HERPESVIRUS INVOLVEMENT IN LYMPHOMA/LEUKAEMIA AND RELATED DISEASE

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ABBREVIATIONS

ACIF	- anti-complement immunofluorescence
AIDS	- acquired immune deficiency syndrome
ALL	- acute lymphoblastic leukaemia
AML	- acute myeloid leukaemia
Ara-C	- cytosine arabinoside
BL	- Burkitt's lymphoma
CBMCs	- cord blood mononuclear cells
CI	- confidence interval
CLL	- chronic lymphocytic leukaemia
CML	- chronic myeloid leukaemia
CMV	- cytomegalovirus
DMSO	- dimethyl sulphoxide
e	- endemic
EA	- early antigen
EBER	- Epstein-Barr encoded RNA
EBNA	- Epstein-Barr nuclear antigen
EBV	- Epstein-Barr virus
ECM	- extracellular matrix
ELISA	- enzyme linked immunosorbent assay
EHV	- equine herpesvirus
EM	- electron microscopy
FCS	- fetal calf serum
FITC	- fluorescein isothiocyanate
GMT	- geometric mean titre
HBSS	- Hank's balanced salts solution
HBLV	- human B lymphotropic virus
HD	- Hodgkin's disease
HHV-6	- human herpesvirus-6
HHV-7	- human herpesvirus-7
HIV	- human immunodeficiency virus
HLA	- human leukocyte antigen
HPV	- human papilloma virus
HRES-1	- HTLV-1 related endogenous sequence-1
HSV	- herpes simplex virus
HTLV-1	- human T-lymphotropic virus type 1
IFA	- indirect immunofluorescence assay

IFN-γ	- interferon-γ
Ig	- immunoglobulin
IM	- infectious mononucleosis
IL-2	- interleukin-2
LMP	- latent membrane protein
LP	- lymphocyte predominant
MC	- mixed cellularity
MHC	- major histocompatibility complex
MLL	- malignant lymphocytic leukaemia
MoAb	- monoclonal antibody
NHL	- non-Hodgkin's lymphoma
nm	- nanometres
NPC	- nasopharyngeal carcinoma
NS	- nodular sclerosing
OR	- odds ratio
PBMCs	- peripheral blood mononuclear cells
PBS	- phosphate buffered saline
PCR	- polymerase chain reaction
PFU	- plaque forming unit
pmol	- picomoles
PRV	- pseudorabies virus
Rb	- retinoblastoma gene product
RS	- Reed-Sternberg
sBL	- sporadic Burkitt's lymphoma
SS	- Sjögren's syndrome
SV40	- simian virus 40
µg	- micrograms
µl	- microlitres
UV	- ultra-violet
VCA	- viral capsid antigen
VZV	- varicella-zoster virus

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All the work presented in this thesis was carried out by myself except the statistical analyses in Chapters 2 and 3.

ABSTRACT

Human herpesvirus-6 (HHV-6) was first isolated from the peripheral blood of six individuals with lymphoproliferative or immunosuppressive disorders. Further studies identified HHV-6 DNA sequences in tumour biopsy samples from persons with lymphoma. Thus, initial findings suggested a link between HHV-6 and lymphoproliferative disease.

This study was designed to examine further the association of HHV-6 with lymphomas and leukaemias by seroepidemiological investigations. Higher antibody titres to HHV-6 were identified in acute myeloid leukaemia, low-grade non-Hodgkin's lymphoma, and Hodgkin's disease (HD).

Epidemiological evidence has lead to the proposition that HD, particularly in young adults, may have a viral aetiology and Epstein-Barr virus (EBV) has consistently been associated with this malignancy. Further analysis of both HHV-6 and EBV serology was carried out and the results suggested that the associations of both viruses with HD were relatively independent of each other. Thus, HD may be the result of an abnormal response to virus infection including EBV and HHV-6. To date there is little molecular evidence linking HHV-6 to HD, although recent studies have clearly demonstrated the presence of EBV within the tumour cells of the disease.

In 1990, a human herpesvirus, distinct from those previously recognised, was isolated and named human herpesvirus-7 (HHV-7). The seroepidemiology of this virus in children and adults is presented in this dissertation. Infection with HHV-7 is widespread and infection occurs usually early in life. A likely vehicle for transmission is saliva.

