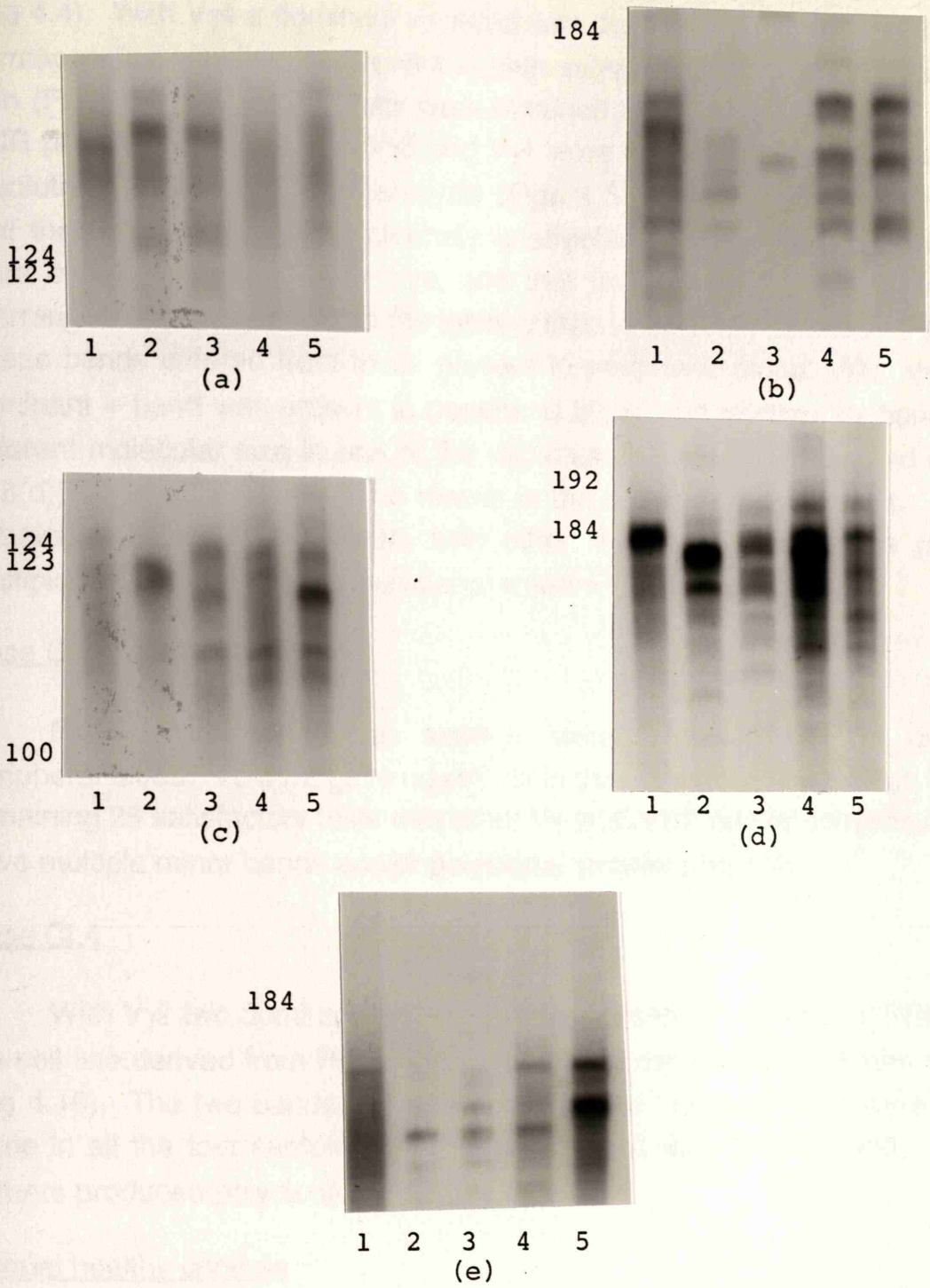


Figure 4.8 PCR analysis of TCRγ gene rearrangements in cutaneous T-cell lymphoma.
a)V2-J1/2 (b) ΨV5-J1/2 (c) V8-J1/2 (d) V9-J1/2 (e) V10-J1/2 rearrangements in case CL2.
Lane 1: PBMC. Lanes 2-3: samples of two areas of clinically unaffected skin. Lanes 4-5: samples of two plaque lesions. Left MW in nucleotides. With V9-J1/2 a dominant ++ band is present in peripheral blood and a second ++ band of smaller molecular size is present in one sample of clinically unaffected skin.

(Fig 4.4). With Vγ4 a dominant ++ band was present in both lesions and a corresponding + band was present in both samples of clinically unaffected skin (Fig 4.6). The same results were obtained in two separate tests. The PCR products obtained with Vγ3 and Vγ4 were diluted in order to improve resolution on electrophoretic analysis (Figs 4.5 and 4.7). This confirmed that the dominant bands in clinically unaffected skin and plaque lesions were of the same molecular size, and that much more of the dominant rearrangement was present in the lesions than in clinically unaffected skin. These bands differed from those present in peripheral blood. With Vγ9 a dominant + band was present in peripheral blood and another ++ band of different molecular size in one of the samples of clinically unaffected skin (4.8(d)). V11-J1/2 produced no results in the single test carried out. The remaining 25 satisfactory tests with other Vγ and Jγ1/2 primers gave multiple minor bands and/or polyclonal smears (Fig 4.8).

Case CL3

No dominant band was seen in lesions, unaffected skin or in peripheral blood. V5-J1/2 gave no results in the single test carried out. The remaining 25 satisfactory tests with other Vγ and Jγ1/2 primer combinations gave multiple minor bands and/or polyclonal smears (Fig 4.9).

Case CL4

With Vγ2 two dominant ++ bands were present in samples of PBMC, the cell line derived from PBMC, skin lesion and cell line derived from skin (Fig 4.10). The two bands of 130 nucleotide and 138 nucleotide were the same in all the four samples. PBMC DNA tested with other Vγ and Jγ1/2 primers produced polyclonal smears.

Normal healthy controls

Detailed results of tests obtained with 8 Vγ and Jγ1/2 primer combinations in five normal controls are given in Appendix 4.2. The results are summarised in Tables 4.1 and 4.4. Illustrative examples are given in Figs 4.11-4.15).

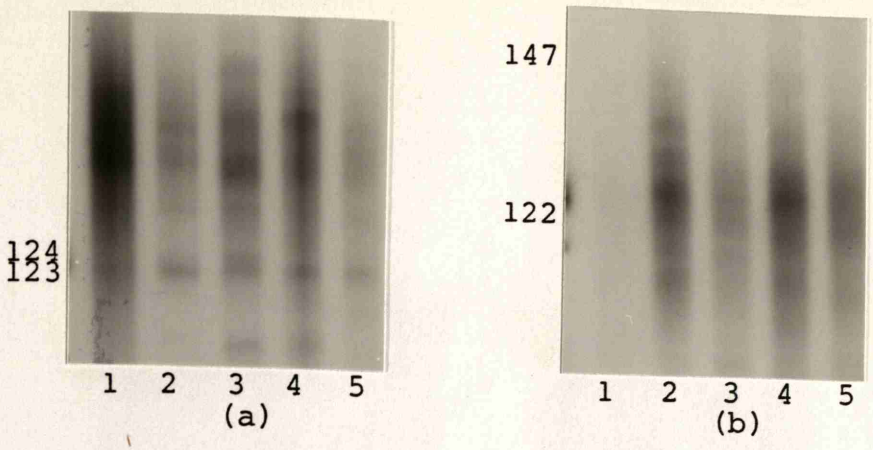


Figure 4.9 PCR analysis of TCRγ gene rearrangements in cutaneous T-cell lymphoma.
a) V2-J1/2 (b) V8-J1/2 rearrangements in case CL3.
Lane 1: PBMC. Lanes 2-3: samples of two areas of clinically unaffected skin. Lanes 4-5: samples of two plaque lesions. Left MW in nucleotides.
No dominant rearrangements are present.

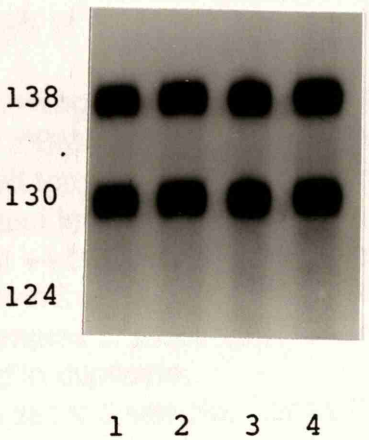


Figure 4.10 PCR analysis of TCRγ gene rearrangements in cutaneous T-cell lymphoma.
V2-J1/2 rearrangements in a case of Sezary syndrome (case CL4).
Lane 1: PBMC. Lane 2: cell line derived from peripheral blood. Lane 3: cell line derived from skin. Lane 4: skin lesion. Left MW in nucleotides. Two dominant ++ bands (representing each allele of the Vγ2 gene) are similar in all four samples.

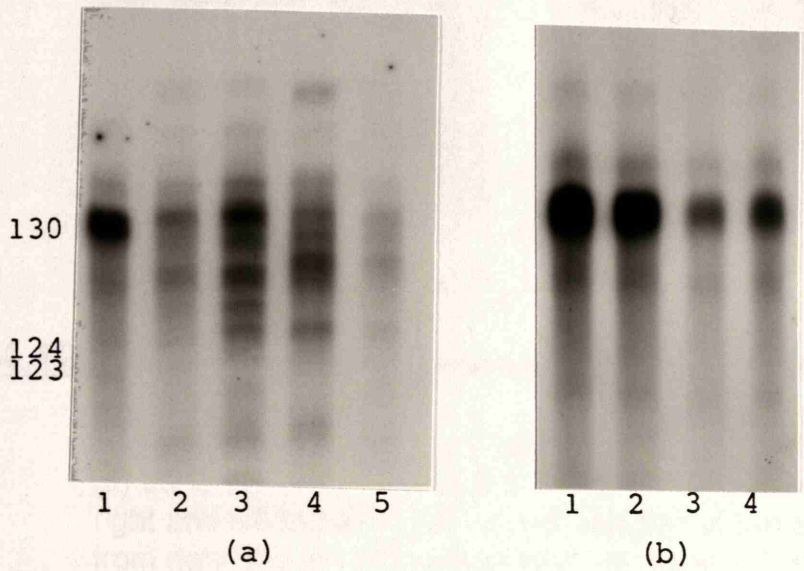


Figure 4.11 PCR analysis of TCRγ gene rearrangements in normal healthy control.
V2-J1/2 rearrangements in control NC1.
(a) Lane 1: PBMC. Lanes 2-3: samples of two areas of skin from right and left forearm. Lanes 4-5: samples of two areas of skin from right and left thorax. Left MW in nucleotides.
A dominant ++ band is present in blood. In this preparation band patterns from blood and skin are generally similar.
(b) Two samples of blood taken at one year interval from control NC1 tested in duplicate.
Lanes 1-2: second sample. Lanes 3-4: first sample. Dominant ++ band is still present in second sample of blood taken a year later.

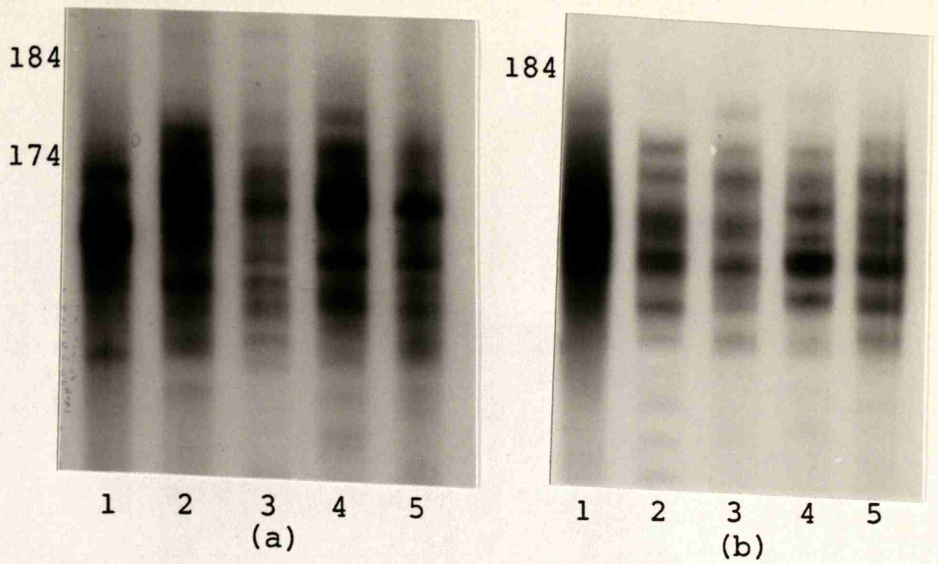


Figure 4.12 PCR analysis of TCRγ gene rearrangements in normal healthy controls.
V10-J1/2 rearrangements in controls NC1 and NC2.
(a) Lane 1: PBMC. Lanes 2-3: samples of two areas of skin from right and left forearm. Lanes 4-5: samples of two areas of skin from right and left thorax from NC1. (b) Lane 1: PBMC. Lanes 2-3: samples of two areas of skin from right and left forearm. Lanes 4-5: samples of two areas of skin from right and left elbow from NC2. Left MW in nucleotides.
No dominant band is present. Band patterns tend to be similar in samples of skin but differ from those in blood in each subject. Strikingly similar band pattern is present between two samples of DNA from forearms and between the two samples from elbows in control NC2.

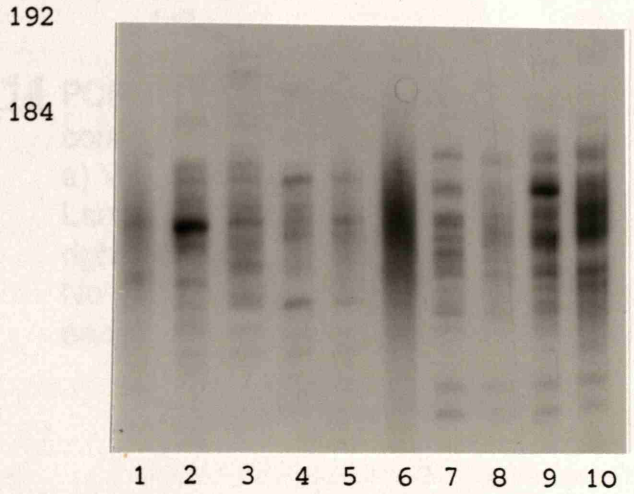


Figure 4.13 PCR analysis of TCRγ gene rearrangements in normal healthy controls.
V9-J1/2 rearrangements in controls NC1 and NC2.
Lane 1: PBMC. Lanes 2-3: samples of two areas of skin from right and left forearm. Lanes 4-5: samples of two areas of skin from right and left thorax in NC1. Lane 6: PBMC. Lanes 7-8: samples of two areas of skin from right and left forearm. Lanes 9-10: samples of two areas of skin from right and left elbows in NC2. Left MW in nucleotides.
No dominant rearrangements are present. In each subject band patterns in skin differs from those in blood. Possible differences are seen when band patterns from different anatomical skin sites are compared in the same subject. Band patterns in blood and skin vary from subject to subject.

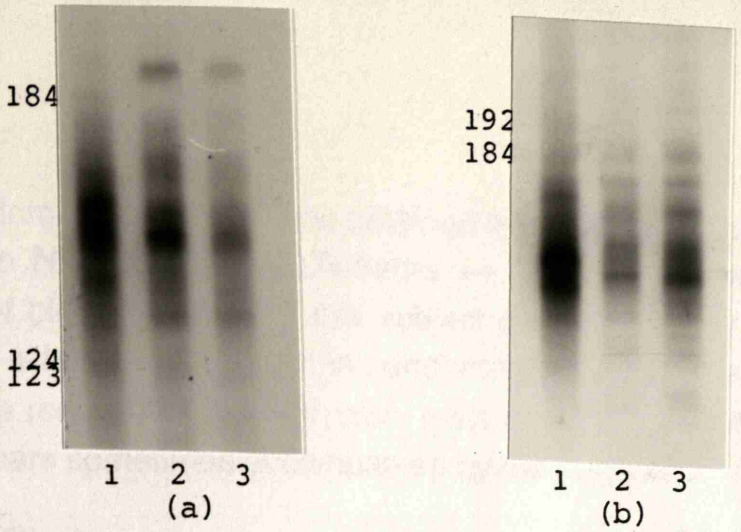


Figure 4.14 PCR analysis of TCRγ gene rearrangements in normal healthy control.
a) V2-J1/2 (b) V9-J1/2 rearrangements in control NC3.
Lane 1: PBMC. Lanes 2-3: samples of two areas of skin from right and left ear.
No dominant band is present. The band patterns in skin from each ear are markedly similar but differ from those in the blood.

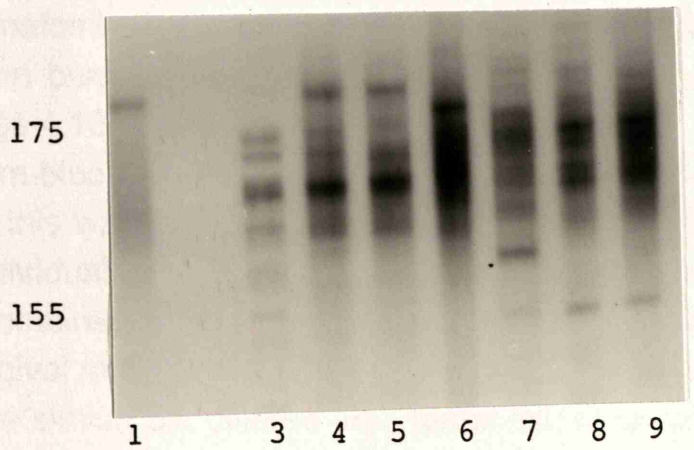


Figure 4.15 PCR analysis of TCRγ gene rearrangements in normal healthy control.
V10-J1/2 rearrangements in controls NC4 and NC5.
Lane 1: PBMC. Lane 3: sample of skin from right arm. Lanes 4-5: samples of right and left gingival mucosa from NC4. Lane 6: PBMC. Lane 7: sample of skin from right arm. Lanes 8-9: samples of right and left gingival mucosa from NC5. Left MW in nucleotides. The band patterns differ in blood, skin and gingival mucosa, but are similar in gingival mucosa from corresponding sites on each side of mouth in each control. Band pattern in blood, skin and gingival mucosae differ from subject to subject.

Blood

A dominant ++ band was obtained in peripheral blood with the primer for Vγ2 in NC 1 (Fig 4.11). The same ++ band was present in a second sample of blood taken from this subject one year later (Fig 4.11(b)). A dominant + band was present in peripheral blood with the primer for Vγ2 in NC 5. The remaining 25 satisfactory tests on 5 samples of peripheral blood gave smears sometimes accompanied by minor bands.

Skin and Gingival mucosa

No dominant bands were found in 65 satisfactory tests performed on 12 skin biopsies from 5 normal controls. No dominant bands were found in 23 satisfactory tests on four biopsies of gingival mucosa from two normal controls. With each primer combination multiple discrete bands were seen extending over 30-40 nucleotides in all DNA samples from each case. The molecular sizes and relative intensities of the bands in multiple skin biopsies from the same individuals were often similar (Figs 4.11, 4.12, 4.13 and 4.14). Band patterns were similar in two samples of DNA taken from symmetrical anatomical skin sites (Fig 4.12). Possible differences were also seen when band patterns from different anatomical skin sites were compared (Figs 4.12 and 4.13).

DNA from blood and skin usually gave different band patterns (Figs 4.12-4.14) but this was not always so (Fig 4.11(a)). When DNA from skin of different individuals was tested with the same primer combination the band pattern obtained differed from subject to subject (Fig 4.13). Band patterns in gingival mucosa taken from corresponding sites on each side of the mouth were similar but differed from peripheral blood and/or skin. The gingival mucosa tended to have additional bands to skin (Fig 4.15). When tested with the same primer combination band patterns in peripheral blood, skin and buccal mucosa were different from subject to subject (Fig 4.15).

Discussion

This is the first reported study of TCR gene rearrangements in normal human skin (Goudie et al. 1991). On PCR amplification most DNA samples produce multiple minor bands of appropriate molecular size for the Vγ and Jγ primer combinations used (Figs 4.11-4.15). F.R. Imrie (1991) has provided convincing evidence that these bands are γ gene

rearrangement products. DNA eluted from individual minor bands which had been carefully excised from the gel was reamplified by PCR, then sequenced with the appropriate Vγ gene primer. In 3 of 6 minor bands examined from normal skin a clear unambiguous sequence ladder was obtained across the relevant V-J junction, indicating the presence of a single PCR amplified γ gene rearrangement (Fig 4.16 left). In the 3 other bands investigated unambiguous sequences were obtained for the V gene but over the N region and J gene no clear ladder was visible (Fig 4.16 right) a pattern consistent with a mixed group of γ gene rearrangements each with the same V and J gene usage but with different rearrangement breakpoints and N region insertions. It is therefore likely that the multiple discrete bands obtained in normal skin by PCR represent different γ gene rearrangements and the same is probably true in abnormal skin, in gingival mucosa, in blood and other tissues.

Comparison of band patterns in normal blood, skin and gingival mucosa from the same control subject (Fig 4.15) reveals prominent differences in the lymphocyte populations in these tissues. The occurrence of less smearing and fewer bands than in the other tissues examined suggests less clonal heterogeneity in normal skin. This may be due to the relatively small total number of lymphocytes in normal skin compared with the other tissues and the fact that most cutaneous lymphocytes belong to the HECA-452+ skin-homing T-cell subset which account for only about 10 per cent of the T-cells in peripheral blood. Band patterns in normal skin vary considerably from individual to individual but are often remarkably similar in multiple skin biopsies from the same subject (Figs 4.12-4.14) further evidence of selective homing to the skin of a small subset of T-cell clones. These findings emphasise the importance of choosing appropriate normal controls for the study of T-cell clones in pathological lesions. Ideally, uninvolved parts of the diseased tissue from the same individual should be selected as has been done in this chapter.

Two dominant bands were found in CTCL case CL1 (Fig 4.3). Both were present in much larger amounts in the two plaques studied than in the control samples of clinically unaffected skin from the same patient and presumably reflect the presence of two γ gene rearrangements in the malignant T-cell clone. Similarly the dominant Vγ3 and Vγ4 rearrangements found in much larger amounts in the lesions than in the clinically unaffected skin in CTCL case CL2 are probably markers of the malignant clone (Figs

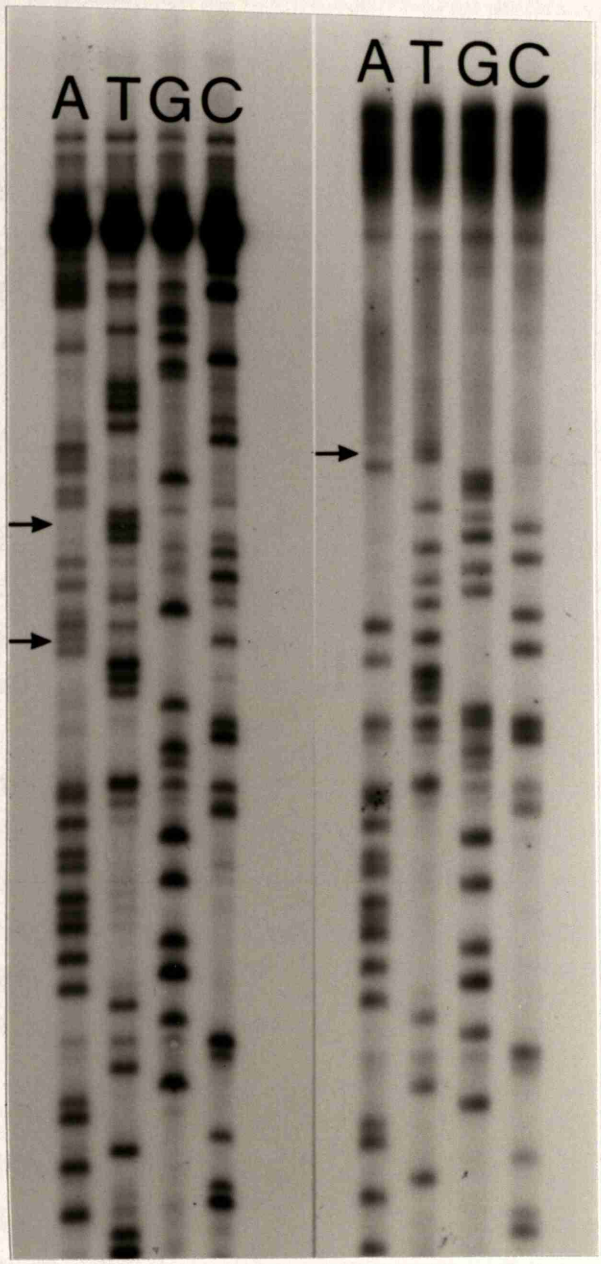


Figure 4.16 Nucleotide sequences of minor bands excised from gel following electrophoresis of PCR-amplified TCR V10-J1/2 rearrangements in DNA from normal human skin. (Left) An unambiguous (monoclonal) nucleotide ladder in uncoded N region between arrows (V sequence below, J sequence above). (Right) Ambiguous (polyclonal) nucleotide sequence above arrow due to N region differences and J gene sequences which are out of phase.
Photographs by permission of F.R. Imrie (1992).

4.4-4.7). The dominant Vγ9 band in blood and in one sample of clinically unaffected skin from case CL2 (Fig 4.8) are not associated with the skin lesions. They may indicate the existence of additional neoplastic clones which as yet are subclinical or unusually large clones of reactive T-or B-cells. The same explanations may apply to the dominant bands found in peripheral blood of control cases NC1 (Figs 4.11) and NC5.

The presence of dominant TCRγ gene rearrangements in mycosis fungoides plaques indicate that these lesions are almost certainly neoplastic especially in view of the fact that they generally precede the development of frankly malignant CTCL in the later stages of the disease. Previous attempts to demonstrate a large monoclonal T-cell population in plaques by karyotypic analysis and Southern blotting of TCR gene rearrangements have presumably been unsuccessful due to inadequate sensitivity of these methods, the relatively small number of lymphocytes available for testing and the presence of multiple clones of non-neoplastic T-cells in the lesions. In the sample from case CL3, despite histological evidence of mycosis fungoides, no dominant clone was found with primers for the more common rearrangements (Fig 4.9). Multiple minor electrophoretic bands were obtained like those in normal skin. Since J1/2 was the only Jγ primer tested a possible explanation is that the malignant clone has rearranged Jγ gene(s) other than J1/2. Other explanations may be that no neoplastic clone is present or only a minority of the cells are neoplastic or there was an error in diagnosis which is sometime difficult even on histological examination of biopsied lesions.

Biopsies of clinically unaffected skin and plaque lesions all showed the presence of the same dominant rearrangements in case CL1. This was also so in case CL2. The detection of rearranged bands of identical molecular size in all four skin samples in such patients strongly suggests that the clone of neoplastic T-cells seen histologically in the lesions was also present in the clinically unaffected skin. This points to widespread subclinical involvement of skin in plaque stage mycosis fungoides. This has long been suspected since patients receiving PUVA therapy who initially have an excellent response sometimes develop new lesions at "sanctuary sites" which do not receive an adequate dose of radiation. These include upper eyelids, between the fingers, under the breasts, natal cleft and groin (du Vivier & Vollum 1980). The finding is also in keeping with the existence of a particular subset of memory T-cells which

recirculate and selectively home to skin. CTCL is a tumour derived from these cells (Picker et al. 1990). Interestingly no evidence of the abnormal clone was found in the blood of cases CL1 or CL2.

In case CL4 with Sezary syndrome, Southern blot analysis had shown the TCR Vγ2 gene to be rearranged in DNA from peripheral blood and cell lines derived from the patient's blood and skin. PCR analysis showed identical Vγ2 gene rearrangements in all four samples including the skin biopsy where Southern blot analysis was unsuccessful presumably because of the small amount and fragmented condition of the DNA. The resolving power of PCR/high resolution gel electrophoresis also allowed it to be shown that both alleles of Vγ2 gene were rearranged. It also confirmed that the cultured cell line from skin is derived from the dominant (neoplastic) T-cell clone and not from other cells which infiltrate the lesions and often grow in culture better than those of the neoplastic cells in cases of CTCL(Ho et al. 1990).

This study of a small series of cases CTCL illustrates well the sensitivity and discriminating power of PCR amplification of TCRγ gene rearrangements in the detection of dominant T-cell clones in small tissue samples, and the value of using unaffected autologous tissue of the same type in control studies.

CHAPTER 5

T-cell Receptor γ Gene Rearrangements in Vitiligo and Psoriasis

Introduction

The mechanisms responsible for the initiation and persistent nature of the spontaneously occurring autoimmune diseases and other poorly understood immunological disorders and for the patchy and symmetrical distribution of some of those affecting the skin are important unsolved problems in human pathology.

T-lymphocytes are now known to have a key role in inducing and sustaining immune responses. It has been suggested (as described in Chapter 1) that the autoimmune and certain other obscure chronic inflammatory diseases such as psoriasis may result from the unregulated and inappropriate functional activity of benign T-cell neoplasms. On the assumption that benign T-cell neoplasms are likely to be monoclonal like the T-cell lymphomas and leukaemias and benign tumours of the other tissues, I have looked for evidence of T-cell monotypia in psoriasis and the organ-specific autoimmune disease vitiligo, two common skin disorders whose lesions are infiltrated with T-lymphocytes. Vitiligo and psoriasis present particularly suitable models for the study of naturally occurring T-cell associated diseases since the lesions are easily seen and recorded and can readily be biopsied under direct vision. I have also attempted to determine whether any clonotypes selectively localise to the areas of affected skin in either of these conditions, in view of their unexplained tendency to cause patchy rather than generalised cutaneous involvement.

Vitiligo

Vitiligo is a common and distressing skin disease characterised by the loss of melanocytes and the development of areas of cutaneous depigmentation. Its incidence has been variously estimated but is probably about 1 per cent (El Mofty 1968). It affects all races and the incidence is

thought to be higher in the more deeply pigmented races. Both sexes seem equally vulnerable to the disease though in most series females predominate probably because of greater awareness and concern about cosmetic defects among women. The onset of vitiligo has been observed from early childhood (Lerner 1959) to old age (El Mofty 1968).

Clinically the disease commonly appears as well demarcated white areas that may be round, oval or irregular in shape and vary in size from several millimetres to several centimetres in diameter. In some cases they remain unchanged for long periods of time but the disease tends to progress slowly or rapidly and occasionally it may lead to amelanosis of the whole skin (Fitzpatrick et al. 1987).

Complete absence of melanocytes has been demonstrated in the lesions of vitiligo by the dopa-reaction and electron microscopy (Birbeck, Breathnach & Everall 1961). The border of the lesion may be hyper-pigmented. It has been reported by Morohashi and associates (1977) that melanocytes at the periphery of vitiligo lesions show signs of cytoplasmic degeneration, such as vacuolization, fatty degeneration, aggregation of melanosomes and pyknosis. There is significant increase in the number of lymphocytes in the epidermis and superficial dermis around the margin of the zone of melanocyte depletion (Nordlund & Lerner 1978, Lever Schaumburg & Lever 1983). The infiltrate has been shown to be composed of T-cells (CD3+, CD4+ and CD8+). Many of the T-cells are activated (MHC class II+, Interferon γ+) of the UCHL1+ memory subset and many express the cutaneous lymphocyte-associated antigen (HECA-452+) typical of skin homing T-cells (Al Badri et al. 1993). Antigen presenting Langerhans cells are also reported to be increased in number in the skin lesions of vitiligo (Birbeck, Breathnach & Everall 1961, Riley 1967, Zelickson & Mottaz 1968).

The nature of the disease is unclear. Genetic factors are recognised to be important. In about 30 per cent of vitiligo patients there is a history of vitiligo in another family member (El Mofty 1968). There are published photographs of supposedly monozygotic twin pairs concordant for vitiligo (Mohr 1951, Siemens 1953, V Mayenburg, Vogt & Ziegelmayer 1976) and

one supposedly monozygotic twin pair discordant (Schachter 1947). When both members of a monozygotic pair are affected the extent, age of onset and course may be similar or dissimilar. Havez, Sharaf & Abd El Nabi (1983) studied the mode of inheritance in 150 families and concluded that the disease is polygenic and does not demonstrate simple Mendalian ratios. An incompletely penetrant autosomal dominant gene has been suggested (Lerner 1959) as has a polygenic autosomal recessive disorder (Carnevale et al. 1980) possibly involving a set of four unlinked loci (Majumder, Dassk & Li 1988). Goudie, Wilkieson & Goudie (1983) studied 2 families with multiple cases of vitiligo and found an increased incidence of other autoimmune diseases (pernicious anaemia, thyrotoxicosis, diabetes mellitus etc). It was suggested that among genetic factors predisposing to vitiligo those determining the development of organ-specific autoimmunity are probably important (Goudie, Jack & Goudie 1985).

An interesting feature of the vitiligo is that skin depigmentation is usually patchy rather than generalised and often shows a roughly symmetrical pattern at corresponding sites on opposite sides of body. The anatomical distribution of lesions on the face and hands were compared in ninety-two patients with vitiligo (Goudie, Wilkieson & Goudie 1983). Many of the lesions were found to be unrelated to conventional anatomical boundaries and there was a degree of symmetry from the earliest to the most advanced lesions. Remarkable similarity in patterns was observed on both sides of the body in the individual patients (Fig. 1.1). It was also noted that the skin pattern in vitiligo may mimic the anatomical distribution of the internal lesions of human autoimmune disease (Goudie, Spence & Mackie 1979). Eight patients had lesions on front of neck and eyelids simulating the goitre and exophthalmos of thyrotoxicosis, and two of the patients were actually suffering from thyrotoxicosis.

Vitiligo is regarded as a member of the family of organ-specific autoimmune disorders which includes Hashimoto's disease, Graves' disease, primary hypothyroidism, atrophic gastritis, pernicious anaemia and Type-1 diabetes mellitus. An increased incidence of vitiligo has been reported in most of these conditions (Cunliffe et al. 1968, Grunnet et al.

1970, Dawber 1970, McGregor, Katz & Doe 1972, Goudie, Jack & Goudie 1985). The reality of this association is supported by the demonstration of an increased incidence of symptomless organ-specific autoantibodies in patients with vitiligo. A study that compared 80 patients with vitiligo with an age and sex matched control series uncovered an increased incidence of organ-specific autoantibodies. Twenty-two patients had autoantibodies directed against thyroid cytoplasm compared to 5 controls, 7 had autoantibodies against thyroglobulin (none in controls) and 17 patients against gastric parietal cells compared with one control (Brostoff, Bor & Fiewel 1969). Another study of 96 patients with vitiligo compared with one hundred age and sex matched normal individuals showed an increased incidence of thyroid microsomal (20%) gastric parietal (13.7%) and pancreatic islet cell (7.2%) autoantibodies (Betterle et al. 1976).

The most convincing evidence in support of the autoimmune hypothesis is the demonstration of antimelanocyte antibodies in patients with vitiligo. Early attempts to demonstrate these autoantibodies gave conflicting results (Langhof, Feuerstein & Schabinski 1965, Woolfson et al. 1971, Betterle et al. 1976, Hertz et al. 1977). The recent use of human melanocytes established in tissue culture has permitted more sensitive and specific antibody assays to be performed. Using extracts of these cells for immunoprecipitation studies, it has been shown that the majority of patients with vitiligo have antibodies to surface antigens on melanocytes. Bystryn and Naughton (1985) examined the incidence of antibodies to melanocytes in 162 patients with vitiligo and unrelated diseases and found antibodies to melanocytes in 84 per cent with vitiligo including 22 of 22 patients with common vitiligo, 5 of 5 patients with vitiligo associated with chronic mucocutaneous candidiasis, 76% with vitiligo associated with other immune diseases and in only 2% (1 of 46) of individuals with non-pigmentary skin diseases. Galbraith et al. (1988) studied the sera from 13 patients with vitiligo for antibody reactivity to melanocyte derived cells by the use of immunofluorescence microscopy, and by Western blot analysis of solubilized membrane antigens of the human melanoma cell line (M14). Reactivity was detected in the 8 of 13 patients. Direct evidence that the sera from vitiligo patients contain autoantibodies has also been provided by Norris et al. (1988) who have also shown that they can directly damage

human melanocytes in vitro by both complement activitation and antibody-dependent cellular cytotoxicity.

Attempts have been made to find an HLA antigen link to vitiligo. Preliminary evidence suggestive of an association between vitiligo and HLA-DR4 has been reported by Foley et al. (1983) in 48% of caucasian vitiligo patients as compared to 28% observed in the control group. This finding has not been confirmed in other racial groups and the evidence for linkage disequilibrium between vitiligo and MHC is less convincing than in other organ-specific autoimmune diseases.

Apart from the MHC association the above observations suggest that vitiligo is an organ-specific autoimmune disease. As in other members of the group there is loss of specialised cells (melanocytes) of the target organ, associated with local lymphoid infiltration at the site of continuing cellular damage, but it is not known whether the primary defect is in the target organs themselves, in the immune system, or both.

Psoriasis

Psoriasis is a poorly understood chronic inflammatory diseases of the skin with a relapsing course and variable clinical features. About 2 per cent of the general population are affected by the disease (Hellgren 1967). It has a universal occurrence, predominantly affecting adults of both sexes (Farber & Nall 1974). The disease can be disabling due to pruritus, concomitant joint disease and pain, especially when palms and soles are involved.

The distinctive lesions of psoriasis are erythrosquamous indicating involvement of both cutaneous vasculature (erythema) and epidermis (increased scale). The clinical lesions first appear as a pinpoint spot of erythema which enlarges and stabilises. The following are the most prominent features of established lesions. 1. They are sharply demarcated with clear cut borders. 2. The surface consists of non-coherent silvery scales. 3. The skin has a glossy homogeneous erythema underneath the scale. 4. When scales are mechanically removed from a psoriatic plaque

by scratching, small blood droplets appear on the erythematous surface within few seconds (also called Auspitz sign diagnostic for the disease) (Fitzpatrick et al. 1987). In the most frequent clinical presentation circular plaques are prominent on elbows, knees and scalp often in symmetrical distribution (Fig. 5.1). There is constant production of large amounts of scales with little alteration in shape or distribution of individual plaques which may persist for months to years. Single small plaques may extend laterally and become confluent. Hyperproliferation of the skin first defined by VanScott & Ekel (1963) is a characteristic feature of psoriasis. Evidence suggests that there is more than eight fold shortening of epidermal cell cycle in psoriatic skin (36 hours compared to 311 hours for normal). One hundred percent of the germinative cells of the epidermis appear to enter the growth fraction as compared to 60-70% in normal subjects (Weinstein & Frost 1968). These changes result in thickening of epidermis three to five times normal. There is parakeratosis and acanthosis and absence of granular layer in epidermis. In the dermis elongated papillae are prominent. These contain dilated tortuous capillaries. Mononuclear cell infiltrate is present around the blood vessels. In early and fully developed psoriatic lesions the dermal mononuclear infiltrate has been shown to consist almost exclusively of T-lymphocytes and macrophages with very few B-lymphocytes or neutrophils (Bjerke, Krough & Matre 1978).

The majority of T-lymphocytes present are activated helper T-cells (CD4+/HLA-DR+) (Baker et al. 1984a). It has been shown that in the infiltrate the ratio of T-helper (CD4+) to T-suppressor (CD8+) cells is greater than the corresponding ratio in the blood of psoriatic patients. This suggests an active and selective migration of helper T-lymphocytes from the blood into established psoriatic lesions (Baker et al. 1984b). T-lymphocytes are also present in the epidermis of psoriatic lesions but are small in number. An increased epidermal entry and activation of helper T-lymphocytes (CD4+) in early psoriasis has been reported. It is also shown that there is an increased epidermal influx and activation of suppressor T-lymphocytes (CD8+) during resolution of lesions. These activated CD4+ and CD8+ T-lymphocytes are mainly found in close proximity to the

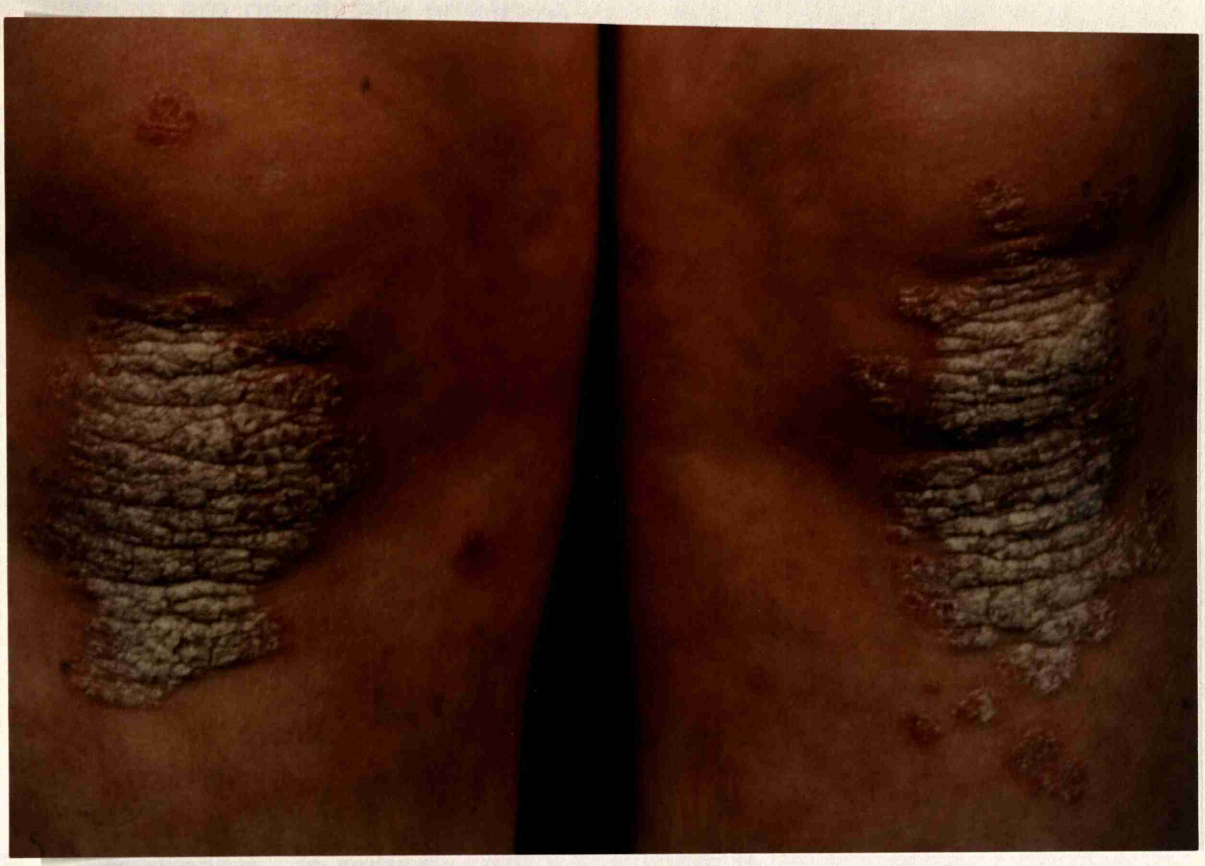


Figure 5.1 Psoriasis. Symmetrical lesions on both knees. There is often a striking mirror-image symmetry, as shown in this patient.

dendritic processes of epidermal antigen-presenting Langerhans cells which are also increased in total number (Baker et al. 1984a).

The cause of the disease is unknown. Several studies suggest that patients are genetically predisposed to psoriasis. The major support for this conclusion came from studies showing an increased incidence of psoriasis among relatives of affected individuals. Approximately one third of patients with psoriasis report some relatives with the disease (Farber & Nall 1974). Twin studies have shown 65-70% concordance in monozygotic twins and 15-20% concordance among dizygotic twins (Krueger & Eyre 1984). It has been concluded that the disease does not follow a typical Mendelian dominant or recessive pattern of inheritance but inheritance is multifactorial (Watson 1972).

As compared to non-psoriatic individuals there is greater than expected frequency of certain MHC antigens in psoriasis (linkage disequilibrium), see Table 5.1. MHC molecules have a key role in the presentation of antigen to T-lymphocytes and the association of particular alleles with psoriasis suggests that T-cells may be involved in the pathogenesis of the disease.

Environmental factors are also considered important in pathogenesis. In patients with active disease psoriasis often develops at the site of injury to the skin such as surgical incisions (Koebner phenomenon) (Eyre & Krueger 1984).