Herpesviruses have not only been associated with malignancy, but also chronic disease such as autoimmune conditions. EBV involvement has been proposed in the autoimmune disease, Sjogren's syndrome (SS). More recently HHV-6 has been linked to this condition. Viruses present within the epithelium of the salivary gland may play a role in the inflammation of such glands which is characteristically found in SS. This project studied possible viral involvement in SS by utilising the *in vitro* tissue culture of epithelial cells derived from salivary gland biopsies. The model proved useful as a means to study cytokine-

mediated mechanisms of autoimmunity in particular the role of interferon-γ and in the identification of putative retroviral antigens in salivary tissue.

CHAPTER 1

GENERAL INTRODUCTION

1.1 HERPESVIRUSES

1.1.1 The Family Herpesviridae

Herpesviruses are highly disseminated in nature and have been isolated from many animal species. The family herpesviridae comprises over 100 viruses with similar biochemical and morphological characteristics (Roizman and Baines, 1991; Roizman *et al.*, 1992).

Inclusion of viruses into the family herpesviridae is based on the architecture of the virion, which can be separated into 4 distinct morphological elements, by electron microscopy. A typical herpesvirion consists of: (i) a core containing the linear double-stranded DNA; (ii) an icosadeltahedral capsid of 100-120 nanometres (nm) in diameter (Wildy *et al.*, 1960) containing 12 pentameric and 150 hexameric capsomeres; (iii) an amorphous sometimes asymmetric tegument which surrounds the capsid; (iv) an envelope derived from host cell internal membranes in which viral glycoprotein spikes are embedded and project from its surface (Fig. 1.1.1). The size of herpesvirions ranges from 120 to 300nm (Roizman and Furlong, 1974).

Herpesviruses are further characterised by the presence of a linear double-stranded DNA genome ranging from approximately 120 to 230 kilobase pairs in size (reviewed by Roizman, 1990). A property of herpesviruses is their ability to remain in a latent state within the host following primary infection. The sites of latency vary between herpesviruses, but include lymphocytes, monocytes and neuronal tissue.

Amongst the most fully characterised herpesviruses are 5 of the 7 isolated from humans, herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (CMV), and Epstein-Barr virus (EBV). Recent and ongoing studies continue to clarify the properties of HHV-6. The most recently identified human herpesvirus-7 (HHV-7) is the least characterised of the human herpesviruses. Other animal herpesviruses that have been the subject of much investigation are pseudorabies virus (PRV), bovine herpesvirus type 1, and equine herpesviruses-1 and -4 (EHV-1 and -4) which can cause economically important diseases in their hosts.