The persistence of the disease throughout life once it has manifested suggest the existence of a memory and the spontaneous exacerbations and remissions of disease activity are consistent with a chronic immune response. The presence of an increased number of helper T-lymphocytes and antigen presenting Langerhans cells and the linkage disequilibrium between certain HLA antigens and psoriasis strongly support an ongoing immune response in psoriasis. There are several reasons to believe that infiltrating T-cells are involved in the pathogenesis of the disease. Abnormalities in T-cell activity have been reported in patients with psoriasis. The proliferative response of peripheral blood lymphocytes in

TABLE 5.1 Disease association of HLA with psoriasis in caucasians (data from Tiwari & Terasaki 1985)

Antigen	Studies N	Frequency in patients (%)	Frequency in controls (%)	Relative risk
B13	36	19	5	4.1
B17	36	19	7	5.3
B37	15	7	2	3.9
CW6	7	56	15	7.5
DR7	5	48	23	3.2

N = Number of studies

psoriasis to certain mitogens such as concanavalin A (Con A) has been shown to be depressed (Guilhou, Clot & Meynadier 1977). Impaired γ interferon production by PBMC's in psoriatic patients has been reported when activated by Con A. Other reports have also shown a marked decrease in lymphocyte response to Con A (Levantine & Brostoff 1975) and it has been proposed that this impairment of activity might indicate a deficiency of T-suppressor cells (Goan et al. 1986). There is evidence in psoriasis of production by activated CD4+ T-cells of the cytokines IL-2, IL-6 and IL-8 which regulate keratinocyte growth (Valdimarsson et al. 1986). IL-6 a major mediator of the host response to injury and infection, and IL-8 a potent T-cell and neutrophil chemoatractant are present in increased amount in psoriatic lesions (Grossman et al. 1989, Nickoloff et al. 1991) and have been shown to be stimulatory for normal keratinocyte proliferation in vitro (Grossman et al. 1989, Reusch et al. 1990). IL-2 has been shown to stimulate both normal and lesional psoriatic keratinocyte proliferation (Baker & Fry 1992). Further support for a probable role of helper T-cells in psoriasis comes from the clinical improvement which may result from treatment with immunosuppressive agents which act on T-cells. Cyclosporin-A which is effective in clearing psoriasis has been shown to have a selective role on activated CD4+ T-cells (Baker et al. 1987), by blocking their production of cytokines such as IL-2, IL-6, and γ interferon. Other immunosuppressive drugs similar in mechanism of action to cyclosporin A, like FK506 supports the primary role of cyclosporin-A on helper (CD4+) T-lymphocytes. FK506 inhibits the production of IL-2, IL-3, IL-4 and γ interferon by helper (CD4+) T-lymphocytes. (Ackerman et al. 1991). Important recent evidence that helper T-cells have a pathogenic role in this disorder is the recent report of clearance of psoriasis by treatment with monoclonal antibodies to CD4 antigen (Poizot-Martin et al. 1991, Nicolas et al. 1991).

Benign T-cell neoplasia hypothesis

The basic pathological abnormalities underlying vitiligo and psoriasis are obscure and it is unknown why the lesions in these disease tend to affect some parts of the skin and spare others in a symmetrical fashion. In a report on a series of patients with symmetrical cutaneous lymphoma, a

disorder in which the lesions (localised B or T-cell tumours of low grade malignancy) affect corresponding areas of the skin on the two sides of the body Goudie et al. (1990) reasoned that the involvement of mirror-image areas of skin in these cases is probably due to spread of the tumour cells by the bloodstream with specific homing or selective proliferation at particular anatomical sites. It was also noted that the patterns formed by some of these tumours are reminiscent of the patchy symmetrical distribution of the T-cell associated skin lesions of psoriasis and vitiligo and postulated that these conditions may be benign variants of symmetrical cutaneous lymphoma which function autonomously and lead to stimulation of epidermal growth with cytokines in psoriasis or autoimmune destruction of autologous melanocytes in vitiligo.

If vitiligo and psoriasis are indeed benign variants of symmetrical cutaneous T-cell lymphoma identical dominant T-cell clones should be demonstrable in all the skin lesions from each individual patient with either of these diseases.

In this chapter an attempt is made to detect such clones in vitiligo and psoriasis using TCRγ gene rearrangements, amplified by PCRs as clonal markers. Initially, tests were performed on lesions and dominant clones were found in only a minority of biopsies. Biopsies of clinically affected and unaffected skin from the same patient were then studied to detect possible examples of selective localisation of T-cell clones in the lesions. Finally a comparison was made of T-cell clones in the lesions of vitiligo and psoriasis in patients who simultaneously had both diseases, in order to distinguish disease-specific clones from disease non-specific clones of skin-homing memory T-cells which are present in normal skin and accumulate in large numbers at sites of chronic cutaneous inflammation (Picker et al. 1991).

Subjects and Methods

Subjects Studied

Nineteen patients were studied, six with vitiligo, nine (including a pair of monozygotic twins) with psoriasis and four with both vitiligo and psoriasis simultaneously affecting different areas of skin. The diagnosis in each was confirmed by the dermatologist who took the biopsies. Details of age, sex and diagnosis of the patients studied and samples taken are given in Appendix 5.1.

Samples of venous blood in anticoagulant were obtained from all but three of the cases. Full thickness incisional or punch biopsies of skin were taken under local anaesthesia from the edge of vitiligo lesions (10 x 5mm ellipses) and from psoriatic plaques (6mm cores), at corresponding sites on the two sides of the body. In two cases with vitiligo a third biopsy was taken from a site elsewhere and in one case two biopsies were taken each from clinically unaffected and affected skin.

In three cases with psoriasis one biopsy was obtained from clinically unaffected skin and one from affected skin.

Biopsies of two vitiligo lesions, two psoriasis lesions and two areas of clinically unaffected skin were taken from three cases (Cases VP1 - VP3) and from two vitiligo lesions and two psoriatic lesions from one patient (Case VP4). A further set of samples from two vitiligo lesions, two psoriatic lesions and two areas of unaffected skin were taken from three cases (Cases VP1, VP3 and VP4) about one year after the first set.

DNA Extraction

From peripheral blood

PBMC were separated and DNA extracted and purified according to the standard protocol given in Appendix 2.2.

From skin biopsies

After biopsy of the skin, fresh tissue was rapidly frozen in liquid nitrogen, wrapped in foil and stored at -70ºC until used for DNA extraction. DNA was extracted from 5 micron cryostat sections of the whole biopsy specimens by the modified protocol for small amounts of tissue given in Appendix 2.7.

PCR Amplification

PCR amplification of 5µl aliquots of each DNA sample was carried out for 35 cycles according to the modified radiolabelled PCR protocol as given in Appendix 2.3 with primers specific for 8 Vγ and Jγ1/2 genes given in Tables 2.3 and 2.4.

PCR products were analysed on 6% polyacrylamide gel run under denaturing conditions and autoradiographs prepared as given in Appendix 2.6.

Results

Detailed PCR results obtained with primers for 8 Vγ and Jγ1/2 genes in patients with vitiligo are given in Appendix 5.2 and psoriasis in Appendix 5.3. Table 5.2 shows the number of patients, samples and successful PCR tests in the groups studied.

Vitiligo

Blood

The results are summarised in Table 5.3.

Dominant + bands were obtained with primers for Vγ3 (Fig 5.2) and Vγ9 (Fig 5.3a) in case V4, Vγ3 in case V5 and ++ bands were obtained with primers for Vγ8 in case V1 and V4 and ψVγ5 (Fig 5.4) in case V5. The remaining 21 satisfactory tests gave smears sometimes accompanied by minor bands. No dominant bands were found in 22 satisfactory tests on blood samples from four patients with vitiligo plus psoriasis.

TABLE 5.2 Number of patients, samples and successful PCRs ( ) in groups studied

Diagnosis	Patients	Blood	Skin lesions	Clinically unaffected skin
Vitiligo	6	4 (28)	14 (91)	2 (16)
Psoriasis	9	8 (46)	15 (66)	3 (15)
Vitiligo plus psoriasis	4	4 (22)	14 (75) 14 (76)	12 (57)

TABLE 5.3 Proportion of patients and PCR tests in which + or ++ dominant rearrangements were found in peripheral blood mononuclear cells in vitiligo, psoriasis and normal controls

Diagnosis	Patients	PCR tests +	PCR tests ++
Vitiligo	3/4	3/28	3/28
Psoriasis	2/8	2/46	0/46
Vitiligo plus psoriasis	0/4	0/22	0/22
Normal controls	2/5	1/30	1/30

\+ = Borderline dominant band
++ = Unequivocally dominant band

Same normal healthy controls as in Chapter 4.

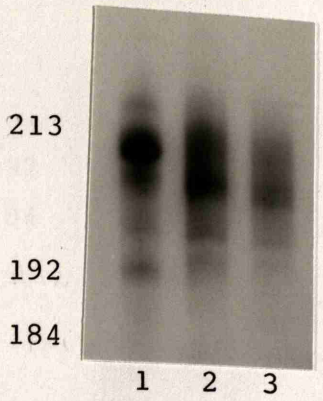


Figure 5.2 PCR analysis of TCRγ gene rearrangements in vitiligo.
V3-J1/2 rearrangements in case V4.
Lane 1: PBMC. Lanes 2-3: samples of two vitiligo lesions. Left MW in nucleotides. A dominant + band is present in blood.

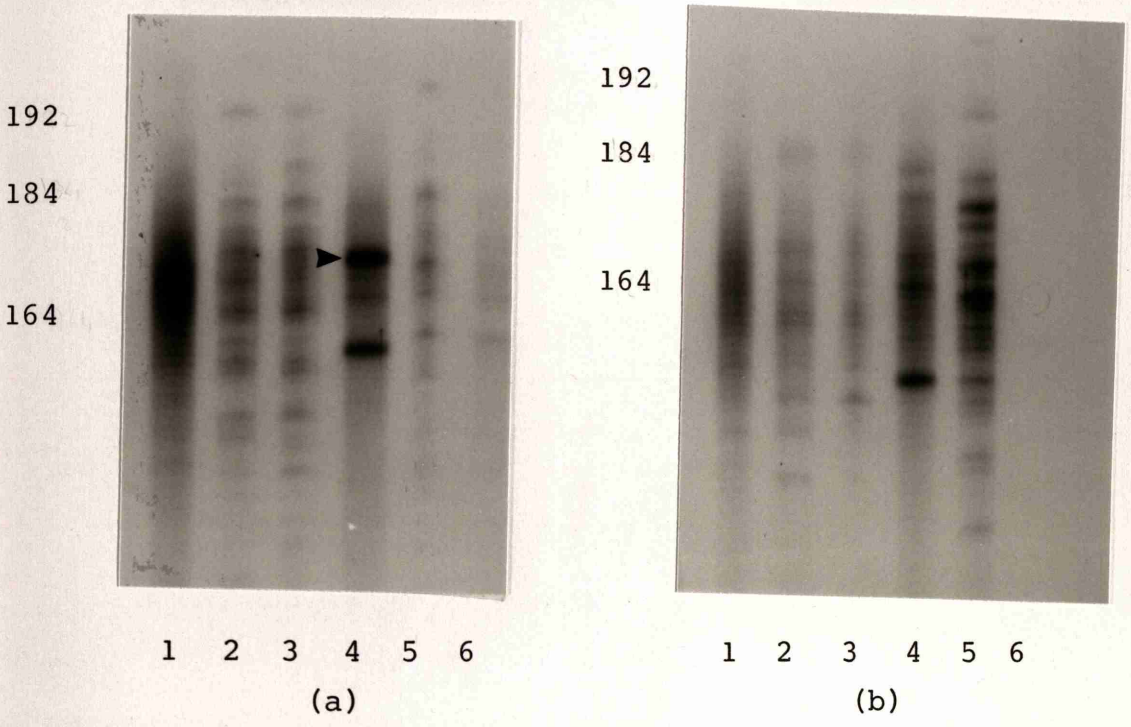


Figure 5.3 PCR analysis of TCRγ gene rearrangements in vitiligo.
a) V9-J1/2 (b) V10-J1/2 rearrangements in cases V3 and V4.
Lane 1: PBMC. Lanes 2-3: samples of two vitiligo lesions from case V3.
Lane 4: PBMC. Lanes 5-6: samples of two vitiligo lesions from case V4.
Left MW in nucleotides.
With V9-1/2 a dominant + band is present in blood of case V4 (arrow).
Band patterns in blood and lesions are different in each individual but are very similar in skin lesions from case V3. Band pattern is different in different individuals. No reaction is obtained in lane 6 (b).

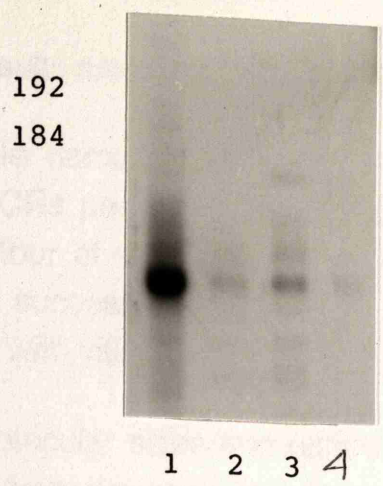


Figure 5.4 PCR analysis of TCRγ gene rearrangements in vitiligo.
ΨV5-J1/2 rearrangements in case V5.
Lane 1: PBMC. Lanes 2-4: samples of three vitiligo lesions.
A dominant ++ band is present in blood, traces of which are also present in the vitiligo lesions.

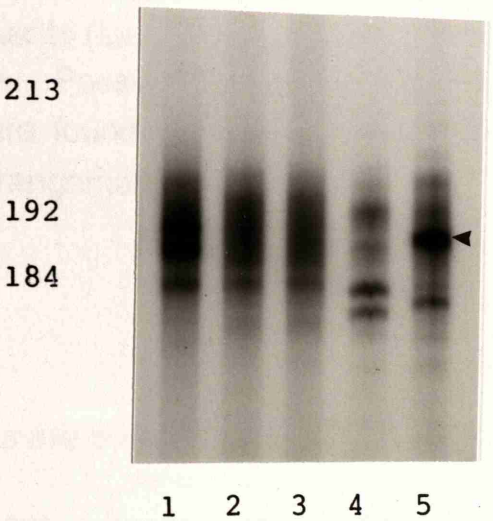


Figure 5.5 PCR analysis of TCRγ gene rearrangements in vitiligo and psoriasis.
V4-J1/2 rearrangements in case VP4.
Lane 1: PBMC.Lanes 2-3: samples of two psoriasis lesions. Lanes 4-5: samples of two vitiligo lesions.
A dominant + band is present in one vitiligo lesion (arrow).

Skin

The results are summarised in Tables 5.2 and 5.4.

A single dominant (+) band (Fig 5.5) was found in 1 of the 166 successful PCRs performed on 28 skin lesion samples from ten patients with vitiligo, four of whom also had psoriasis. No dominant bands were present in 73 successful PCRs on 14 biopsies of unaffected skin from five patients, one with vitiligo and four with vitiligo plus psoriasis.

The molecular sizes and relative intensities of the bands in multiple skin biopsies from the same individual were often similar but band patterns differed markedly when DNA from the skin of different patients was tested with the same primer combination (Fig 5.6). DNA from skin and PBMC from the same individual usually gave different band patterns (Fig 5.6,5.7) but this was not always so (Fig 5.4).

An attempt was made to detect minor "lesion specific" rearrangements which were consistently present in lesions but absent from clinically unaffected skin from the same individual. It was possible to compare the rearrangements in 2 lesions and 2 areas of unaffected skin from five patients (four of whom also had psoriasis) with a total of 29 primer combinations. Possible examples of Vγ2 vitiligo-specific rearrangements (Fig 5.8) were found in case V6 and of Vγ11 and Vγ3 (Fig 5.9) vitiligo-specific rearrangements in case VP2.

Psoriasis

Blood

Results are summarised in Tables 5.2 and 5.3.

Dominant + bands were obtained with primers for Vγ4 in case P2 and with Vγ9 in case P9. The remaining 44 satisfactory tests gave smears sometimes accompanied by minor bands.

TABLE 5.4 Proportion of patients, samples and PCR tests in which + or ++ dominant rearrangements were found in skin lesions of vitiligo and psoriasis and normal controls

	Patients		Skin biopsies		PCRs tests	
	+	++	+	++	+	++
Vitiligo lesions	1/10	0/10	1/28	0/28	1/166	0/166
Psoriasis lesions	0/13	0/13	0/29	0/29	0/142	0/142
Normal controls	0/5	0/5	0/12	0/12	0/67	0/67

\+ = Borderline dominant band
++ = Unequivocally dominant band

Same normal healthy controls as in Chapter 4.

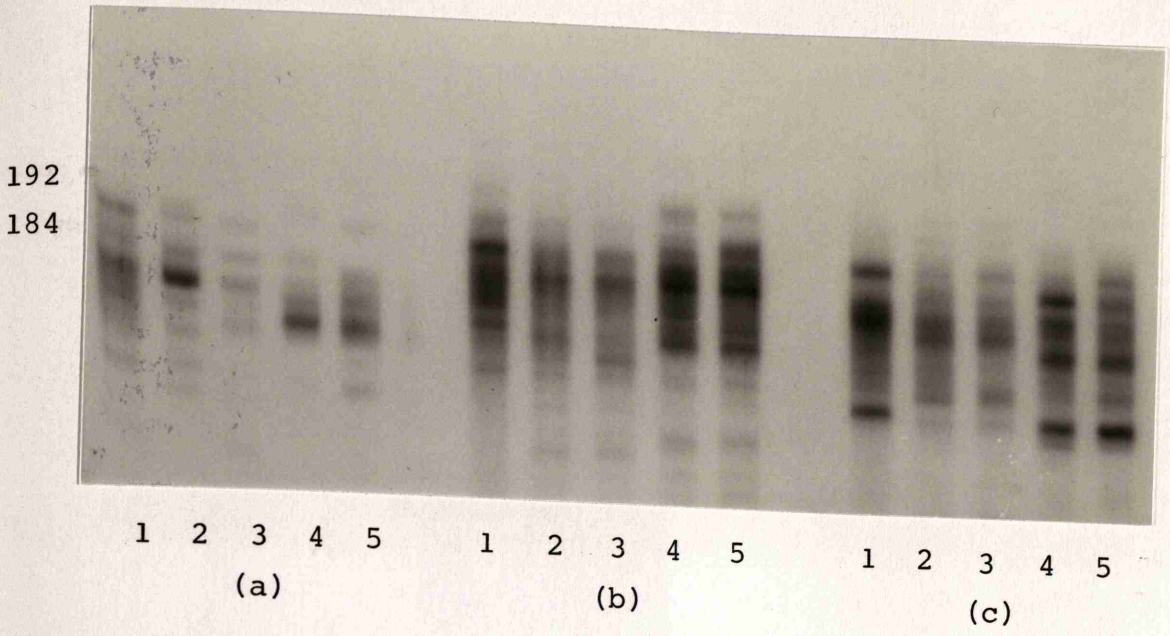


Figure 5.6 PCR analysis of TCRγ gene rearrangements in vitiligo.
(a) V5-J1/2 (b) V9-J1/2 (c) V10-J1/2 rearrangements in cases V1 and V2.
Lane 1: PBMC. Lanes 2-3: samples of two vitiligo lesions from case V1. Lanes 4-5: samples of two vitiligo lesions from case V2. Left MW in nucleotides.
Band pattern is different in blood and skin lesions but is similar in skin lesions from the same patient. Band pattern in skin lesions is different in the two patients.

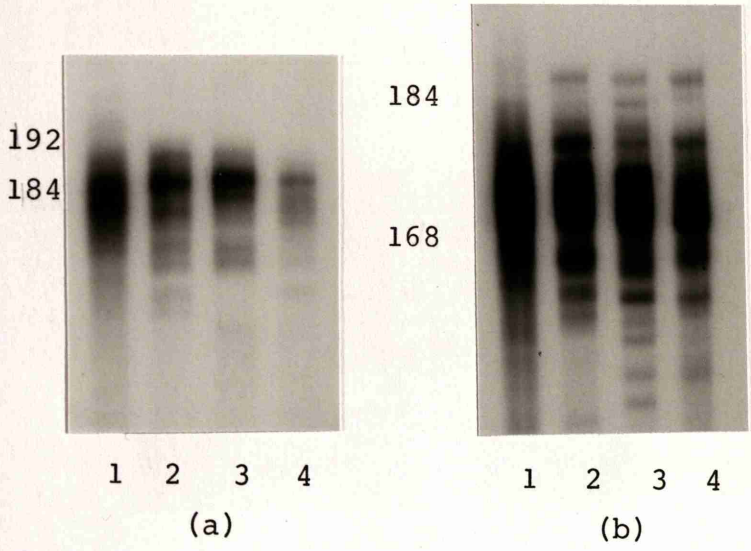


Figure 5.7 PCR analysis of TCRγ gene rearrangements in vitiligo.
(a) V3-J1/2 (b) V9-J1/2 rearrangements in case V3.
Lane 1: PBMC. Lanes 2-4: samples of lesions from right and left anterior shin and right upper thigh. Left MW in nucleotides.
Band pattern is similar in three lesions but is different in corresponding blood.

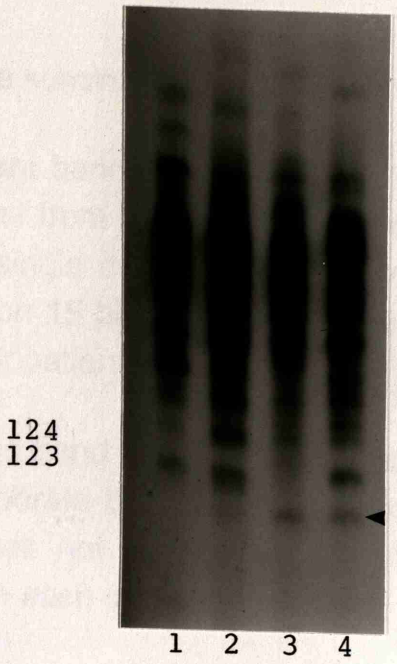


Figure 5.8 PCR analysis of TCRγ gene rearrangements in vitiligo.
V2-J1/2 rearrangements in case V6.
Lanes 1-2: samples of two areas of clinically unaffected skin. Lanes 3-4: samples of two vitiligo lesions. Left MW in nucleotides. A faint possibly lesion specific band (arrow) is present in both skin lesions.

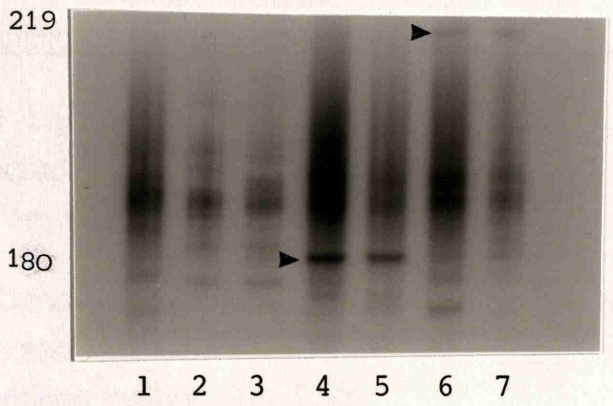


Figure 5.9 PCR analysis of TCRγ gene rearrangements in vitiligo and psoriasis.
V3-J1/2 rearrangements in case VP2.
Lane 1: PBMC. Lanes 2-3: samples of two areas of clinically unaffected skin. Lanes 4-5: samples of two psoriasis lesions. Lanes 6-7: samples of two vitiligo lesions. Left MW in nucleotides. A band (arrow) specific to psoriasis and a faint band (arrow) specific to vitiligo is present.

Skin

Results are summarised in Tables 5.2 and 5.4.