Figure 1.1.1

Herpesvirus Virion Structure

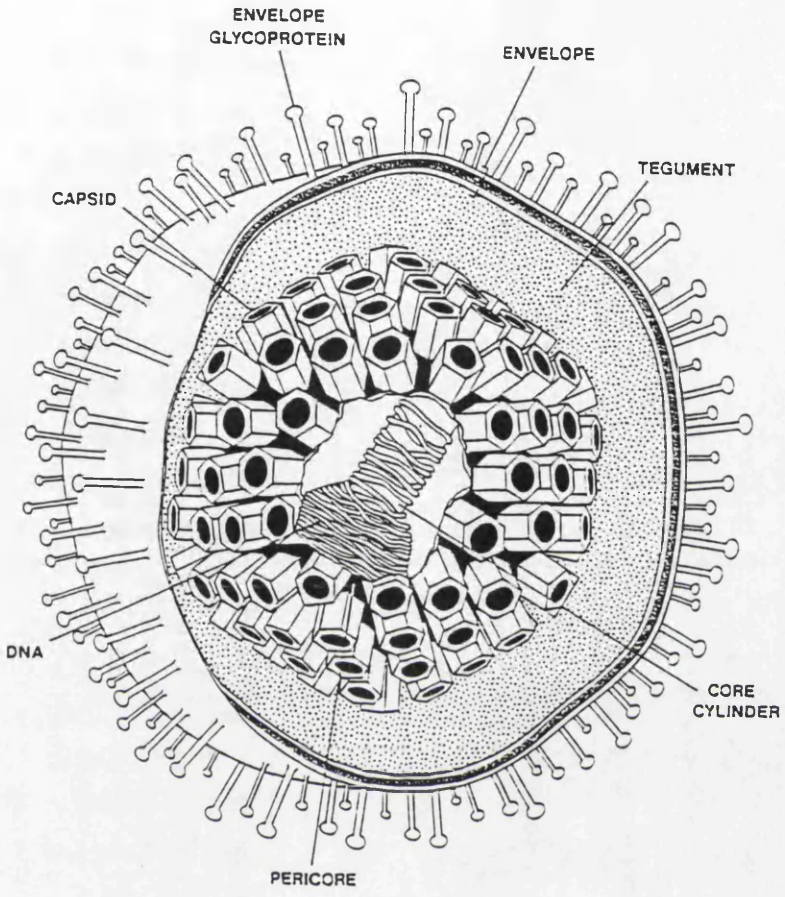


Figure 1.1.1 Schematic diagram of a herpesvirus virion

1.1.2 Classification Of Herpesviruses

Herpesviruses can be classified into subgroups based on different criteria including genome structure or biological properties. As the genome structures of the majority of herpesviruses are not well defined, the present classification scheme is based on the biological behaviour of the isolate.

(i) Classification by biological properties

The majority of herpesviruses have been assigned to 3 subfamilies based on biological criteria. These are the alpha, beta and gamma herpesviruses (Roizman, 1982; Roizman *et al.*, 1992). This classification is based on evidence that infection by herpesviruses can vary with respect to: (i) host range and cell tropism; (ii) the rate at which the virus multiplies in culture and at which infected cells are destroyed; (iii) site of latency.

a. Alphaherpesviruses

Alphaherpesviruses have a variable host range, a relatively short replication cycle, rapid infection in culture, efficient destruction of infected cells, and establish latency primarily within neurons or lymphoid tissue. This subfamily includes HSV-1, HSV-2, VZV, PRV, and EHV-1 and -4.

b. Betaherpesviruses

Betaherpesviruses exhibit a restricted host range. The viral replicative cycle is slow and latency is often maintained in lymphoid cells and secretory glands. Members of this subfamily include CMV. More tentatively assigned to this group are HHV-6 and HHV-7.

c. Gammaherpesviruses

Gammaherpesviruses have a limited host range. All members replicate in lymphoblastoid cells *in vitro* and may cause lytic infections in epithelioid and fibroblastic cells. Viruses tend to be lymphotropic, infecting either T or B cells. Gammaherpesviruses can be sub-divided into two groups based on their lymphotropism. $\gamma 1$ viruses preferentially are B cell lymphotropic and $\gamma 2$ viruses T cell lymphotropic. In lymphocytes, the replicative cycle is often pre-lytic or

lytic, but without the production of infectious virus. Latency frequently occurs in lymphoid tissue. This subfamily of herpesviruses includes EBV.

(ii) Classification by genome structure

Herpesviruses can be sub-divided into different groups based on their genome sequence arrangement. Herpesviruses contain copies of terminal sequences greater than 100 base pairs in size. According to the variation in the presence and location of these sequence, herpesviruses can be divided into 6 groups, designated A to F (Roizman *et al.*, 1992) (Fig. 1.1.2). Based on this genome structure, HHV-6 appears to fall into group A. EBV is categorised as group C, and VZV as group D. Group E viral genomes are exemplified by those of HSV-1, HSV-2, and CMV.

(iii) Classification by nucleotide sequence

The comparison of nucleotide sequence data will probably result in the most accurate classification of herpesviruses. However to date, as for genome structure, nucleotide sequences are available from only a minority of herpesviruses. At present the sequences of at least 10 herpesviruses are known or almost completed, including the human viruses HSV, VZV, EBV, CMV, and HHV-6 (McGeoch *et al.*, 1988; Davison and Scott, 1986; Chee *et al.*, 1990; Baer *et al.*, 1984; Lawrence *et al.*, 1990). According to biological properties, HHV-6 was initially tentatively assigned to the gammaherpesviruses based on its lymphotropism. Further characteristaion of the virus has suggested properties more in line with the betaherpesviruses and sequence data (Lawrence *et al.*, 1990) suggests a closer homology to CMV at the molecular level. Although the current classification scheme is based on biological properties, classification by nucleotide sequence is becoming increasingly common. As for HHV-6, limited sequencing of herpesviruses may allow adequate comparison with more complete sequence data from others to allow categorisation in the appropriate taxonomic group.

1.1.3 Herpesvirus Life Cycle

Lytic infection by herpesviruses consists of a number of stages beginning with viral entry into the cell and finally resulting in the release of progeny virus and host cell death (Fig. 1.1.3).