No dominant bands were found in 142 satisfactory tests performed on 29 skin lesions from thirteen patients with psoriasis four of whom also had vitiligo. A single dominant + band was found in 1 of 72 satisfactory tests performed on 15 biopsies of unaffected skin from three patients with psoriasis and four patients with vitiligo plus psoriasis.

As in vitiligo band patterns were usually different in different patients but similar in separate biopsies from the same patient, (eg. Figs 5.9 and 5.10) but this was not so in the pair of monozygotic twins who gave different results in each of the skin biopsies examined (Fig 5.11).

In an attempt to detect minor "lesion specific" rearrangements, it was possible to compare the rearrangements of 2 psoriatic lesions and 2 areas of unaffected skin from four patients (who also had vitiligo) with a total of 22 primer combinations. Possible examples of disease-specific Vγ3 and V γ11 rearrangements were found in three patients case VP1 (Fig 5.12), VP2 (Fig 5.9), and case VP4 (Fig 5.10).

This result was confirmed in cases VP1 and VP4 (Fig 5.10b) in a second set of biopsies obtained a year later (Fig 5.12).

Comparison of vitiligo and psoriatic lesions in the same patient

The Vγ11 band present in the psoriatic biopsies but absent from clinically unaffected skin in case VP1 was not present in 4 samples of vitiligo lesions from this patient (Fig 5.12).

The Vγ3 band present in the psoriatic biopsies but absent from the clinically unaffected skin in case VP4 was present in much smaller amounts in the vitiligo lesions (Fig 5.10).

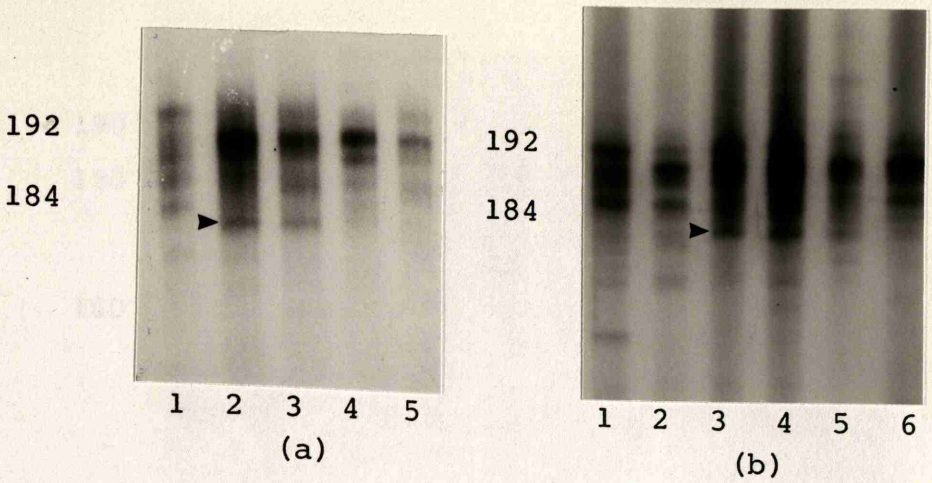


Figure 5.10 PCR analysis of TCRγ gene rearrangements in vitiligo and psoriasis.
V3-J1/2 rearrangements in Case VP4.
(a) Lane 1: PBMC. Lanes 2-3: samples of two psoriasis lesions. Lanes 4-5: samples of two vitiligo lesions. A possible psoriasis-specific band (arrow) is present.
(b) Repeat biopsies taken 20 months later.
Lanes 1-2: samples of two areas of clinically unaffected skin. Lanes 3-4: samples of two psoriasis lesions. Lanes 5-6: samples of two vitiligo lesions. Left MW in nucleotides.
The band apparently specific to psoriasis lesions (a) is present in small amounts in one of the vitiligo lesions.

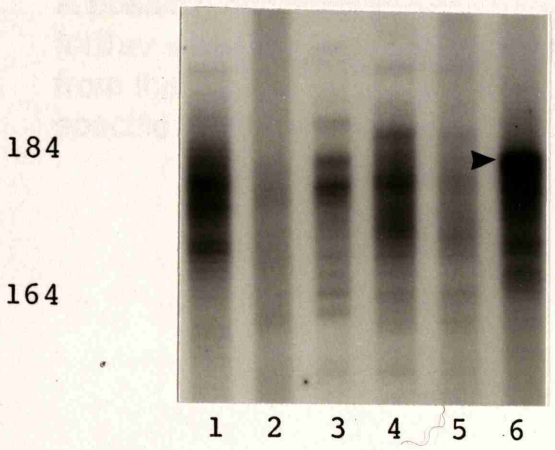


Figure 5.11 PCR analysis of TCRγ gene rearrangements in psoriasis.
V9-J1/2 rearrangements in monozygotic twins.
Lane 1: PBMC, Lanes 2-3: samples of two psoriasis lesions from twin P8. Lanes 4-5: samples of two psoriasis lesions.
Lane 6: PBMC from twin P9. Left MW in nucleotides.
The band pattern is different in two lesions from each twin and is also different in the two individuals. A dominant + band (arrow) is present in blood of case P9.

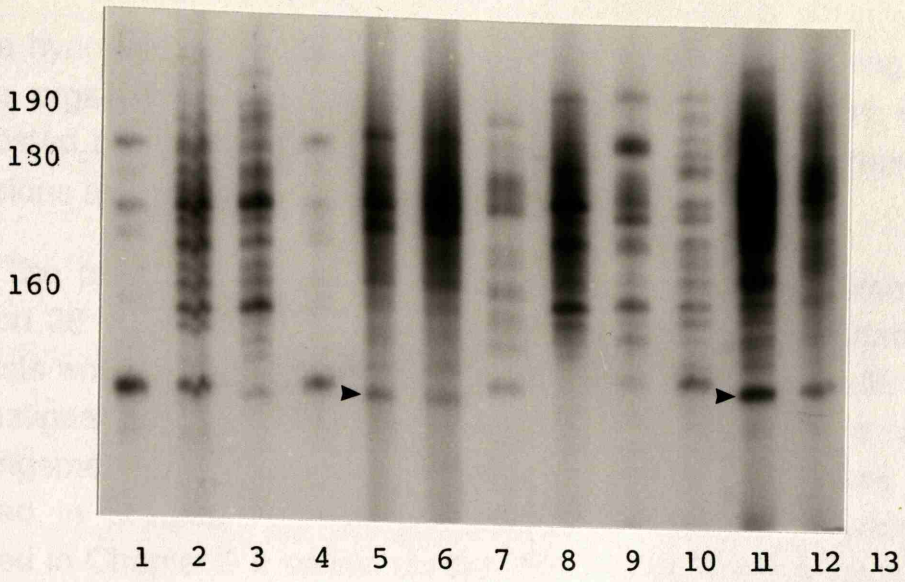


Figure 5.12 PCR analysis of TCRγ gene rearrangements in vitiligo and psoriasis.
V11-J1/2 rearrangements in case VP1.
Lanes 1-2: samples of two areas of clinically unaffected skin. Lanes 3-4: samples of two vitiligo lesions. Lanes 5-6: samples of two psoriasis lesions. Left MW in nucleotides. A possible psoriasis-specific band (arrow) is present. A further set of six biopsies (lanes 7-12) taken after a year from the same case confirms the presence of psoriasis-specific band.

Discussion

The hypothesis that benign T-cell neoplasia is the underlying cause of certain organ-specific autoimmune and chronic inflammatory disorders, was tested by looking for dominant clonal TCRγ gene rearrangements in the lesions and blood of patients with vitiligo and/or psoriasis.

The presence of one borderline + dominant rearrangement in 166 tests on 28 vitiligo lesions and none in 192 tests on 29 psoriatic lesions contrasts with the finding of 10 + and 11 ++ rearrangements in 261 tests on 33 malignant T-cell lymphomas reported in Chapter 3 and 6++ rearrangements in 33 tests on skin lesion from 3 of 4 cases of CTCL reported in Chapter 4. From the dilution experiment (Experiment 18) reported in Chapter 2 it seems unlikely that clones forming more than one per cent of the total T-cell population are present in the skin lesions of vitiligo and psoriasis. In spite of the complexity of the band patterns obtained and the subjective way in which they have been assessed it can be concluded that clonal dominance of the extent encountered in CTCL does not occur in the lesions of vitiligo and psoriasis.

The absence of obvious dominant TCR gene rearrangements does not exclude the possibility of selective localisation of benign neoplastic T-cells in the lesions in these conditions, since a benign clone would be expected to have fewer cells (possibly < 1 per cent) than a clinically apparent malignant T-cell lymphoma. Band patterns were therefore compared in diseased and clinically unaffected skin from the same patient in the hope of finding "lesion-specific" bands selectively localised to the lesions. It was found that the band patterns in different skin samples from the same patient were usually remarkably similar in keeping with the existence of a subset of clones of skin-homing T-cells (Picker et al. 1991, Mackay et al. 1991). Possible examples of selective localisation of one band to the lesions of vitiligo and another to the lesions of psoriasis were found in case VP2 (Fig 5.9) but it has not been possible to exclude the possibility that these are chance findings since the patient was unwilling to provide further confirmatory biopsies. A more convincing example of a possible Vγ11 psoriasis-specific rearrangement was seen in case VP1 (Fig 5.12) in whom the findings in the original 2 psoriatic biopsies were

confirmed in 2 biopsies taken a year later. The absence of the psoriasis-specific band from the 4 vitiligo lesions obtained from this patient is further evidence that it is not a non-specific effect of skin-homing T-cells which accumulate in large numbers in the lesions of both diseases (Baker et al. 1984, Al Badri et al. 1993). Nucleotide sequencing of this band is required in order to show whether it is monoclonal and identical in each of the psoriatic lesions. In conclusion there is as yet no convincing evidence of selective localisation of putatively pathogenic neoplastic T-cell clones in the lesions of vitiligo and psoriasis as would be expected if these diseases are benign variants of symmetrical T-cell lymphoma. On the other hand this possibility cannot yet be excluded.

Dominant bands were detected in DNA tested from peripheral blood in three of eight patients with vitiligo (4 of whom had psoriasis) and two of twelve patients with psoriasis (4 of whom had vitiligo) (Table 5.3). It is unknown whether these represent a limited clonal response to antigenic stimulation or reflect the presence of circulating clones of neoplastic lymphocytes at the benign end of the spectrum. Small numbers of these cells may enter the lesions from the peripheral blood (Fig 5.4) and may be of pathogenic importance but this seems unlikely since similar bands have been found in the blood in 2 of 5 healthy normal control subjects described in Chapter 4.

CHAPTER 6

T-Cell Receptor γ Gene Rearrangements in Synovial Lymphocytes in Rheumatoid Arthritis.

Introduction

Rheumatoid arthritis is defined as a chronic or subacute systemic inflammatory disorder, principally involving the joints, with peripheral symmetrical inflammatory non-suppurative arthritis (Jayson & Grennan 1983). The disease occurs in all racial groups and in all parts of the world affecting about 1 percent of people. It is estimated to affect approximately one and a half million patients in the United Kingdom (Thompson 1971). The disease may occur at any age but the onset is most frequent in the fourth and fifth decade of life (Christian 1982). Rheumatoid arthritis is a disorder unique to man (Boyle & Buchanan 1971). The disease seems to be relatively new as it has not been seen in any ancient skeletal remains and there are no clear biblical or literature references before the 19th century. The first classical description of rheumatoid arthritis is commonly attributed to Landre-Beauvais (1800). Garrod used the term rheumatoid arthritis in 1858 to describe a syndrome which he recognised as distinct from both gout and acute rheumatic fever. In 1940 Waller discovered a factor in the serum of patients with rheumatoid arthritis now known as rheumatoid factor (RF). Numerous reports on the rheumatoid synovium have shown the spontaneous production of large quantities of immunoglobulins and RF both in vivo and in vitro. Immune complexes are presumably generated by the synovium and are deposited within the synovial tissue and fluids as well as on the free cartilaginous surfaces. These synovial complexes activate complement and generate chemotactic factors which attract polymorphonuclear cells which are found in increased number in rheumatoid joint fluids (Bunch et al. 1974, Ward & Zvaifler 1971, Bourke et al. 1982, Sabharwal et al. 1982). Phagocytosis of the complexes results in the release of lysosomal enzymes capable of degrading collagen, elastin and other proteins as well as prostaglandins and activated oxygen radicals (Hensen 1971, Fridovich 1978). This series of events contributes to the erosive destructive changes seen in the joints in rheumatoid arthritis.

Histologically in established rheumatoid arthritis hypertrophy of synovial lining cells occurs increasing from 1 or 2 cell layers up to 10 cell layers in depth. The synovium is oedematous and contains villous extensions projecting in the joint cavity. Beneath the hypertrophied synovial lining, infiltration with mononuclear cells is observed. The mononuclear cell infiltrate is composed of macrophages, plasma cells and predominantly lymphocytes(Loewi, Darling & Howard 1974).

The aetiology of rheumatoid arthritis remains one of the major medical mysteries. Many causes have been considered and several factors may possibly operate together to produce the disease with environmental conditions provoking the onset in genetically predisposed individuals. The suggested causes include psychological stress, infection and autoimmunity. There is no clear evidence that rheumatoid arthritis is due to nutritional factors, a metabolic error, an endocrine abnormality or imbalance in the autonomic nervous system, nor that occupational factors, cold, damp or injury play a major role (Lawrence 1970). Hereditary predisposition has often been considered as important in the aetiology of rheumatoid arthritis. Family studies have shown a slightly increased risk of rheumatoid arthritis in first degree relatives of rheumatoid arthritis patients (Aho et al. 1986). An increased frequency of concordance of rheumatoid arthritis exists in monozygotic twins compared with dizygotic twins (Lawrence 1970, Aho et al. 1986) but there is no evidence either of a simple dominant or recessive trait.

The similarities of rheumatoid arthritis to other arthritides such as Lyme disease for which an infectious cause has been established suggests the possibility that an exogenous agent such as bacteria, mycoplasma or virus may be involved. Although there have been scattered reports suggesting the importance of one or another of these agents, no single agent has proven to be a constant or likely candidate for an aetiological role in rheumatoid arthritis. E.B virus has received great attention as a potential causative agent in rheumatoid arthritis (Alspaugh et al. 1981). Antibodies to E.B virus antigens have been observed more frequently or in higher concentrations in patients with rheumatoid arthritis compared to controls (Tan 1979). However examination of synovial tissues have failed to reveal evidence of E.B virus antigenic particles within synovial tissues (deChamplain et al. 1983).

Many investigators strongly believe that autoimmunity has a major role in the pathogenesis of the rheumatoid arthritis. The concept that it is an autoimmune disease actually originated from the observation almost half a century ago that the sera of rheumatoid arthritis patients will agglutinate many organisms and inert particles (Waller 1940). This agglutination has been shown to be due to the presence of RF which acts as autoantibody to the patient's own gamma globulin (Milgrom et al. 1962). Other autoantibodies including antinuclear antibodies directed against single stranded DNA (Koffler et al. 1971), histones (Hannestad & Stollar 1978) non-histone soluble cellular proteins (Venables, Erhardt & Maini 1980) collagen (Trentham 1985) and cytoskeletal filamentous proteins (Osung, Chandra & Holborow 1980) have also been detected in a significant proportion of rheumatoid arthritis patients. This observation emphasises that multiple autoimmune phenomena are frequently observed in patients with rheumatoid arthritis. Although autoimmunity is thought to have an important role in the pathogenesis, it is not yet known how self tolerance breaks down or why autoantibody production persists for many years in patients affected by the disease.

Examination of the phenotypic characteristics of lymphocytes from patients with rheumatoid arthritis have shown that there are more T-lymphocytes than B-lymphocytes in the synovial membrane (Van Boxel & Paget 1975) and that the infiltrating T-lymphocytes are predominantly helper inducer CD4+ cells (Klareskog et al. 1982). The T-lymphocytes in the synovial tissue, fluid and blood of patients with rheumatoid arthritis stain positively for MHC class II antigens indicating that the majority of these cells are in activated state (Burmester et al. 1981, Klareskog et al. 1982). The MHC class II staining has been shown in both helper inducer CD4+ and cytotoxic CD8+ subsets but predominantly in CD4+ cells (Pincus, Clegg & Ward 1985). It has been noted that the percentage of CD4+ cells is greater than that of CD8+ in the peripheral blood of rheumatoid arthritis patients while these cells are approximately equal in the synovial fluid (Burmester et al. 1981, Veys et al. 1982). Several studies have examined the organisation of cells within rheumatoid synovium. Infiltration of T-lymphocytes adjacent to HLA-DR positive macrophage-like or dendritic-like cells has been observed (Klareskog et al. 1982, Meijer et al. 1982, Burmester et al. 1981). Activated B-lymphocytes have been observed surrounded by T-lymphocytes (Young et al. 1984). Another study

suggests that the rheumatoid synovium is composed of lymphocyte-rich areas composed primarily of CD4+ helper inducer cells and transitional, primarily CD8+ suppressor cytotoxic cell areas (Kurosaka & Ziff 1983). The transitional areas with CD8+ predominance contain lymphocytes with a blastic appearance, and many macrophage like cells. Together these observations suggests ongoing cellular interactions in the rheumatoid synovium. It is suspected that T-lymphocytes that accumulate in the synovial membrane have a crucial role in the development of rheumatoid arthritis. An association with HLA-DR4 (MHC class II) antigen has been reported in the majority of seropositive rheumatoid arthritis patients (Stastny 1978, Winchester 1981). The MHC association of rheumatoid arthritis suggests that CD4+ T-cells may participate in the pathogenesis of rheumatoid arthritis, since $CD4_+$ T-cells recognise class II molecules. T-cells are also shown to be activated in rheumatoid synovial tissue and there is evidence that therapies relatively specific for T-cells, such as cyclosporin and monoclonal anti-CD4 antibodies cause significant improvement in rheumatoid arthritis patients (Horneff et al. 1991, Weinblatt et al. 1987). These observations are also supported by studies on animal models in which a rheumatoid arthritis like disease can be transferred with T-cells specific for mycobacterial antigens (Holoshitz, Matitiau & Cohen 1984, Van Eden, Holoshitz & Cohen 1987).

As described in Chapter I, it has been suggested that benign neoplasia of T-lymphocytes may be primarily responsible for the defect in self tolerance which leads to symmetrical autoimmune articular damage in rheumatoid arthritis, and if this hypothesis is correct the presence of abnormally large T-cell clones should be detected in the diseased joints and perhaps in peripheral blood.

It has been known for some time that apparently classical seropositive rheumatoid arthritis occurs in as many as a third of patients with an uncommon form of T-cell neoplasia lying at the benign end of the spectrum (Newland et al. 1984, Loughran et al. 1988). The condition is variously known as chronic T-cell lymphocytosis with neutropenia, (Loughran et al. 1985, Aisenberg et al. 1981) LGL lymphocytosis (Chan, Winton & Waldmann 1986), chronic Tγ lymphoproliferative disease (Reynolds & Foon 1984) and granulated T-cell lymphocytosis with neutropenia (McKenna et al. 1977). The patients have a peripheral blood lymphocytosis consisting predominantly of large granular lymphocytes

(LGL). The lymphocytosis is typically mild to moderate but many of these patients have severe neutropenia and recurrent infections. The lymphocytes in most of these patients bear CD3+, CD8+ markers characteristically expressed by T cytotoxic lymphocytes (McKenna et al. 1977, Aisenberg et al. 1981). There are three lines of evidence that the condition is neoplastic. (1) In most cases which have been investigated, monoclonal rearrangements of TCRβ genes have been demonstrated in peripheral blood by Southern blot analysis (Chan et al. 1986, Berliner et al. 1986, Foroni et al. 1988, Loughran et al. 1988). (2) Cytogenetic studies often show clonal chromosomal abnormalities strongly suggestive of neoplasia (Loughran et al. 1985, Brito-Babapulle et al. 1986, Catovsky et al. 1979). (3) A small proportion of cases progress to frank T-cell leukaemia (Brouet et al. 1981, Newland et al. 1984). The connection between the neoplastic T-cells and the production of RF is unknown and it has not been established to what extent the diseased synovium is infiltrated by the neoplastic clone.

Studies regarding the clonal origin of infiltrating T-lymphocytes have been done in patients with ordinary rheumatoid arthritis. In 1987 Savill et al. using Southern blot analysis demonstrated clonal TCRβ gene rearrangements in synovial fluid mononuclear cells but not in peripheral blood from 3 of 12 patients with the common form of rheumatoid arthritis. In experiments by Stamenkovic et al. (1988) IL-2 responsive T-lymphocytes were grown out of synovial fragments from 14 patients undergoing surgery for advanced destructive inflammatory joint disease. Eleven of the samples were from rheumatoid arthritis patients and three from osteoarthritis. Southern blot analysis of TCRβ chain gene in 13 of 14 cultures showed distinct rearrangements indicating that the cultures were characterised by predominance of a limited number of clones. Evidence of oligoclonality was found in all the 11 cases of rheumatoid arthritis. No evidence of oligoclonality was found in prolonged cultures of peripheral blood lymphocytes from five normal controls. In another study by this group (Chatila et al. 1990) TCRβ gene rearrangement was demonstrated in synovial tissue specimens from 12 patients with rheumatoid arthritis. Identical rearrangements were noted in freshly isolated synovial tissue and the corresponding IL-2 propagated T-cell culture. In one case the same rearrangement was found in samples from five different joints and an identical rearrangement was found in three samples from different joints in

another case, providing evidence of specific homing or selective proliferation of one dominant T-cell clone in synovial tissue. Van Laar et al. (1990) studied cultured T-cell populations from five synovial membranes and two synovial fluids from rheumatoid arthritis patients. Southern blot analysis of TCRβ gene rearrangement showed multiple dominant T-cell clones (one to three) in all the patients, suggesting an oligoclonal origin of the expanded T-cell population in rheumatoid arthritis.

Cooper et al. (1991) showed that T-cells within the actively inflamed rheumatoid arthritis joint are of diverse clonal origin but a small number of clonal multiples and oligoclonal populations are present. Using Southern blotting they analysed 205 clones derived with IL-2 alone or with phytohaemagglutinin directly from four synovial samples obtained by synovectomy, 92% of the clones obtained showed distinct TCR gene rearrangement patterns indicating marked polyclonality. However a few clones (1 quadruplicate and 6 pairs) with identical rearrangements were identified. Other authors have failed to confirm mono- or oligoclonality of synovial T cells in rheumatoid arthritis. Analysis of TCRβ chain gene rearrangement in synovial fluid samples from 15 rheumatoid arthritis patients by Keystone et al. (1988) demonstrated unrearranged (germline) TCR gene fragments of DNA in all of the cases, indicating marked clonal heterogeniety. Duby et al. (1989) showed that only a minor degree of oligoclonality can be demonstrated among T-lymphocytes from synovial fluid. Southern blot analysis of TCR gene rearrangements was performed on 40 rheumatoid arthritis synovial fluid T-cell clones as well as on fresh T-cells from rheumatoid arthritis synovial fluid, rheumatoid arthritis peripheral blood and normal peripheral blood. Identical rearrangement was demonstrated in only two of the clones and the remainder were unique. No dominant clonal T-cell population was observed among normal peripheral blood T-cells or in rheumatoid arthritis samples which had not been cultured. Hylton et al. (1992) studied 19 clones cultured from needle synovial biopsies obtained from seven patients with rheumatoid arthritis. No dominant clones with identical rearrangements of TCRβ gene were detected by Southern blotting indicating marked clonal heterogeneity.