Figure 1.1.2 The herpesviruses can be divided into six groups designated A to F depending on the presence and reiterations of terminal sequences. Group F viruses, which are not represented in the figure, have no reiterations. Group A viruses have a large sequence from one terminus repeated at the other. In group B viruses the terminal sequence is repeated at both termini although the number of repeats can vary. Group C viruses have a number of reiterations at both termini and there may be unrelated sequences directly repeated within the unique sequences of the genome. Group D viruses have a terminal sequence that is repeated in an inverted orientation within the genome. Group E viruses have sequences from both termini that are repeated and arranged together in an inverted orientation within the unique sequence of the genome.

CCV=channel catfish virus, HVS=herpesvirus saimiri, H.=herpes, HV=herpesvirus, MDV=Marek's disease virus, HVT=herpesvirus of turkeys.

Figure 1.1.2
Herpesvirus Genome Structures

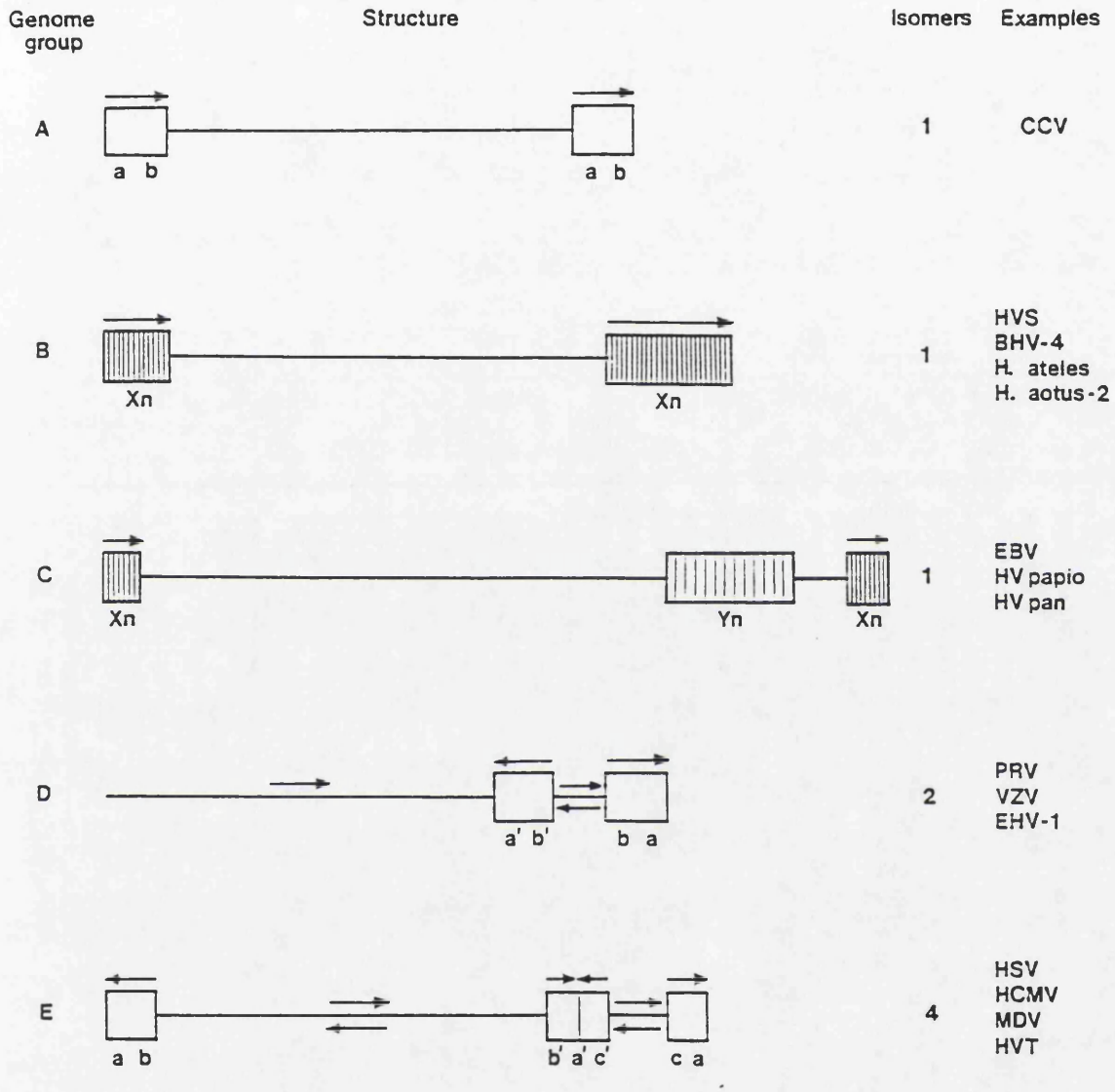


Figure 1.1.2 Schematic representation of the five types of herpesvirus genome structure as defined in Roizman *et al.* (1992) .

Figure 1.1.3
Herpesvirus Life Cycle

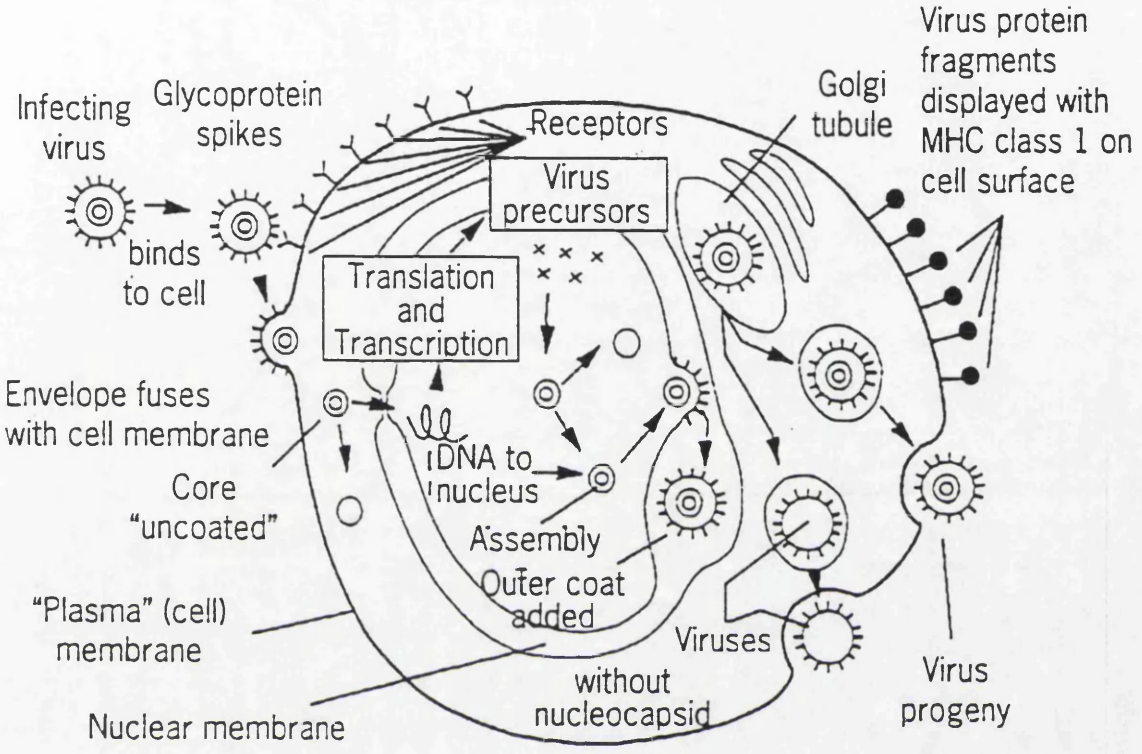


Figure 1.1.3 Schematic representation of the herpesvirus lytic cycle (Subak-Sharpe, 1992).

The virion adsorbs and enters the cell by virtue of an interaction between viral envelope glycoproteins and receptors at the cell surface. Amongst the cellular receptors identified to date is the C3d receptor for complement (CR2 or CD21) (Fingeroth *et al.*, 1984) used by EBV. Entry of viruses within phagocytic vesicles (Dales and Silverberg, 1969) is less likely.

Following entry of the virion into the cell the capsid is removed. Transcription of the viral genome in the nucleus is followed by translation of viral proteins in the cytoplasm in a co-ordinated fashion that can be temporally separated into 3 groups (Honess and Roizman, 1974). The first group, immediate-early proteins, activate transcription of the second group, the early proteins, which consist of enzymes such as deoxypyrimidine kinase and DNA polymerase required for the replication of viral DNA. Expression of late proteins including the structural components of the virion may be activated by early proteins.