PCR amplification of cDNA has been used in a number of ways to detect specific TCR V genes in synovial lymphocytes from rheumatoid joints. These studies were not carried out to detect monoclonality but to determine whether there is a restricted V gene usage by T-cells as in

experimental allergic encephalomyelitis induced in mice and rats by immunising with myelin basic protein (Acha-Orbea et al. 1988). Conflicting results were obtained (see Table 6.1) and there was no evidence of single dominant monoclonal rearrangement.

In this Chapter I have studied clonal heterogeneity of lymphocytes from rheumatoid joints, using PCR amplified TCRγ gene rearrangements as the clonal markers. Because of the sensitivity of the technique it has been possible to make a detailed comparison of the TCRγ rearrangements present in fresh and cultured synovial fluid and to assess the validity of using cultured T-cells for studies of this kind.

Subjects and Methods

Subjects studied

Four synovectomy specimens removed for treatment of rheumatoid arthritis and 18 synovial fluid samples from 16 cases of rheumatoid arthritis, one of systemic lupus erythematosus (SLE) and one of psoriatic arthritis were obtained from the patients attending the Centre for Rheumatic Diseases, Glasgow Royal Infirmary. Clinical details of age and sex of the patients are given in Appendix 6.1.

Culture of Synovial Fluid T-cells

Synovial fluid T-cells were cultured for 1-4 weeks in the presence of IL-2 seeded at an original cell density of 10^6 cells/ml in round-bottomed tubes in RPMI-1640 with 10% foetal calf serum. Cultures were incubated in 5% CO_2 in a humidified atmosphere at 37ºC. The medium was changed three times a week by half replacement, adding new IL-2 each time. 2 x 10^6 cells were removed for PCR studies at different intervals. The cultures were carried out by Dr. E. Murphy at the Centre for Rheumatic Diseases, Glasgow Royal Infirmary.

DNA Extraction

From Synovial Tissue

High molecular weight DNA was extracted from several hundred 5 micron cryostat sections from each frozen synovectomy specimen

TABLE 6.1 PCR studies of TCR V gene usage bias in rheumatoid arthritis

Reference	Source of cDNA	No. of cases	Biased TCR expression	Clonality
Paliard et al. (1991)	Cultured SF mononuclear cells	9	Vβ14 (9 cases)	Oligoclonal
Sottini et al. (1991)	Uncultured SF T-cells	3	Vβ 7 (2 cases)	
Uematsu et al. (1991)	Cultured SF T-cells	1	Vβ 2, Vβ 3	Polyclonal
Howell et al. (1991)	Cultured SF T-cells	5	Vβ 3, Vβ 14 Vβ 17 (5 cases)	Oligoclonal
Pluschke et al. (1991)	Uncultured SF T-cells	2	Vα14, Vα15, VαFR1 (2 cases)	Polyclonal
Olive, Gatenby & Sergeantson (1991)	Uncultured SM T-cells	5	Vα none Vβ none	Polyclonal
Bucht et al. (1992)	Uncultured SF T-cells	9	Vα1, Vα2, Vα5 Vα8, Vα11, Vα13 and Vα16 (7/9) Vβ none	

SF = Synovial fluid
SM = Synovial membrane

according to the standard protocol for proteolytic digestion, phenol/chloroform extraction and ethanol precipitation and DNA content was determined by UV spectrophotometry as described in Appendix 2.2.

From Synovial Fluid

Mononuclear cells from fresh and cultured synovial fluids were separated and DNA was extracted using the protocol given in Appendix 2.7.

PCR Amplification

PCR was performed with primers specific for 8 Vγ genes and Jγ1/2 (Tables 2..3 and 2.4) using the modified radiolabelled PCR protocol given in Appendix 2.3 for 35 cycles. The reaction products were separated electrophoretically in denaturing 6% polyacrylamide gels and autoradiographs were prepared as described in Appendix 2.6

Results

Detailed results obtained with 8 Vγ and Jγ1/2 primer combinations in 23 cases of arthritis are given in Appendix 6.1 and analysed in Tables 6.2, 6.3 and 6.4.

Synovial membrane

Twenty satisfactory tests were performed on DNA from synovectomy specimens from four rheumatoid arthritis patients. Examples of results are shown in Fig 6.1. No dominant bands were found.

Uncultured Synovial Fluid

Sixty nine satisfactory tests were performed on DNA from synovial fluid from joints affected by rheumatoid arthritis in seventeen patients. DNA from Case RA6 gave no reaction with any of the primers and is excluded from the analysis of results (Table 6.2). Dominant bands were seen in four cases, ++ with Vγ8 in case RA5, ++ with Vγ8 and + with Vγ10 in case RA9 (Fig 6.2), + with Vγ3 and Vγ8 (Fig 6.3) in case RA15 and + with Vγ5 in case RA17. A dominant ++ band with Vγ8 was present in the

TABLE 6.2 Frequency of dominant TCRγ gene rearrangements detected in arthritic joints

Sample	Diagnosis	Patients with dominant bands	PCR tests with dominant bands	
			+	++
SM	RA	0/4	0/20	0/20
SF				
Uncultured	RA	4/16	4/69	2/69
	SLE	1/1	0/6	1/6
	Pso	0/1	0/6	0/6
Cultured	RA	7/14	10/91	0/91
	SLE	1/1	1/6	0/6

+	=	Borderline dominant band
++	=	Unequivocally dominant band
RA	=	Rheumatoid arthritis
SLE	=	Systemic lupus erythematosus
Pso	=	Psoriasis
SM	=	Synovial membrane
SF	=	Synovial fluid

TABLE 6.3 Analysis of paired samples showing effects of in vitro culture with IL-2 on γ gene rearrangements in pathological synovial fluid

Case	DNA Nos.	Period of culture (days)	V2	V3	V5	V8	V9	V10
RA8	299, 302	0 - 4				S		
RA9	297, 306	0 - 5				S		S
RA10	312, 318	0 - 7	G/L					
RA11	313, 317	0 - 7	S			S		
RA12	314, 316	0 -7	L	G/L	G/L		S	G/L
RA13	408, 415	0 - 7		G/L		S		G/L
RA15	409, 414	0 - 7		S		S	S	S
RA16	410, 413	0 - 7		G		S	L	S
RA14	311, 319	0 - 13	L			S		
RA18	290, 304	0 - 14	S	L		S	S	S
RA17	407, 412	0 - 14	L	G/L	L	G/L	G/L	L

Table 6.3 (continued)

Case	DNA Nos.	Period of culture (days)	V2	V3	V5	V8	V9	V10
RA21	330, 332	0 - 26	G	G/L	G/L	G/L	G/L	G/L
RA13	415, 419	7 - 15	G	G	G	G	G/L	G
RA15	414, 418	7 - 15	G/L	G	S	G/L	G	G
RA16	413, 417	7 - 15	S		G/L	S	G	G
RA17	412, 416	14 - 21	S	G/L	S	L	G/L	G

S = Similar
G = Gain in band(s) following culture
L = Loss of band(s) following culture

TABLE 6.4 Effects of duration of culture of synovial fluid cells on band patterns

Duration of culture	Unchanged	Loss of bands	Gain in bands
4 - 7 days	15	6	5
>7 days	8	17	24

Figure 6.1 PCR analysis of TCRγ gene rearrangements in rheumatoid arthritis.
V3-J1/2 rearrangements.
Lanes 1-4: samples of synovial membrane from cases RA1,2,3 and 4. Left MW in nucleotides.
No dominant bands are present.

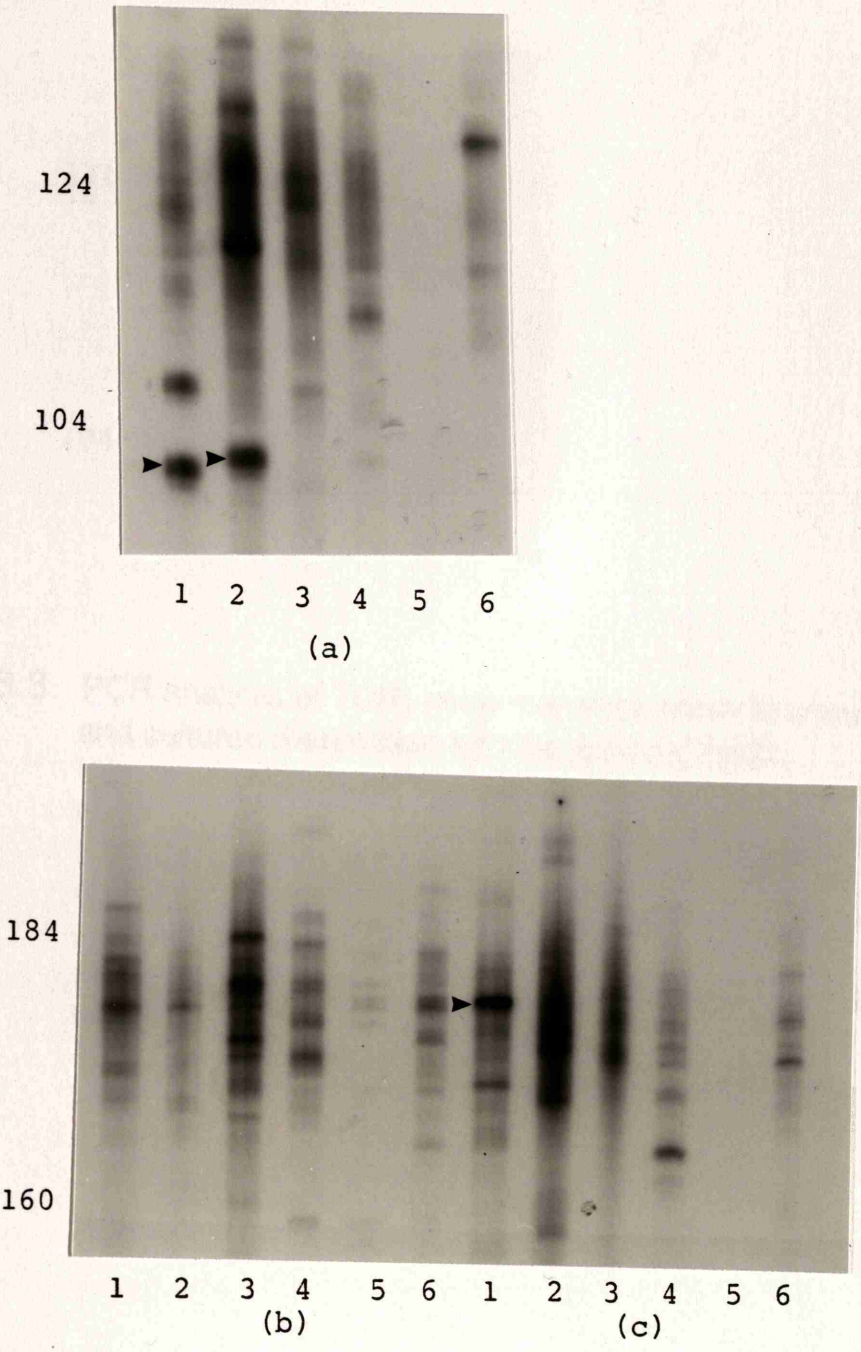


Figure 6.2 PCR analysis of TCRγ gene rearrangements in uncultured synovial fluid from rheumatoid arthritis and SLE patients. (a) V8-J1/2 (b) V9-J1/2 (c) V10-J1/2 rearrangements. Lanes 1-6: cases RA9, LE1, RA8, RA7, RA20 and RA19. Left MW in nucleotides. With V8-J1/2 a dominant ++ band (arrow) is present in case RA9 and case LE1. With V10-J1/2 a dominant + band (arrow) is present in case RA9. Little or no reaction is obtained in case RA20.

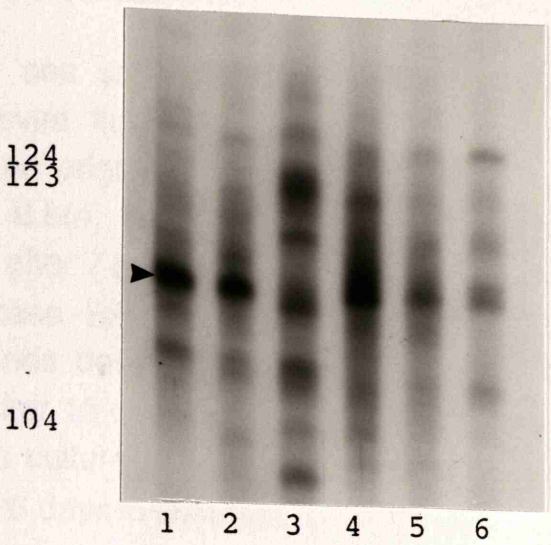


Figure 6.3 PCR analysis of TCRγ gene rearrangements in uncultured and cultured rheumatoid arthritis synovial fluids.
V8-J1/2 rearrangements.
Case RA15. Lane 1: uncultured. Lane 2: cultured 7 days.
Lane 3: cultured 15 days.
Case RA16. Lane 4: uncultured. Lane 5: cultured 7 days.
Lane 6: cultured 15 days. Left MW in nucleotides.
In case RA15 a dominant + bands present in uncultured fluid is found after 7 but not after 15 days culture.

only case of SLE studied (Fig 6.2) and no dominant bands were found in one case of psoriatic arthropathy.

Cultured Synovial Fluid

Ninety one satisfactory tests were performed on 14 rheumatoid arthritis synovial fluids following culture in IL-2 for 4-26 days. The dominant band originally present in case RA9 persisted after 5 days in culture (Fig 6.5(a) lanes 5-6) but those present in cases RA15 had disappeared after 7 (with Vγ3) and 15 (with Vγ8) days culture (Fig 6.3). The + band in case RA17 disappeared following culture for 7 days. New dominant bands developed in cases RA13 (Vγ8 after 15 days culture), RA15 (Vγ3 after 15 days culture), RA17 (Vγ8 after 14 days culture and Vγ3 after 21 days culture) and RA21 (Vγ2 (Fig 6.4a and b) and Vγ8 (Fig 6.5a and b) after 26 days in culture).

In case LE1 the dominant Vγ8 band persisted after culture (Fig 6.5) lanes 7-8). Gains and/or losses of minor bands were frequently encountered in culture (Table 6.3) and are illustrated in Figs 6.3 - 6.5. Table 6.4 suggests that such changes become increasingly common with increasing duration of culture.

Discussion

The hypothesis that benign T-cell neoplasia is the underlying cause of rheumatoid arthritis was tested by looking for dominant clonal TCRγ gene rearrangements in the synovial tissue and synovial fluid from rheumatoid joints.

On PCR amplification most DNA samples produced multiple minor bands or smears of appropriate molecular weight. No dominant bands were detected in uncultured synovectomy specimens from four patients. When uncultured synovial fluids were tested from 16 patients with RA, dominant bands were detected in four (Figs 6.2, 6.3). Two of the dominant bands were categorised ++ and four were borderline +.

The findings of other workers who have investigated the clonal origin of synovial T-cells in patients with rheumatoid arthritis are summarised in Table 6.5. Savill et al. (1987) using Southern blot analysis detected dominant TCRβ rearrangements in 27 per cent (3 of 11) of uncultured

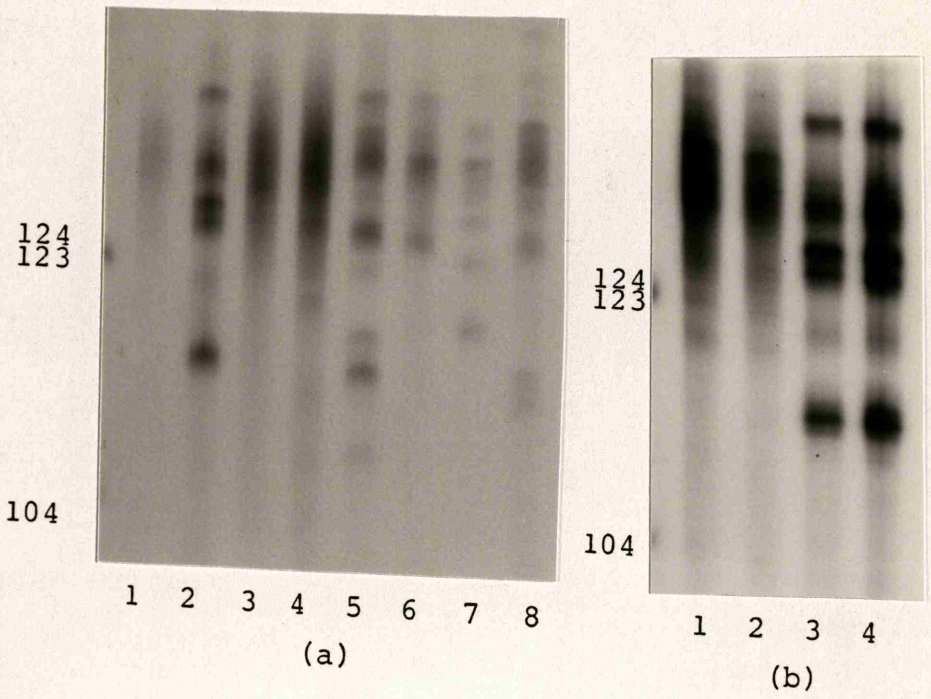


Figure 6.4 PCR analysis of TCRγ gene rearrangements in uncultured and cultured rheumatoid arthritis synovial fluid.
(a) V2-J1/2 rearrangements.
Lane 1,3,5 and 7: uncultured. Lanes 2,4,6 and 8: cultured samples from cases RA21, RA18, RA14 and RA10. Left MW in nucleotides.
Gain of bands in cases RA21 and RA10 and loss of bands in case RA14 is seen following culture.
(b) Repeat testing in duplicate in case RA21.
Lanes 1-2: uncultured. Lanes 3-4: cultured samples.Results in case RA21 are confirmed.

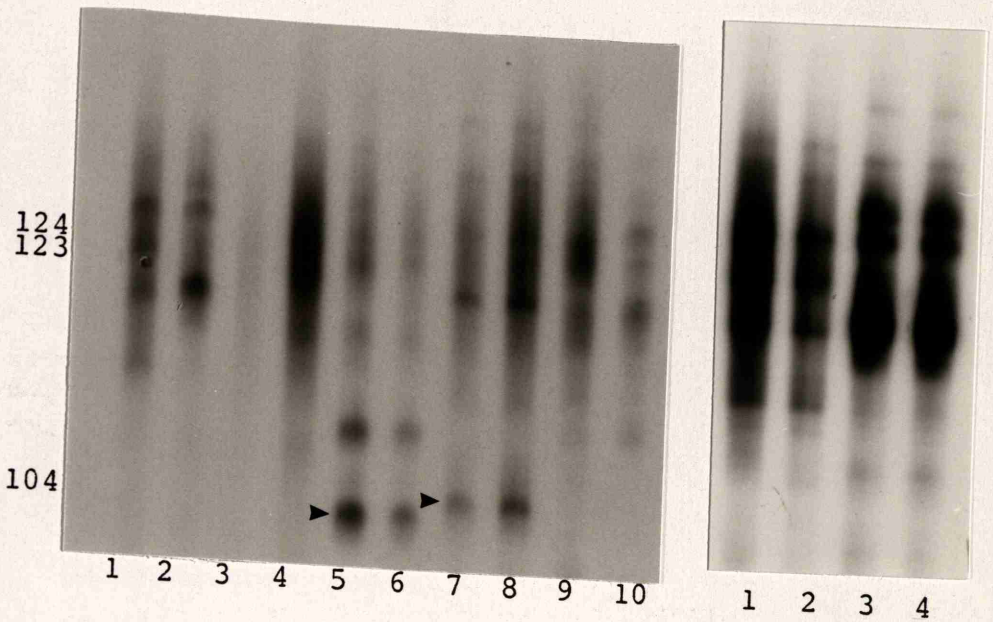


Figure 6.5 PCR analysis of TCRγ gene rearrangements in uncultured and cultured rheumatoid arthritis and SLE synovial fluid.
(a) V8-J1/2 rearrangements.
Lanes 1,3,5,7 and 9: uncultured. Lanes 2,4,6,8 and 10: cultured samples from cases RA21, RA18, RA9, LE1 and RA8. Left MW in nucleotides.
A dominant + band (arrow) is present in RA9 and LE1 in both uncultured and cultured samples. A dominant + band and gain and loss of band is seen in case RA21 following culture. Gain of band is seen in case RA8 following culture.
Note similarity of results to those seen in Fig 6.2a.
(b) Repeat testing in duplicate in case RA21.
Lanes 1-2: uncultured. Lanes 3-4: cultured samples.
Results in Fig 6.5(a) lanes 1 and 2 are confirmed.

TABLE 6.5 Reported frequency (no. of positive cases/no. of cases tested) of rheumatoid arthritis patients with dominant TCRβ or γ gene rearrangements in synovium or synovial fluid

Reference	Method	Type of specimen		No. postive/No. cases tested	
		RA	Control	RA	Control
Savill et al. (1987)	Southern blot β	Uncultured SF	Uncultured PBMC	3/11	0/11
		Cultured SF		1/4	
Keystone et al. (1988)	Southern blot β	Uncultured SF	No control	0/15	
Stamenkovic et al. (1988)	Southern blot β	Cultured SM	Cultured SM from osteoarthritis	11/11	2/3
VanLaar et al. (1990)	Southern blot β	Cultured SM	No control	5/5	
		Cultured SF		2/2	
Chatila et al. (1990)	Southern blot β	Cultured SM	Cultured SM from JRA and SLE	12/12	3/3
Hylton et al. (1992)	Southern blot β	Cultured SM	No control	0/7	
Present study	PCRγ	Uncultured SM and SF	Uncultured SF from SLE and Pso	4/20	1/2
		Cultured SF	Cultured SF from SLE	7/14	1/1

RA = Rheumatoid arthritis
JRA = Juvenile rheumatoid arthritis
SLE = Systemic lupus erythematosus
Pso = Psoriasis
SF = Synovial fluid
SM = Synovial membrane

synovial fluid samples, a finding similar to that in the present study. Keystone et al. (1988) however failed to confirm mono- or oligoclonality of uncultured synovial T-cells in 15 cases of RA. These results differ from those reported by Stamenkovic et al. (1988), Van Laar et al. (1990) and Chatila et al. (1990) who have found dominant TCRβ gene rearrangements in cultured synovial tissue or cultured synovial fluid in 90-100% of cases studied. This has led to the speculation that if dominant T-cell clones are present in cultured synovial samples they are artefacts resulting from the selective growth advantage of particular clones in vitro . It is possible that cells which have recently been activated in vivo may be refractory to further growth stimulation in vitro.

Our pre- and post culture studies show that investigations based on analysis of cultured synovial lymphocytes are likely to be misleading. In only two of our cases did a dominant + band which was present before culture persist in culture. In two cases the dominant bands originally present had disappeared after 7 and 14 days culture. In addition new dominant bands which were not apparent in the uncultured samples were found in these two and four other cases after culture. Minor differences in band pattern were frequently observed in cultures of different duration (Fig 6.3). To our knowledge these studies of PCR amplified TCRγ gene rearrangements provide the first direct evidence of the effects of in vitro culture in IL-2 on the relative abundance of T-cell clones in samples of synovial fluid.

The significance of the dominant bands in uncultured samples from rheumatoid joints in the present study is difficult to assess. It is uncertain whether they reflect γ gene rearrangements in B or T-lymphocytes, since both cell types are present in large numbers in rheumatoid synovium unlike the skin diseases reported in Chapter 5, where lesions are infiltrated almost exclusively by T-cells. A further difficulty is the lack of information about the clones of lymphocytes in clinically unaffected joints from these patients or other individuals and our very limited experience of TCRγ gene rearrangements in other joint diseases (one patient with the non-organ specific autoimmune disease SLE gave a dominant ++ band in uncultured synovial fluid, and one with psoriatic arthropathy gave no dominant bands).

Finally the finding of 4 of 20 rheumatoid arthritis cases with dominant bands in affected joints is probably an underestimate of the true frequency

in view of our testing with an incomplete set of PCR primers. While it is unlikely that dominant bands were present but not detected in the remaining 16 cases, the possibility cannot be excluded that small neoplastic T-cell clones at the benign end of the spectrum are regularly present in the joint lesions in rheumatoid arthritis.

CHAPTER 7

General Discussion

The mechanisms responsible for the initiation and persistent nature of the spontaneously occurring autoimmune diseases and other poorly understood immunological disorders are among the major unsolved problems in immunopathology. In 1958 Burnet postulated that forbidden (autoreactive) clones of neoplastic lymphocytes "approaching or reaching malignancy" may be responsible but this seemed to be so in only a few rare disorders such as chronic cold haemagglutinin disease and mixed essential cryoglobulinaemia in which the pathogenic B-cells are monoclonal and prone to become frankly malignant.

T-lymphocytes are now known to have a key role in inducing and sustaining immune responses. In 1989 Goudie and Lee revived Burnet's hypothesis and postulated that the autoimmune and certain other poorly understood chronic inflammatory diseases may result from the unregulated and inappropriate functional activity of T-cell neoplasms at the benign end of the spectrum. It was suggested that such tumours are presently misdiagnosed as reactive T-cell hyperplasia because neoplastic lymphocytes are migratory cells which do not form circumscribed tumour masses and are morphologically similar to reactive T-cells from which they might be distinguished by their monoclonality. For example the monoclonal T-cell populations found in angioimmunoblastic lymphadenopathy, pityriasis lichenoides et varioliformis acuta and chronic T-cell lymphocytosis with neutropenia may be neoplastic and the underlying cause of the tissue damage which occurs in these diseases. Southern blotting had been used to demonstrate the monoclonality in these conditions and in malignant lymphomas and leukaemias of T-cell origin but the method was not suitable for the study of benign T-cell clones forming only a minority of the cells present in small samples of diseased tissue.

The object of the present study was to devise a sensitive method for the detection of T-cell clones in small tissue samples and to use the method in pilot studies on examples of human diseases in which T-cells are known or thought to be of pathogenic importance.

TCRγ genes were selected as clonal markers since they are clonally rearranged in all T-cells (including those expressing αβ receptors) and can be specifically amplified by PCR with a relatively small set of V and J gene-specific primers. The method was developed and validated with model T-cell lines whose γ gene rearrangements were already known. It was shown that the method is very sensitive and with the appropriate primer combinations can detect nanogram quantities of monoclonal T-cell DNA in the presence of 20-100 parts of polyclonal DNA from reactive (hyperplastic) lymph nodes, monoclonal T-cell DNA giving PCR products which can be visualised as one or two sharply defined dense electrophoretic bands on polyacrylamide gel electrophoresis. When polyclonal DNA from reactive lymph nodes is amplified a smear of PCR products varying in size by ± 20 nucleotides is seen on polyacrylamide gel electrophoresis.

The extent to which the new method can distinguish malignant from reactive T-cell clones in clinical samples was shown in a study of 36 histologically proven T-cell lymphomas and 12 cases of reactive lymph node hyperplasia. Employing a battery of 8 PCRs for the most common TCRγ gene rearrangements on 0.5μg aliquots of DNA dominant clonal bands were found in 67 per cent of the tumours and in only 8 per cent (one in twelve) of the reactive controls. Subjective criteria were used for assessment of the results. It was decided not to use densitometric analysis since much research would be required to take account of factors such as quality of DNA, efficiency of primer combination, batch of Taq polymerase and specific activity of 32P dCTP which are all likely to influence the absolute density of the autoradiographic bands obtained.

In contrast to previous studies with less sensitive techniques such as karyotype or Southern blot analysis in which evidence of monoclonality has seldom been found in the plaque stage of cutaneous T-cell lymphoma, dominant clonal bands were obtained in 2 of 3 patients with this condition. In each case biopsies from two separate lesions and two areas of clinically unaffected skin contained the dominant clonotype, the unaffected skin in smaller amounts. No trace of the neoplastic clonotype was found in the blood of these cases. This is the first demonstration of widespread cutaneous dissemination of the neoplastic clonotype in apparent focal clinical disease. The finding is in keeping with an origin of the neoplasm from skin homing T-cells and provides a likely explanation for recurrence of

the disease at sanctuary sites such as natal and interdigital clefts, which may receive inadequate radiation dosage. As expected, blood and diseased skin from a case of Sezary syndrome (cutaneous T-cell lymphoma plus T-cell leukaemia) showed the same dominant clonotype. In contrast to the lesions of cutaneous T-cell lymphoma, the skin of five controls with no skin disease gave rise to multiple minor electrophoretic bands representing different clonotypes sometimes against a confluent background smear.

The validity of the results is confirmed by their reproducibility on repeated testing of the same specimen, the appropriate molecular size of the PCR products and evidence from nucleotide sequencing (by F. R. Imrie) that individual bands are derived from V-J junctional regions of one or more TCRγ gene rearrangements.

Based on the experience obtained with normal skin and cutaneous T-cell lymphoma, studies were undertaken to investigate the presence of dominant T-cell clones in the lesions of vitiligo, an organ-specific autoimmune disease of epidermal melanocytes and psoriasis, a poorly understood skin disorder in which T-cells are though to have a pathogenic role. As in normal skin multiple bands and smears were found in skin biopsies from vitiligo and psoriasis. A dominant clonal band was demonstrated in only 1 of 28 lesions from 10 patients with vitiligo and in none of 29 lesions from 13 patients with psoriasis. Since only a limited set of primers was used and a number of tests failed it is possible that some dominant clones were overlooked in this study but overall the findings obtained strongly suggest that large clones of neoplastic T-cells are not associated with the lesions of vitiligo and psoriasis. Further tests could have been performed if the available DNA samples had been diluted but this was not done in case it resulted in loss of signals from small neoplastic clones.

It is possible that clones of neoplastic T-cells at the benign end of the spectrum are much smaller than their malignant counterparts and do not give rise to dominant electrophoretic bands. An attempt was therefore made to demonstrate selective localisation of minor clones to the lesions of vitiligo or psoriasis on the premise that selectively localised clones are likely to be implicated in the pathogenesis of the disease with which they are associated. Because of the general tendency of skin homing T-cells to

localise in chronic inflammatory skin lesions, disease-specific localisation was investigated by comparing the clonotypes present in the lesions of vitiligo and psoriasis in patients who simultaneously had both diseases affecting different areas of skin. Of the four patients studied three showed possible examples of a psoriasis-specific and one of a vitiligo-specific band. It is possible that these findings occurred by chance and it remains to be shown whether the bands are mono- or polyclonal and, if monoclonal, whether the clone can be isolated in vitro to study markers of neoplasia and its ability to initiate appropriate lesions when injected into unaffected skin. The findings are thus inconclusive and the possibility has not been excluded that vitiligo and psoriasis are benign variants of cutaneous lymphoma in which small clones of inappropriately functioning neoplastic T-cells are responsible for the development of the lesions.

In the non-organ-specific autoimmune disease, rheumatoid arthritis dominant bands were detected in diseased joints in 4 of 20 cases studied. This study was also performed with a limited set of primers and some of the tests failed. It is therefore possible that some dominant bands were not detected but unlikely that dominant clones are regularly present in vivo in the diseased joints. The interpretation of these findings is difficult due to lack of information about clones present in unaffected or other diseased joints in these patients. Important direct evidence was obtained that in vitro culture in IL-2 has significant effects on the relative abundance of T-cell clones in samples of synovial fluid and that investigations by other workers based on analysis of cultured synovial lymphocytes are likely to have given misleading results.

An interesting incidental finding was the presence of dominant clonotypes in 7 of the 21 blood samples studied including those from normal control subjects and patients with vitiligo or psoriasis. Their significance is unknown but they may reflect the frequent occurrence of clinically inapparent low grade T-cell neoplasia, a possibility which needs further investigation.

The preliminary studies described above have been restricted to T-cell malignancies, vitiligo, psoriasis and rheumatoid arthritis. Additional conditions worthy of investigation for dominant or disease-associated T-cell clones include systemic lupus erythematosus (in which a dominant band was detected in the only specimen studied), other auotimmune diseases

like thyroiditis, gastritis, type I diabetes mellitus and pemphigus and other poorly understood T-cell-associated immunopathological disorders such as multiple sclerosis. A comparison of TCRγ gene rearrangements in diseased joints with those in the blood in chronic T-cell lymphocytosis with neutropenia and in the skin lesions of psoriasis would also be of interest.

APPENDIX 2.1

Selection of Vγ and Jγ gene-specific PCR primers

Original primers

These were selected in the hope that the use of multiple primer combinations in the same PCR reaction mixture would give distinguishable reaction products for each of the common V-J rearrangements and so reduce the number of tests needed for each DNA sample. For example, a mixture of the V2, V8, J1/2 and JP2 strategically located primers shown in Table 2.1 and illustrated in Figs 2.1 and 2.2 would give reaction products which could be separated by electrophoresis according to their molecular size, V2-J1/2 159bp, V2-JP2 135bp, V8-J1/2 109 bp and V8-JP2 85bp (Table 2.2). The estimated PCR product sizes in Table 2.2 assume no junctional deletions or N region sequences and are calculated as follows. Number of nucleotides from 5' end of V primer to 5' end of V gene heptamer sequence (Fig 2.1) plus number of nucleotides from 3' end of J primer to 3' end of J heptamer sequence as illustrated in Fig 2.2.

At the same time an attempt was made to obtain V and J gene specificity by locating primers across sequences which differed from the corresponding parts of homologous genes by as many nucleotides as possible (Figs 2.1 and 2.2).

The composition of each primer (number of nucleotides, percentage of GC) was adjusted to give similar melting points (see Table 2.1) which were calculated according to the following formula.

$$Tm = 0.41\ [Pgc] + 81.5 - 675/L$$

Where Pgc is the percentage of G or C bases in the oligonucleotide (between 30 and 70) and L is the length of the oligonucleotide in bases (Davis, Dibner & Battey 1986).

Modified Primers

The modified primers shown in Table 2.3 and Figs 2.3 and 2.4 were selected for use in pairs, one V-J combination per reaction mixture. Care was taken to locate the 3' end of primers so that the last three bases were gene-specific and differed from the corresponding parts of homologous genes by as many nucleotides as possible. Melting points of complementary hybridization (Table 2.3) and sizes of PCR products (Table 2.4) were calculated as for original PCR primers.

APPENDIX 2.2

Standard protocols for isolation, purification and quantification of DNA

The methods used are based on standard procedures used in the Leukaemia Research Fund Laboratories, Glasgow Royal Infirmary. Special modifications for small samples are given in Appendix 2.7. Buffers and reagents used are given Appendix 2.9.

Step 1: Preliminary treatment of sample

This varies depending on type of sample (suspension culture of T-cells, peripheral blood, unfixed frozen tissues) and in the case of synovial fluid and tissue sample the size of the available specimen.

a) Cultured T-cells

Centrifuge 10ml aliquots of suspension culture at 400g (1900 rpm in MSE bench centrifuge) for 10 minutes at room temperature.

Resuspend pellet in 10ml PBS and repeat centrifugation.

Proceed to Step 2 with pellet.

b) Fresh peripheral blood

Separation of mononuclear cells (T and B-lymphocytes, monocytes, platelets)

Starting material
20ml blood in anticoagulant (heparin, EDTA, or citrate dextrose). May be kept overnight at 4ºC but then requires rotation mixing for 15 minutes before use.

Put approximately 10ml PBS in two graduated universal containers, then add 10ml whole blood, to each. Mix gently by inversion.
Put 6ml Lymphoprep in each of 5 Falcon 15ml tubes and layer on 8 ml of blood - PBS mixture with Pasteur pipette and rubber bulb, taking care not to mix layers. Five tubes required for 20ml blood.

Centrifuge at 400g for 30 minutes .
Discard upper (serum) layer. Transfer mononuclear cell-rich serum/lymphoprep interface (about 3ml) to 4 fresh 15ml Falcon tubes sucking round the wall of the tube with Pasteur pipette and bulb. Discard remaining Lymphoprep, red cells and granulocytes at bottom of the tube.

Add PBS to 14ml mark on tube.
Centrifuge at 400g for 10 minutes.
Remove PBS with Pasteur pipette and bulb without disturbing pellets, resuspend cells by flicking tubes then add 2ml PBS to each tube and transfer all cells to one of the tubes. Make up to 14ml with PBS.
Centrifuge at 400g for 10 minutes.
Proceed to Step 2 with pellet.

c) Frozen tissue

Cut 5-10micron sections by cryostat at -20ºC and collect in a disposable plastic tray.
Transfer 100-500 sections in 15ml Falcon tubes.
Proceed to step 2.
To avoid cross contamination of samples use disposable scalpels and forceps, clean microtome chucks by scrubbing and microtome blades carefully by wiping with acetone between samples. Label tubes with permanent ink.

Step 2: Lysis and digestion

Add 5.5ml 0.2M sodium acetate pH7. Shake or whirlmix.
Add 375 µl 10% SDS. Shake or whirlmix.
Add 150µl proteinase K (10mg/ml) (Stored at - 20ºC).
Shake or whirlmix.
Incubate at 56ºC for 1 hour or overnight at 37ºC.

Step 3: Extraction of DNA

Using disposable plastic pipette add 6ml Phenol/chloroform/-isoamyl alcohol (25:24:1) mix well for 5 minutes.
Centrifuge at 600g for 2 minutes.
Holding tube at 45º transfer clear supernatant (with DNA) with a pipette into 50ml Falcon tube. If any precipitate from interface is accidentally transferred then repeat. Dispose of phenol in stoppered tube by incineration.

Step 4: Precipitation of DNA

Add 2½ volumes (approx 15ml) absolute alcohol.
DNA precipitates at once.
Remove DNA with sealed Pasteur pipette, squeeze precipitate against side of tube to remove alcohol and allow to dry in air for 5 minutes.

Dip pipette tip with DNA in chloroform and dry in air for 5 minutes.
Place tip of pipette with DNA precipitate in Eppendorf tube with 200µl TE buffer x 1.
Seal with parafilm and leave at room temperature overnight for DNA to redissolve.
Store DNA at 4ºC.

Step 5: Measurement of concentration of DNA

DNA quantitation is done with standard spectrophotometric analysis at 260nm and 280nm. The ratio between the reading at 260nm and 280 nm provides an estimate of the purity of the DNA extracted.

Sometimes measurement of concentration of DNA is difficult because of the high viscosity and poor mixing of DNA in TE buffer.

Procedure

Pipette 10µl of DNA in 1.5 ml Eppendorf tube containing 500µl of water.
Read in spectrophotometer at 260nm and 280nm.

Calculation:
At 260nm 1 unit of OD = 45µg DNA/ml.
DNA µg/ml = reading x 45 x 50.

Note: The ratio of 260:280 reading is ideally 1.8. If less than 1.3 there is excess of protein in the DNA preparation.

APPENDIX 2.3

PCR methods

For buffers, reagents and equipment used see Appendix 2.9.

1. Original PCR protocol (IBI)

Reaction mixture

50µl reaction mixture consisting of :

	Components	Amount added/finalconcentration
a)	Deionized water	28µl
b)	Template DNA	1µl (1 µg)
c)	Primer (1)	1µl/50µM
d)	Primer (2)	1µl/50µM
e)	dNTP	4µl/2.5mM (each)
f)	Taq reaction buffer x 10	5µl/x1
g)	Taq Polymerase	10µl (4 units)

Reactions are performed in 50µl of reaction mix in 1.5ml capped Eppendorf tubes.Sterile tubes and pipette tips are used all the time. dNTPs are prepared as individual 25mM stock solutions of each type (dATP, dGTP, dTTP, dCTP) in distilled water. Aliquots are stored at -20ºC. Working solutions are prepared by diluting to 2.5mM from thawed, well stirred vials of the stock. Taq polymerase and other recently frozen reagents are vortexed and spun down briefly.

As far as possible master mix of reagents for samples (water, Taq reaction buffer, dNTP, primers) are prepared and aliquoted and the mixtures are pipetted into individual reaction tubes. When this is not possible and one of the constituents has to be added by itself (eg. when each reaction tube contains a different DNA sample) the variable constituent is diluted with water to give a minimum pipetting volume of 5µl.

To reduce evaporation or refluxing, the mixture is overlaid by 2 drops (~50µl) of liquid paraffin.

The tubes are heated for initial denaturation of DNA at 99ºC for 5 minutes on the thermalcycler. Taq DNA polymerase is added to the tubes and the tubes are spun down briefly on a centrifuge. The tubes are then subjected to 30 PCR cycles (Appendix 2.4).

2. Modified PCR protocol for hot start procedures

In this method the starting reaction mixture (water, dNTP, primers, Taq reaction buffer, DNA template) is the same as in original PCR protocol but it is prepared on ice to avoid mispriming of target DNA.

Eppendorf tubes containing 40µl of starting reaction mixture are layered with liquid paraffin and heated at 99ºC for 5 minutes on the thermalcycler. At the end of this time the temperature of the tubes is brought to 69ºC and held there. The bulk working dilution of Taq polymerase (reduced to 2 units/10µl of H_20) is then heated to 69ºC for 1-2 minutes and 10µl aliquot pipetted into the starting reaction while the tubes are still in the thermalcycler. This is done by quickly sliding the pipette tip down the side of the tube through liquid paraffin to the bottom of the tube and pipetting up and down a few times to mix the reagents, withdrawing the tip from the tube along the side with the pipette depressed. The liquid paraffin layer is not disturbed, and tubes are not taken out of the heating block for centrifugation. The tubes are then subjected to 30 PCR cycles (Appendix 2.4).

3. Modified radiolabelled PCR protocol

Reaction mixture

25µl of reaction mixture consisting of:

	Components	Amount added/final concentration
a)	Deionised water	To 25µl
b)	Template DNA	5µl (~ 0.5µg)
c)	Primer (1)	0.5µl/0.8µM
d)	Primer (2)	0.5µl/0.8µM
e)	dATP, dTTP, dGTP	2µl/200µM
f)	Taq reaction buffer x 10	2.5µl/x l
g)	Taq polymerase	0.25µl (0.25 units)
h)	dCTP mixture	
	(cold dCTP)	2µl/4 µM
	(32P dCTP)	0.4µCi

Reactions are performed in 25µl of reaction mix in Eppendorf tubes. Master mix of reagents for samples water, Taq reaction buffer, dNTP (dATP, dTTP and dGTP) and primers are prepared and aliquoted and the mixture is pipetted into individual tubes with 5µl of DNA sample.

The mixture is overlaid with 2 drops of liquid paraffin and the tubes centrifuged briefly. The tubes are then heated at 99ºC for 5 minutes after that the temperature is then brought to 69ºC and held there.

dCTP mixture is prepared by adding 49µl of cold dCTP (50µM),and 1 µl (10mci/ml) of 32P dCTP. The bulk working solutions of Taq polymerase, dCTP mixture and reaction buffer are heated to 69ºC for 1-2 minutes, 10µl aliquots are pipetted into other reagents held at 69ºC as described in modified PCR protocol for hot start procedure. The tubes are then subjected to 30 PCR cycles (Programme 9 (Appendix 2.4)) when DNA is from large amount of starting sample and 35 cycles (Programme 10 (Appendix 2.4)) when the DNA samples are very small as from skin biopsies and fresh and cultured synovial fluid cells, DNA concentration is not measured.

APPENDIX 2.4

PCR - Cycle programmes

Programmes 1,2 and 3

Comparison of annealing temperatures. Cold start. Taq polymerase added at hold phase and tubes centrifuged briefly at room temperature before cycling.

	Denaturation	99ºC	5 minutes
	Hold	65ºC	
	(Denaturation	94ºC	1 minute
30 cycles	(Annealing	64ºC	2 minutes (Programme 1)
		62ºC	2 minutes (Programme 2)
		60ºC	2 minutes (Programme 3)
	(Extension	72ºC	2 minutes

Programme 4

Sampling of PCR products after 30, 40 and 50 cycles. Cold start. Taq polymerase added at hold phase and tubes centrifuged briefly at room temperature before cycling.

	Denaturation	99ºC	5 minutes
	Hold	65ºC	
	(Denaturation	94ºC	1 minute
30 cycles	(Annealing	60ºC	2 minutes
	(Extension	72ºC	2 minutes
	(Hold	60ºC	Sample PCR product
	(Denaturation	94ºC	1 minute
10 cycles	(Annealing	60ºC	2 minutes
	(Extension	72ºC	2 minutes
	(Hold	60ºC	Sample PCR product
	(Denaturation	94ºC	1 minute
10 cycles	(Annealing	60ºC	2 minutes
	(Extension	72ºC	2 minutes

Programmes 5 and 6

Comparison of duration of extension time. Taq polymerase added before initial denaturation of DNA.

	Denaturation	94ºC	5 minutes
	(Annealing	62ºC	2 minutes
30 cycles	(Extension	72ºC	12 seconds (programme 5)
	(		2 minutes (programme 6)
	(Denaturation	94ºC	1 minute

Programmes 7, 8 and 9

Comparison of annealing temperatures. Hot start. Taq polymerase added at 69ºC without centrifugation.

	Denaturation	99ºC	5 minutes
	Hold	69ºC	
	(Denaturation	94ºC	1 minute
30 cycles	(Annealing	64ºC	1 minute (programme 7)
		63ºC	1 minute (programme 8)
		62ºC	1 minute (programme 9)
	(Extension	72ºC	12 seconds

Programme 10

For amplification of DNA from skin biopsies and small samples of DNA from fresh and cultured synovial cells. As for programme 9 but 35 cycles.

APPENDIX 2.5

Agarose gel electrophoresis

For buffers, reagents and equipment used see Appendix 2.9

Preparation and running

2.5g of agarose in 100ml of TBEX1 buffer.
Dissolve in microwave oven for 2-3 minutes with occasional stirring.
Allow to cool to handling temperature (60ºC) and add 10µl ethidium bromide.
Mix thoroughly and pour the gel solution into the taped tray and comb(s) placed into the slots with the flat side of the comb facing the nearest end of the unit.
Allow to set for 20 minutes. When gel is solidified and fully opaque remove the tape from the ends of the tray.
Place the cast gel into the unit.
Fill the unit with buffer TBEX1 (~ 800-900ml) covering the gel to a depth of 1-5mm above the gel surface carefully remove the comb with a gentle wiggling upward motion, and add 40ul of ethidium bromide into the tank.
Add blue dye (1/10 the volume of PCR sample) into the samples, load the samples with a Gilson pipette (taking care not to puncture bottom of wells).
Close the cover and connect power leads with negative lead to the same end of unit as the sample wells.
Run the gel at 100V for about 1 hour.
Note proposed slots for each PCR samples.
To photograph the gel place the gel on the transilluminator and photograph under UV light.
Note: Ethidium bromide is carcinogenic. Gloves should be worn all the time while handling gel.

APPENDIX 2.6

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis is carried out following manufacturers instructions on 2010 macrophore sequencing system (Pharmacia).

Preparation of 6% denaturing polyacrylamide gel

Solutions, buffers and reagents used for preparation of 6% denaturing polyacrylamide gel are given in Appendix 2.9. The gel is cast by the sliding plate method using a 55cm long notched glass plate and 0.4mm thick spacers.

1. Bind silane treatment of notched glass plate

Place the clean 55cm long notched glass plate with small bevelled area facing upwards in a fume hood.
Using a lint free tissue spread bind silane solution (Appendix 2.9) evenly over the top surface of the plate and leave to dry for 1-2 minutes.
Rinse the plate with ethanol, while still wet, polish with another tissue. Leave to dry once more before giving a final polish with another tissue.

2. Repel-silane treatment of the thermostatic plate

Place the clean thermostatic plate on macromould gel casting table, so that protruding edge of the plastic frame is turned downwards.
Using a lint free tissue spread approximately 5ml of Repel-silane over the entire top surface of the plate and leave to dry for a few minutes.
Polish the plate with a fresh tissue and repeat the Repel Silane treatment. After the second polish rinse the plate with ethanol and while it is still wet, polish the plate once more. Leave to dry for a few minutes and then polish with another lint free tissue.
Use a spirit level to ensure that the thermostatic plate is level and check that the table does not rock.

3. Preparation of 0.4mm thick 6% gel

Place the spacer strips (0.4mm thick) on the thermostatic plate with the cut out sections at the end closest to the moveable plate support and facing outwards. Carefully align each spacer against the inside edge of the plastic rim down the side edges of the plate and the inside edge of the plastic frame at the top of the plate.

Secure each spacer with clamps at approximately 10cm intervals,using the clamp at the top end of the thermostatic plate to prevent gel solution polymerising under the comb when it is inserted later.
Incline the support at the low position by lifting it at the end opposite to the moveable plate support and securing with the angle bracket.
Place the notched glass plate with Bind-Silane treated face downwards between the clamps on thermostatic plate.
Draw both notched glass plate and moveable plate support backwards, so that only half of the notched plate rests on the side spacers. Continue to draw back the notched plate alone so that only 3-4cm of the plate remains resting on the spacers.
Pour the gel solution (Appendix 2.9) onto the thermostatic plate. Apply slight pressure on the glass plate and allow the space between the notched and thermostatic plates to fill to the bottom of the side spacers with gel solution. Then start to slide the notched plate uphill pouring more gel solution. Continue to apply slight pressure on the glass plate while sliding the plate.
When gel casting is complete i.e. when the ears of the notched plate have reached the top of the side spacers, stop sliding and quickly return the thermostatic plate to horizontal position by releasing the angle support bracket.
Clamp the lower end of the notched plate and thermostatic plate. Carefully insert the sample application comb to a depth not more than the width of the teeth. Then use the remaining clamps to hold the two plates.
Finally attach the comb clamp positioning it directly over the teeth of the sample application comb.
Leave the gel to polymerise for 15-20 minutes.

4. Pre-electrophoresis

Place the gel in the macrophore unit.
Switch on the thermostatic circulator and set it to run at +50ºC.
Pour TBEX1 buffer into the reservoires of electrophoresis tanks.
Remove the comb and thoroughly wash out the wells in order to remove unpolymerised gel solutions and excess urea.
Run the gel for 20 minutes at approximately 1800-2000 volts. This pre-electrophoresis step removes any charged contaminants from the gel as well as allowing the gel to come to temperature.

5. Sample preparation

While the pre-electrophoresis step is being performed the samples should be prepared.

Add 10µl of formamide dye mix (Appendix 2.9) to each PCR sample.
Heat samples at 99ºC for 2-3 minutes.

6. Loading and running the samples

Once the temperature of the circulating water bath has reached the required level, switch off the high voltage power supply and open the macrophore unit.
Rinse out the sample wells again.
Immediately load the samples into the well using a drawn-out micropipette.
Once all the sample are loaded, start the run at constant voltage of 2000 volts for two hours.
Note proposed slot for each PCR sample.

7. Autoradiography

After electrophoresis is complete the radiolabelled PCR products are detected by autoradiography using a film.

Before performing the autoradiography step the urea must be washed out of the gel.

Removal of urea

Place the class plate with the gel still attached, in a box containing approximately 2 litres of 10% V/V acetic acid. Agitate for 15 minutes to remove the urea.

Drying of the gel

Dry the gel with a hot air drier. Make sure the gel is completely dry before the film is applied.

Application of the film

Take the dried gel attached to the glass plate to the dark room. Place the right size film on the gel, and cover with the second glass plate. Clamp the glass plates together. Place the plates into the exposure box and fit the light-proof lid.

Exposure

The film should be exposed at room temperature for 6-20 hours. The film is then developed.

Preparation of 6% non-denaturing polyacrylamide gel

Solutions used for preparation of 6% non-denaturing polyacrylamide gel are given in Appendix 2.9.

Prepare the gel by the sliding plate method using 55cm long notched glass plate and 0.4mm thick spacers according to the method described above.

Leave the gel to polymerise for 15-20 minutes
Place the gel in the macrophore unit
Pour the TBEX1 buffer into the reservoirs of electrophoresis tanks.
Remove the comb carefully. Immediately rinse the wells with TBEX1 buffer.
Mix the PCR samples with 10µl of blue juice (Appendix 2.9), load the mixture onto the wells, using a drawn-out micropipette.
Once loading is complete connect the electrodes to the power pack. Run the gel at 1800-2000 volts for 1½ hours.
When electrophoresis is complete, turn off the electric power. Remove the glass plates from the unit. Detach the plates using a thin spatula.
Submerge the gel attached to glass plate in ethidium bromide staining solution, just enough to cover the gel completely. After staining for 30-45 minutes at room temperature. Carefully blot excess liquid from the surface of the gel, cover the gel with a piece of saran wrap avoiding to create air bubbles or folds in the saran wrap.
To photograph the gel place the gel on the transilluminator and photograph, under U/V light.

APPENDIX 2.7

Modified protocol for isolation and purification of DNA for small samples of fresh and cultured synovial fluids and skin biopsies

For buffers and reagents used see Appendix 2.9.

Step 1: Preliminary treatment of sample

a) Fresh synovial fluid

Centrifuge heparinised aspirates of synovial fluid at 1500g for 20 minutes at room temperature to isolate the constituent T-cells.
Resuspend pellet in equal volumes of PBS.
Isolate mononuclear cells by density gradient centrifugation on Lymphoprep as for mononuclear cells from peripheral blood (Appendix 2.2). Proceed to Step 2.

b) Cultured synovial fluid

Centrifuge 1ml aliquots of suspension culture in 1.5ml Eppendorf tubes at full speed on bench microfuge for 20 minutes at 4ºC.
Resuspend pellet in equal volume of PBS and repeat centrifugation.
Proceed to Step 2 with pellet.

c) Skin biopsies

Cut 5-10 micron section by cryostat at -20ºC and collect in a disposable plastic tray.
Transfer 25-50 sections in each of 4 Eppendorf tubes.
Label tubes with permanent ink.

Step 2: Lysis and digestion

Into each sample tube add,
1ml of 0.2M sodium acetate pH7.
Shake or whirlimix.
75µl of 10% SDS. Shake or whirlimix.
30µl of proteinase K (10mg/ml). Shake or whirlimix.
Incubate at 37ºC overnight.

Step 2: Extraction of DNA

Add an equal volume of phenol/chloroform/Isoamyle alcohol (25:24:1). Mix well for 5 minutes.
Centrifuge slow on bench microfuge for 20 minutes at 4ºC.
Collect upper aqueous layer, avoiding debris. Discard organic phase.

Step 4: Precipitation of DNA

Add 2 volumes of cold ethanol. Allow to precipitate at -20ºC for 1-2 hours or overnight.
Centrifuge at full speed on bench microfuge for 20 minutes at 4ºC
Pour off ethanol
Cover tubes with parafilm and make several holes with syringe needle (to minimise cross contamination).
Dry pellet for 30 minutes in vacuum desiccator.
Redissolve pellet in TEX1 buffer (~ 10µl per tube but vary according to size of precipitate).
Allow to dissolve overnight at room temperature.
Transfer all DNA to one of the tubes. Make up to 50-100ul with distilled water.

Note: Dilutions should be done in distilled water since excessive EDTA (in TE buffer) will chelate Mg2+ in the PCR buffer.
Use aliquots of the DNA sample for each amplification (typically 4-5µl).

APPENDIX 2.8

Preparation of P32 labelled PBR322-HaeIII digested DNA marker

1. Digestion of PBR-322 DNA -20µg

a)	10X NBL reaction buffer 8	20µl
b)	PBR-322 DNA	
c)	H_20 to final volume	200µl
d)	HaeIII (10u/µl)	5µl

Incubate for 2 hours at 37ºC and check for complete digestion by removing 0.5µg aliquot and running on 2% agarose mini-gel.
If not complete add further 5µl enzyme and incubate for a further 1 hour.

2. Bacterial alkaline phosphatase treatment of digested DNA.
Add 0.5 ul alkaline phosphatase + 20 ul reaction buffer x 5. (BRL)
Add 1/10 volume of 3M Na acetate pH7.0
Add 2½ volumes ethanol.
Allow to precipitate at -20ºC for 1-2 hours.
Centrifuge at full speed on bench microfuge for 20 minutes at 4ºC.
Pour off ethanol.
Dry pellet for 30 minutes in vacuum desiccator.
Resuspend pellet in 50mM Tris pH8.
Incubate for 30 minutes at 37ºC.
Remove phosphate by phenol extraction.
Recover DNA by ethanol precipitation.
Resuspend pellet in TE buffer 0.1mg/ml.

3) Forward reaction for 5' end labelling.

Solutions required:

1) 10X Kinase buffer

   500mM Tris, pH7.4
   100mM Mg Cl_2
   50mM DTT
2) Polynucleotide kinase
3) 10 mCi/ml $\gamma\ ^{32}P$ ATP
4) Dephosphorylated HaeIII cut PBR-322

Make up the following reaction mix:

a)	H_20	11.5µl
b)	Kinase buffer x 5	5µl
c)	10mCi/ml γ 32P ATP	2.5µl
d)	0.5µg dephosphorylated HaeIII cut PBR-322	5µl
e)	6 units polynucleotide kinase	1µl

Mix and incubate for 50 minutes at 37ºC.
Inactivate the enzyme for 5 minutes at 65ºC.
Dilute to appropriate concentrtion for use.

APPENDIX 2.9

List of buffers and commercial sources of reagents and equipments

TBE buffer (10X) (to make 1 litre)
pH7-8

Tris base (IM)	108g
Boric acid (IM)	55g
EDTA-Na_2.H_20 (20mM)	9.5g
H_20 to 1 litre	

TE buffers (10X) (to make 1 litre)
pH7.4-7.6

Tris base (100mM)	2.11g
EDTA-Na_2.$2H_20$ (10mM)	25ml of 0.4M stock

Blue dye (final concentration)

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Ficol FF	15%

Ethidium bromide

10mg/ml of H_20

NIB buffer (IX) (Final concentration)

KCl	50mM
Tris-pH8.3	10mM
MgCl	1.5mM
Gelatin	100µg/ml
NP40	0.45%
Tween 20	0.45%

Taq reaction buffer (1X)
(Final concentration)

Tris-HCl pH8.3	10mM
KCl	50mM
$MgCl_2$	1.5mM
Gelatin	0.01%
Tween 20	0.01%
NP 40	0.01%

10% Ammonium persulphate solution (to make 5ml)

Ammonium persulphate	0.5g
H_2O	to 5ml

Formamide dye mix

Formamide	100ml
Amberlite MB-1	5g
Stir gently for 30 minutes	
Filter to remove resin. Add,	
Bromophenol blue	0.03g
Xylene cyanol FF	0.03g
EDTA-Na_2.2H_2O	0.75g

Bind-silane solution

Absolute ethanol	20ml
10% acetic acid	5ml
Bind-silane	75µl

Acrylamide stock solution (to make 250ml)

Acrylamide	95g
NN'-Methylenebis acrylamide	5g
H_2O	to 250ml

Polyacrylamide gel recipes

(a) 6% denaturing gel

Urea	42gm
Acrylamide stock solution	14.5ml
10 X TBE buffer	10ml
H_2O	40.5ml

Dissolve urea
Make up to 99.2ml with distilled H_2O
Filter through 0.45µm pore size membrane
Add (just before casting gel)

10% Ammonium persulphate	0.8ml
TEMED	80µl

(b) 6% non-denaturing gel

Acrylamide stock solution	14.5ml
10 X TBE buffer	10ml
H_20	99.2ml
Add just before casting gel,	
10% ammonium persulphate	0.8ml
TEMED	80µl

Nick translation buffer (10X)

	(Final concentration)
Tris pH7.2	0.5M
Mg SO4	0.1M
Dithiothreitol	1mM
BSA (fraction 5)	500µg/ml

Commercial sources of reagents and equipment

Lymphoprep

Nycomed Pharma AS,
Oslo, Norway

Proteinase K, dNTP's and Taq DNA Polymerase

IBI Cambridge

32P dCTP

Amersham International
Buckinghamshire, UK.

Primers

Oswel DNA Service,
Edinburgh

Molecular weight marker V, PBR 322 DNA and Hae III

Northumbria Biological Ltd. UK.

Thermal cycler

Hybaid Middlesex

Macrophor sequencing system

Pharmacia LKB Biotechnology
Sweden

Spectrophotometer (PU8620)

Philips, UK.

Multipurpose horizontal gel electrophoresis unit

IBI Cambridge

Centrifuge (L201M, 10205)

Howe-Sigma
Poole,
Dorset

APPENDIX 3.1

Detailed results of tests for dominant TCRγ gene rearrangements in biopsies of T cell lymphomas

Case No.	Age/ Sex	DNA No.	Diagnosis	PCR analysis V2	V3	V4	ΨV5	V8	V9	V10	V11	Southern blot analysis
L1	F	40	T-NHL	-	-	-	-	++*	-	-	-	ND
L2	34/F	53	T-cell lymphoma	-	+	-	-	+*†	-	-	-	ND
L3	NK	54	CTCL	-	-	-	+	++*†	-	-	-	ND
L4	75/F	62	Pleomorphic T-cell lymphoma	-	+	-	-	++*†	+	-	-	ND
L5	20/F	63	Pleomorphic T-cell lymphoma	++†	-	-	-	+*†	-	-	-	ND
L6	16/M	83	T-cell lymphoma	-	-	-	-	+†	-	-	-	ND
L7	62/M	107	AIL	-	++	-	-	-	+†	-	-	TCRβ2R TCRγ R

Appendix 3.1 (continued)

Case No.	Age/ Sex	DNA No.	Diagnosis	PCR analysis V2	V3	V4	ΨV5	V5	V8	V9	V10	V11	Southern blot analysis
L8	43/M	196	TNHL	-	-	-	-		-	-	-	-	No clonal R TCRβG
L9	35/F	200	TNHL	-	+	-	-		+	-	-	+	TCRβ1R TCRγR
L10	70/F	201	TNHL	NR	NR	NR	NR		NR	NR	NR	NR	TCRβ1R TCRγR
L11	15/F	195	TNHL	++	-	-	-		-	-	-	-	TCRβ1R TCRγR
L12	46/M	PP4	T-Large cell lymphoma	-	-	NR		-	-	+†	++†	-	V10
L13	58/F	PP5	CTCL	-	-	NR		-	-	++†	-	-	V2 or 4 and V9
L14	7/F	PP6	Cutaneous lymphoblastic T-cell lymphoma	-	-	NR		-	-	-	-	-	No clonal R
L15	59/M	PP7	Pleomorphic T-cell lymphoma	-	-	NR		-	-	++†	+†	-	No clonal R

Appendix 3.1 (continued)

Case No.	Age/ Sex	DNA No.	Diagnosis	PCR analysis V2	V3	V4	V5	V8	V9	V10	V11	Southern blot analysis
L16	57/M	PP8	Skin T-cell lymphoma	ND	-	NR	-	ND	++†	-	-	No clonal R V9
L17	58/M	PP9	Pleomorphic T-cell lymphoma of skin	-	-	NR	-	-	-	-	-	No clonal R
L17	58/M	F/10	Pleomorphic T-cell lymphoma of skin	-	-	NR	-	-	-	-	-	V3
L18	62/F	RR1	Pleomorphic T-cell lymphoma of skin	-	-	NR	-	-	-	-	-	V11 or V9
L19	64/M	RR4	Pleomorphic T-cell lymphoma	-	+†	NR	-	-	-	-	+	V2 orV4
L20	NK	NN6	EATL	++	-	NR	-	++	-	-	ND	V2 and V8
L21	50/M	HH4	Cerebriform T-cell lymphoma	++	-	NR	-	-	-†	-	+	V2 or V4 and V11
L22	NK	D1	T-cell lymphoma	-	-	+†	-	-	-	-	+	V4

Appendix 3.1 (continued)

Case No.	Age/ Sex	DNA No.	Diagnosis	PCR analysis V2	V3	V4	V5	V8	V9	V10	V11	Southern blot analysis
L23	40/M	F5	Pleomorphic T-cell lymphoma	-	-	NR	-	-	-	-	-	V3 and V2 or V11
L24	20/M	F6	T-ALL	-	-	NR	++	-	-	+	-	V5 and V10
L25	54/M	F7	Pleomorphic T-cell lymphoma	-	+	NR	+	-	-	-	-	Unclear
L26	NK	F8	T-cell lymphoma	-	-	NR	+	-	-	-	-	No clonal R
L27	59/M	F9	T-zone lymphoma	++	-	NR	-	-	-	-	-	V2 orV4
L28	M	HH3	EATL	-	-	NR	-	-	-	-	-	V3 and V5
L29	NK	003	EATL	-	-	NR	+	+	++	NR	-	V8 and V9
L30	29/M	QQ10	Large T-cell lymphoma of skin	-	-	-	-	-	-	-	-	No clonal R

Appendix 3.1 (continued)

Case No.	Age/ Sex	DNA No.	Diagnosis	PCR analysis V2	V3	V4	V5	V8	V9	V10	V11	Southern blot analysis
L31	NK	RR2	EATL	-	-	-	-	-	-	-	-	No clonal R
L32	NK	RR3	Pleomorphic T-cell lymphoma	-	-	-	-	-	-	-	-	No clonal R

-	=	No dominant band	T-NHL	=	T-cell non-Hodgkin's lymphoma
+	=	Borderline dominant band	CTCL	=	Cutaneous T-cell lymphoma
++	=	Unequivocally dominant band	AIL	=	Angioimmunoblastic lymphadenopathy
NR	=	No reaction	EATL	=	Enteropathy associated T-cell lymphoma
ND	=	Not done			
NK	=	Not known			
*	=	Contamination			
†	=	Tests repeated			
R	=	Rearranged			
G	=	Germline			

APPENDIX 3.2

Detailed PCR results with Jγ1/2 and 8 Vγ primer combinations in biopsies of reactive lymph nodes

Case No.	Age/ Sex	DNA No.	V2	V3	V4	V5	V8	V9	V10	V11
RN1	55/F	404	-	-	NR	-	-	-	NR	-
RN2	65/F	405	-	-	NR	-	-	-	-	-
RN3	46/M	406	-	-	NR	-	+	-	-	-
RN4	71/F	438	NR	NR	-	-	-	-	-	-
RN5	80/F	439	-	NR	-	-	-	-	-	-
RN6	31/F	440	NR	NR	-	NR	-	-	-	-
RN7	NK	441	NR	NR	-	-	-	-	-	NR
RN8	NK	442	NR	NR	-	-	-	-	-	-
RN9	92/F	443	-	NR	-	-	-	-	-	-
RN10	NK	444	-	-	-	-	-	NR	-	-

Appendix 3.2 (continued)

Case No.	Age/ Sex	DNA No.	V2	V3	V4	V5	V8	V9	V10	V11
RN11	85/F	445	-	-	-	NR	-	-	-	-
RN12	72/F	446	-	NR	-	-	-	NR	-	-

-	=	No dominant band
+	=	Borderline dominant band
NR	=	No reaction
NK	=	Not known

APPENDIX 4.1

Detailed PCR results with primers for Jγ1/2 and 8 Vγ genes in patients with CTCL

Case No.	DNA No.	Sample	V2	V3	V4	V5	V8	V9	V10	V11
CL1	287	PBMC	-	-	-	NR	-	-	-	ND
	293	Unaffected skin	-	-	-	NR	-	-	-	ND
	294	Unaffected skin	NR	-	-	NR	-	-	-	ND
	295	Lesion	+	-	-	NR	-	-	++	ND
	296	Lesion	+	-	-	NR	NR	-	++	ND
CL2	239	PBMC	-	-	-	-	-	++	-	NR
	248	Unaffected skin	-	++	+	-	-	++	-	NR
	249	Unaffected skin	-	++	+	-	-	-	-	NR
	250	Lesion	-	++	++	-	-	-	-	NR
	251	Lesion	-	++	++	-	-	-	-	NR
CL3	322	PBMC	-	-	-	NR	NR	-	-	-
	324	Unaffected skin	-	NR	-	NR	-	-	-	NR
	325	Unaffected skin	-	-	-	NR	-	-	-	-
	326	Lesion	-	-	NR	NR	-	NR	NR	NR
	327	Lesion	-	-	-	NR	-	NR	NR	NR
CL4	SS1	PBMC	++*	-	-	-	-	-	-	-
	SS2	Cell line (PBMC)	++*							
	SS3	Lesion	++*							
	SS4	Cell line (skin)	++*							

-	=	No dominant band	*	=	Two dominant bands with same primer
+	=	Borderline dominant band	NR	=	No reaction
++	=	Unequivocally dominant band	ND	=	Not done

APPENDIX 4.2

Detailed PCR results with primers for Jγ1/2 and 8 Vγ genes in normal healthy controls

Case No.	DNA No.	Sample	V2	V3	V4	ΨV5	V8	V9	V10	V11
NC1	233	PBMC	++	ND	SMB	SMB	S	S	SMB	ND
	240	Skin right arm	MB	ND	MB	MB	MB	MB	SMB	ND
	241	Skin left arm	MB	ND	MB	MB	MB	MB	MB	ND
	242	Skin right thorax	MB	ND	MB	MB	MB	MB	MB	ND
	243	Skin left thorax	MB	ND	MB	MB	MB	MB	MB	ND
NC2	234	PBMC	S	ND	S	SMB	S	S	S	ND
	244	Skin right arm	SMB	ND	MB	MB	MB	MB	MB	ND
	245	Skin left arm	SMB	ND	MB	MB	MB	MB	MB	ND
	246	Skin right elbow	SMB	ND	MB	NR	MB	NR	MB	ND
	247	Skin left elbow	SMB	ND	MB	MB	MB	MB	MB	ND
NC3	216A	PBMC	S	S	NR	ND	ND	S	S	S
	216B	Skin right ear	MB	MB	NR	ND	ND	MB	NR	S
	216C	Skin left ear	MB	MB	NR	ND	ND	MB	MB	S
NC4	228	PBMC	S	ND	S	SMB	S	SMB	SMB	ND
	230	Skin right arm	MB	ND	SMB	MB	MB	MB	MB	ND
	231	Gingiva right	MB	ND	SMB	MB	MB	MB	MB	ND
	232	Gingiva left	MB	ND	SMB	MB	MB	MB	MB	ND
NC5	229	PBMC	+	ND	S	SMB	SMB	NR	S	ND
	235	Skin right arm	MB	ND	MB	MB	MB	MB	MB	ND
	237	Gingiva right	SMB	ND	MB	MB	MB	MB	MB	ND
	238	Gingiva left	SMB	ND	MB	MB	MB	MB	MB	ND

+	=	Borderline dominant band	S	=	Smear
++	=	Unequivocally dominant band	MB	=	Multiple bands
ND	=	Not done			
NR	=	No reaction			

APPENDIX 5.1

Details of cases and samples studied in Chapter 5

Case No.	Age/ Sex	Diagnosis	Samples	DNA No.
V1	73/M	Vitiligo	Blood	166
			Vitiligo lesion right forearm	207
			Vitiligo lesion left forearm	208
V2	62/F	Vitiligo	Vitiligo lesion right forearm	209
			Vitiligo lesion left forearm	210
V3	32/M	Vitiligo	Blood	172
			Vitiligo lesion right anterior shin	203
			Vitiligo lesion left anterior shin	204
			Vitiligo lesion right upper thigh	215
V4	52/M	Vitiligo	Blood	174
			Vitiligo lesion right upper anterior thigh	205
			Vitiligo lesion left upper anterior thigh	206
V5	50/M	Vitiligo	Blood	211
			Vitiligo lesion right abdomen	212
			Vitiligo lesion left abdomen	213
			Vitiligo lesion right arm	214
V6	46/M	Vitiligo	Unaffected skin right arm	15
			Unaffected skin left arm	3
			Vitiligo lesion right arm	1
			Vitiligo lesion left arm	2
P1	50/M	Psoriasis	Blood	227
			Unaffected skin right buttock	270
			Psoriasis lesion right leg	271
P2	30/F	Psoriasis	Blood	226
			Unaffected skin right buttock	258
			Psoriasis lesion right arm	256
P3	60/F	Psoriasis	Blood	221
			Unaffected skin left buttock	222
			Psoriasis lesion left elbow	223
P4	43/M	Psoriasis	Blood	192
			Psoriasis lesion posterior aspect right thigh	201
			Psoriasis lesion posterior aspect left thigh	202

Appendix 5.1 (continued)

Case No.	Age/ Sex	Diagnosis	Samples	DNA No.
P5	36/F	Psoriasis	Blood	163
			Psoriasis lesion right knee	179
			Psoriasis lesion left knee	180
P6	M	Psoriasis	Psoriasis lesion right	177
			Psoriasis lesion left	178
P7	39/F	Psoriasis	Blood	165
			Psoriasis lesion right thigh	175
			Psoriasis lesion left thigh	176
P8	18/F	Psoriasis	Blood	184
			Psoriasis lesion right elbow	198
			Psoriasis lesion left elbow	199
P9	18/F	Psoriasis	Blood	183
			Psoriasis lesion right elbow	196
			Psoriasis lesion left elbow	197
VP1	50/F	Vitiligo plus psoriasis	Blood	435
			Unaffected skin	436
			Unaffected skin	423
			Vitiligo lesion	437
			Vitiligo lesion	422
			Psoriasis lesion	420
			Psoriasis lesion	421
			Second set of biopsies taken 10 months later	
			Unaffected skin	473
			Unaffected skin	474
			Vitiligo lesion	475
			Vitiligo lesion	476
			Psoriasis lesion	477
			Psoriasis lesion	478
VP2	35/M	Vitiligo plus psoriasis	Blood	432
			Unaffected skin	425
			Unaffected skin	426
			Vitiligo lesion	429
			Vitiligo lesion	430
			Psoriasis lesion	427
			Psoriasis lesion	428

Appendix 5.1 (continued)

Case No.	Age/ Sex	Diagnosis	Samples	DNA No.
VP3	70/M	Vitiligo plus psoriasis	Blood	458
			Unaffected skin	457
			Unaffected skin	456
			Vitiligo lesion	455
			Vitiligo lesion	454
			Psoriasis lesion	453
			Psoriasis lesion	452
			Second set of biopsies taken 12 months later	
			Unaffected skin right abdomen	461
			Unaffected skin left abdomen	462
			Vitiligo lesion right abdomen	463
			Vitiligo lesion left abdomen	464
			Psoriasis lesion right abdomen	465
			Psoriasis lesion left abdomen	466
VP4	34/F	Vitiligo plus psoriasis	Blood	252
			Vitiligo lesion right thigh	255
			Vitiligo lesion left thigh	256
			Psoriasis lesion right elbow	253
			Psoriasis lesion left elbow	254
			Second set of biopsies taken 20 months later	
			Unaffected skin right thigh	467
			Unaffected skin left thigh	468
			Vitiligo lesion right thigh	471
			Vitiligo lesion left thigh	472
			Psoriasis lesion right elbow	469
			Psoriasis lesion left elbow	470

APPENDIX 5.2

Detailed PCR results with primers for Jγ1/2 and 8Vγ genes in patients with vitiligo

Case No.	DNA No.	Sample	V2	V3	V4	ΨV5	V8	V9	V10	V11	Comments
V1	166	PBMC	MB	SMB	SMB	MB	++	SMB	MB	MB	
	207	Vitiligo lesion	MB	MB	MB	MB	MB	MB	MB	MB	
	208	Vitiligo lesion	MB	MB	MB	MB	MB	MB	MB	MB	
V2	209	Vitiligo lesion	MB	MB	MB	MB	MB	MB	MB	MB	
	210	Vitiligo lesion	MB	MB	MB	MB	MB	MB	MB	MB	
V3	172	PBMC	SMB	SMB	NR	S	S	S	S	SMB	
	203	Vitiligo lesion	SMB	MB	NR	MB	SMB	MB	MB	MB	
	204	Vitiligo lesion	SMB	MB	NR	NR	SMB	MB	MB	MB	
	215	Vitiligo lesion	SMB	MB	NR	MB	SMB	MB	MB	MB	
V4	174	PBMC	SMB	+	S	MB	++	+	MB	MB	
	205	Vitiligo lesion	SMB	MB	S	MB	MB	MB	MB	MB	
	206	Vitiligo lesion	MB	MB	S	MB	NR	MB	NR	MB	
V5	211	PBMC	ND	+	SMB	++	ND	SMB	MB	ND	
	212	Vitiligo lesion	ND	MB	S	S	ND	MB	NR	ND	
	213	Vitiligo lesion	ND	NR	NR	S	ND	MB	NR	ND	
	214	Vitiligo lesion	MD	MB	NR	S	ND	NR	MB	ND	

Appendix 5.2 (continued)

Case No.	DNA No.	Sample	V2	V3	V4	V5	V8	V9	V10	V11	Comments
V6	15	Unaffected skin	SMB	SMB	S	SMB	SMB	SMB	MB		
	3	Unaffected skin	SMB	SMB	S	SMB	SMB	SMB	SMB	MB	
	1	Vitiligo lesion	SMB	MB	S	SMB	SMB	SMB	S	MB	
	2	Vitiligo lesion	SMB	MB	S	SMB	SMB	SMB	S	MB	
VP1	435	PBMC	S	S	S	S	ND	NR	NR	S	Patient also
	436	Unaffected skin	MB	MB	MB	MB	ND	NR	NR	MB	has psoriasis
	423	Unaffected skin	MB	MB	MB	MB	ND	NR	NR	MB	
	437	Vitiligo lesion	MB	MB	MB	MB	ND	NR	NR	MB	
	422	Vitiligo lesion	MB	MB	MB	MB	ND	NR	NR	MB	
	473	Unaffected skin	ND	S	ND	MB	ND	ND	ND	MB	Second set of
	474	Unaffected skin	ND	MB	ND	MB	ND	ND	ND	MB	biopsies taken
	475	Vitiligo lesion	ND	MB	ND	MB	ND	ND	ND	MB	10 months later
	476	Vitiligo lesion	ND	MB	ND	MB	ND	ND	ND	MB	
VP2	432	PBMC	MB	MB	MB	MB	MB	NR	S	MB	Patient also
	425	Unaffected skin	MB	MB	MB	MB	S	NR	MB	MB	has psoriasis
	426	Unaffected skin	MB	MB	MB	MB	S	NR	MB	MB	
	429	Vitiligo lesion	MB	MB	MB	MB	S	NR	MB	MB	
	430	Vitiligo lesion	MB	MB	MB	MB	S	NR	MB	MB	
VP3	458	PBMC	NR	NR	ND	NR	ND	NR	S	NR	Patient also
	457	Unaffected skin	MB	NR	ND	MB	ND	NR	S	MB	has psoriasis
	456	Unaffected skin	MB	S	ND	MB	ND	NR	S	MB	
	455	Vitiligo lesion	MB	MB	ND	MB	ND	NR	S	MB	
	454	Vitiligo lesion	MB	MB	ND	MB	ND	MB	S	MB	

Appendix 5.2 (continued)

Case No.	DNA No.	Sample	V2	V3	V4	V5	V8	V9	V10	V11	Comments
VP3	461	Unaffected skin	ND	MB	MB	MB	ND	MB	MB	MB	Second set of biopsies taken one year later
	462	Unaffected skin	ND	MB	MB	MB	ND	MB	MB	MB	
	463	Vitiligo lesion	ND	MB	MB	MB	ND	MB	MB	MB	
	464	Vitiligo lesion	ND	MB	MB	MB	ND	MB	MB	MB	
VP4	252	PBMC	S	MB	MB	MB	SMB	SMB	SMB	ND	Patient also has psoriasis
	255	Vitiligo lesion	MB	MB	MB	MB	MB	MB	MB	MB	
	256	Vitiligo lesion	MB	MB	+	MB	MB	MB	MB	ND	
	467	Unaffected skin	ND	MB	ND	ND	ND	MB	MB	ND	Second set of biopsies taken 20 months later
	468	Unaffected skin	ND	MB	ND	ND	ND	MB	MB	ND	
	471	Vitiligo lesion	ND	MB	ND	ND	ND	MB	S	ND	
	472	Vitiligo lesion	ND	MB	ND	ND	ND	MB	S	ND	

+	=	Borderline dominant band
++	=	Unequivocally dominant band
MB	=	Multiple bands
S	=	Smear
NR	=	No reaction
ND	=	Note done

APPENDIX 5.3

Detailed PCR results with primers for Jγ1/2 and 8 Vγ genes in patients with psoriasis

Case No.	DNA No.	Sample	V2	V3	V4	ΨV5	V8	V9	V10	V11	Comments
P1	227	PBMC	ND	MBS	MBS	ND	ND	S	NR	ND	
	271	Unaffected skin	ND	++	NR	ND	ND	MB	MB	ND	
	270	Psoriasis lesion	ND	NR	NR	ND	ND	MB	NR	ND	
P2	226	PBMC	S	S	+	NR	S	S	S	S	
	258	Unaffected skin	MB	MB	MB	MB	MB	MB	MB	MB	
	259	Psoriasis lesion	MB	MB	MB	MB	MB	MB	MB	MB	
P3	221	PBMC	SMB	SMB	ND	ND	ND	SMB	SMB	SMB	
	223	Unaffected skin	MB	MB	ND	ND	ND	MB	MB	MB	
	222	Psoriasis lesion	MB	MB	ND	ND	ND	MB	MB	MB	
P4	192	PBMC	MB	SMB	S	SMB	SMB	ND	S	S	
	201	Psoriasis lesion	MB	MB	MB	NR	MB	ND	S	S	
	202	Psoriasis lesion	MB	MB	MB	NR	MB	ND	S	S	
P5	163	PBMC	S	NR	SMB		S	SMB	ND	ND	
	179	Psoriasis lesion	MB	NR	NR	NR	SMB	SMB	MB	ND	
	180	Psoriasis lesion	MB	MB	NR	NR	NR	ND	ND	ND	
P6	177	Psoriasis lesion	MB	NR	ND	MB	MB	MB	SMB	ND	
	178	Psoriasis lesion	MB	MB	ND	IB	MB	MB	NR	ND	
P7	165	PBMC	MB	MB	ND	SMB	NR	SMB	SMB	ND	
	175	Psoriasis lesion	MB	MB	ND	MB	MB	SMB	SMB	ND	
	176	Psoriasis lesion	NR	MB	ND		MB	ND	ND	ND	
P8	184	PBMC	SMB	S	S	MB	SMB	SMB	SMB	MB	
	198	Psoriasis lesion	SMB	MB	NR	MB	MB	MB	MB	IB	
	199	Psoriasis lesion	NR	NR	NR	NR	NR	MB	NR	NR	
P9	183	PBMC	SMB	MB	MB	NR	SMB	+	SMB	MB	Monozygotic
	196	Psoriasis lesion	NR	NR	NR	NR	NR	MB	NR	MB	twin of case P8
	197	Psoriasis lesion	SMB	MB	MB	NR	MB	MB	MB	NR	

Appendix 5.3 (continued)

Case No.	DNA No.	Sample	V2	V3	V4	V5	V8	V9	V10	V11	Comments
VP1	435	PBMC	S	S	S	S	ND	NR	NR	S	Patient also has vitilgio
	436	Unaffected skin	MB	MB	MB	MB	ND	NR	NR	MB	
	423	Unaffected skin	MB	MB	MB	MB	ND	NR	NR	MB	
	420	Psoriasis lesion	S	MB	MB	MB	ND	NR	NR	MB	
	421	Psoriasis lesion	S	MB	MB	MB	ND	NR	NR	MB	
	473	Unaffected skin	ND	S	MD	MB	ND	ND	ND	MB	Second set of biopsies taken 10 months later
	474	Unaffected skin	ND	MB	ND	MB	ND	ND	ND	MB	
	477	Psoriasis lesion	ND	MB	ND	SMB	ND	ND	ND	MB	
	478	Psoriasis lesion	ND	MB	ND	SMB	ND	ND	ND	MB	
VP2	432	PBMC	MB	MB	MB	MB	MB	NR	S	MB	Patient also has vitiligo
	425	Unaffected skin	MB	MB	MB	MB	S	NR	MB	MB	
	426	Unaffected skin	MB	MB	MB	MB	S	NR	MB	MB	
	427	Psoriasis lesion	MB	MB	MB	MB	S	NR	MB	MB	
	428	Psoriasis lesion	MB	MB	MB	MB	S	NR	MB	MB	
VP3	458	PBMC	NR	NR	ND	NR	ND	NR	S	NR	Patient also has vitiligo
	457	Unaffected skin	MB	NR	ND	MB	ND	NR	S	MB	
	456	Unaffected skin	MB	S	ND	MB	ND	NR	S	MB	
	453	Psoriasis lesion	MB	NR	ND	MB	ND	S	S	MB	
	452	Psoriasis lesion	NR	NR	ND	MB	ND	S	S	MB	
	461	Unaffected skin	ND	MB	MB	MB	ND	MB	MB	MB	Second set of biopsies taken one year later
	462	Unaffected skin	ND	MB	MB	MB	ND	MB	MB	MB	
	465	Psoriasis lesion	ND	S	S	MB	ND	S	SMB	S	
	466	Psoriasis lesion	ND	S	S	MB	ND	S	SMB	S	

Appendix 5.3 (continued)

Case No.	DNA No.	Sample	V2	V3	V4	V5	V8	V9	V10	V11	Comments
VP4	252	PBMC	S	MB	MB	MB	SMB	SMB	SMB	ND	Patient also
	253	Psoriasis lesion	MB	MB	MB	MB	MB	MB	MB	ND	has vitiligo
	254	Psoriasis lesion	MB	MB	MB	MB	MB	MB	MB	ND	
	467	Unaffected skin	ND	MB	ND	ND	ND	MB	MB	ND	Second set of
	468	Unaffected skin	ND	MB	ND	ND	ND	MB	MB	ND	biopsies taken
	469	Psoriasis lesion	ND	MB	ND	ND	ND	MB	S	ND	20 months later
	470	Psoriasis lesion	ND	MB	ND	ND	ND	MB	S	ND	

+	=	Borderline dominant band
++	=	Unequivocally dominant band
MB	=	Multiple bands
S	=	Smear
NR	=	No reaction
ND	=	Not done

APPENDIX 6.1

Detailed PCR results with primers for Jγ1/2 and 8 Vγ genes in arthritic joints

Case No.	Age/ Sex	Diagnosis	DNA No.	Specimen	Duration of Culture	V2	V3	V4	ΨV5	V5	V8	V9	V10	V11
RA1	60/F	RA	58	SM		S	S	NR	S		S	S	NR	
RA2	43/F	RA	131	SM		S	S	NR	NR		S	NR	NR	
RA3	67/F	RA	135	SM		S	S	S	NR		NR	S	NR	NR
RA4	38/F	RA	137	SM		S	S	S	S		S	S	S	S
RA5	61/F	RA	280	SF uncultured		SMB	SMB	NR		ND	++	S	NR	ND
RA6	60/F	RA	281	SF uncultured		NR	NR	NR		ND	NR	NR	NR	ND
RA7	40/F	RA	300	SF uncultured		SMB	S	S		ND	SMB	SMB	SMB	ND
RA8	48/F	RA	299	SF uncultured		S	S	S		ND	SMB	SMB	SMB	ND
			302	cultured	4 days	S	S	SMB		ND	SMB	SMB	S	ND
RA9	56/F	RA	297	SF uncultured		SMB	SMB	SMB		ND	++	SMB	+	ND
			306	cultured	5 days	SMB	MB	SMB		ND	+	SMB	+	ND

Appendix 6.1 (continued)

Case No.	Age/ Sex	Diagnosis	DNA No.	Specimen	Duration of culture	V2	V3	V4	V5	V8	V9	V10	V11
RA10	52/F	RA	312	SF uncultured		SMB	NR	NR	ND	S	ND	ND	ND
			318	cultured	7 days	SMB	NR	NR	ND	NR	ND	ND	ND
RA11	61/F	RA	313	SF uncultured		SMB	NR	NR	ND	SMB	ND	ND	ND
			317	cultured	7 days	SMB	S	NR	SMB	SMB	ND	ND	ND
RA12	54/F	RA	314	SF uncultured		SMB	MB	ND	SMB	NR	SMB	MB	ND
			316	cultured	7 days	S	S	ND	ND	S	SMB	SMB	ND
RA13	54/M	RA	408	SF uncultured		ND	SMB	ND	ND	SMB	SMB	S	ND
			415	cultured	7 days	S	SMB	ND	ND	SMB	NR	MB	
			419	cultured	15 days	SMB	SMB	ND	ND	+	SMB	SMB	ND
RA14	40/F	RA	311	SF uncultured		SMB	NR	NR	ND	S	ND	ND	ND
			319	cultured	13 days	SMB	ND	NR	ND	S	ND	ND	ND
RA15	69/M	RA	409	SF uncultured		ND	+	ND	ND	+	SMB	SMB	ND
			414	cultured	7 days	SMB	SMB	ND		+	SMB	SMB	ND
			418	cultured	15 days	SMB	+	ND	SMB	SMB	SMB	SMB	ND
RA16	63/F	RA	410	SF uncultured		ND	SMB	ND	ND	SMB	SMB	SMB	ND
			413	cultured	7 days	SMB	SMB	ND	SMB	SMB	SMB	SMB	ND
			417	cultured	15 days	SMB	SMB	ND	SMB	SMB	SMB	SMB	ND

Appendix 6.1 (continued)

Case No.	Age/ Sex	Diagnosis	DNA No.	Specimen	Duration of culture	V2	V3	V4	V5	V8	V9	V10	V11
RA17	57/F	RA	407	SF uncultured		S	SMB	ND	+	SMB	SMB	SMB	ND
			412	cultured	14 days	S	SMB	ND	S	+	SMB	SMB	ND
			416	cultured	21 days	S	+	ND	S	SMB	SMB	SMB	ND
RA17	32/F	RA	290	SF uncultured		SMB	S	ND	S	S	S	SMB	ND
			304	cultured	14 days	SMB	S	ND	SMB	S	S	SMB	ND
RA19	75/M	RA	310	SF cultured		NR	NR	S	ND	SMB	SMB	SMB	ND
			320	cultured	14 days	SMB	+	S	ND	SMB	SMB	SMB	ND
RA20	54/M	RA	309	SF uncultured		S	NR	S	ND	NR	MB	NR	NR
			321	cultured	14 days	SMB	+	SMB	ND	SMB	SMB	SMB	ND
RA21	33/F	RA	330	SF uncultured		S	SMB	ND	SMB	SMB	SMB	SMB	ND
			332	cultured	26 days	SMB	SMB	ND	SMB	+	SMB	SMB	ND
LE1	40/F	SLE	298	SF uncultured		S	S	S	ND	++	SMB	SMB	ND
			305	cultured		S	SMB	SMB	ND	+	SMB	SMB	ND
Pso	69/F	Psoriatic A	329	SF uncultured		S	S	NR	ND	S	S	S	ND

RA	=	Rheumatoid arthritis	S	=	Smear
SLE	=	Systemic lupus erythematosus	MB	=	Mulitple bands
Pso	=	Psoriasis	NR	=	No reaction
SF	=	Synovial fluid	ND	=	Not done
SM	=	Synovial membrane			

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