Finally, viral DNA and structural proteins are assembled into progeny virions. The core and capsid are assembled within the nucleus. Envelopment of the capsid occurs by a budding process through several internal membranes including the inner nuclear membrane (Roizman and Furlong, 1974). Subsequently virions may be transported in vacuoles and secreted from the cell (Roizman, 1978).

Host cell death may occur in two ways. Host cell DNA and protein synthesis can be shut-off in an infected cell (Wagner and Roizman, 1968). Alternatively, the presence of viral peptides in association with major histocompatibility complex (MHC) class 1 molecules in the host cell plasma membrane can target an immune response against the viral antigens leading to the destruction of the cell.

A characteristic of herpesviruses is their ability to persist in their natural host in a non-infectious form. They can remain for years avoiding detection by the host immune response. In cells harbouring latent virus, the viral genomes take the form of closed circular molecules and only a small subset of viral genes, the latency-associated transcripts (LAT), are expressed. In cells infected with HSV, LAT arises from the DNA strand complementary to an immediate early gene ICPO (Stevens *et al.*, 1987) and, as an antisense message, could inhibit transcription of this immediate early gene. However, viral mutants that lack LAT have also been reported to establish latency, which questions their

necessity (Whitley, 1990). EBV differs from the other human herpesviruses in that there is extensive gene expression during latency (discussed 1.1.4.iv). On reactivation from latency, productive infection results in the death of the host cell and release of infectious virus. Mechanisms by which reactivation is triggered are unclear. For HSV-1, external or internal stimuli such as physical trauma, immunosuppressive agents and stress may result in activation (Hill, 1985). Exposure to ultra-violet light is also associated with reactivation from latency possibly mediated through host cellular factors.

1.1.4 Human Herpesviruses

(i) Herpes simplex viruses

The HSVs comprise two viruses, HSV-1 and HSV-2, that are distinguished according to antigenic and nucleotide sequence differences. HSVs are distributed world-wide and have been reported in developing and developed countries, including remote Brazilian tribes (Black, 1975). Records of human HSV infections date to ancient Greek times (Nahmias and Dowdle, 1968).

The epidemiology of HSV infections have been reviewed previously (Rawls and Campione-Piccardo, 1981; Whitley, 1990). HSVs are transmitted from infected to susceptible individuals during close personal contact (reviewed by Whitley, 1990). Virus must come in contact with mucosal surfaces or abraded skin for infection to occur. A consequence of viral replication at the site of infection is the passage of the virus within neurons to the dorsal root ganglia where latency is established. Primary HSV-1 infection rarely becomes systemic although viral dissemination is more common in neonatal HSV infection leading to multiorgan involvement. Similarly virus dissemination may occur in immunosuppressed patients where the host immunity/viral balance is altered.

The mouth and lips are the most common sites of HSV-1 infections, although many organs can become infected (reviewed by Nahmias *et al.*, 1981). Primary infection can occur at any age, although it is particularly common in children. Great variability exists in the clinical symptomatology of HSV infections ranging from asymptomatic infection to combinations of fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, oedema, localised lymphadenopathy, anorexia and malaise. Delayed primary infection by HSV later in life can result in pharyngitis in association with a mononucleosis syndrome. The frequency of

reactivation of HSV-1 varies among individuals although factors such as fever, stress, and exposure to ultra-violet light may be involved (Whitley, 1985; Hill, 1985). Oropharyngeal disease is a common consequence of primary and, less frequently, reactivated HSV-1 infection

Infection with HSV-2 is more commonly the consequence of genital transmission through sexual contact (Josey *et al.*, 1966). Most genital infections are caused by HSV-2. However, a proportion are attributable to HSV-1 although genital HSV-1 infections are usually less severe and less prone to reactivation (Corey *et al.*, 1983). HSV-1 and HSV-2 infections can be present in the eye, the skin, and the nervous system (Whitley, 1990) and HSV infection has also been associated with cervical cancer (discussed 1.2.3).

The method of choice for the diagnosis of HSV infection is specific viral identification either by virus isolation or by direct detection of HSV in lesion tissue (Herrmann and Stewart, 1981). Serological tests are of more limited value. Since HSV infection is common, the detection of HSV antibodies provides little information regarding the time of infection. Serodiagnosis is dependent on the identification of a significant rise in HSV antibody titre or the detection of HSV antibodies of the IgM subclass (Herrmann and Stewart, 1981). Certain serological assays for HSV are of limited efficacy in the identification of HSV type-specific antibodies. Antigenic differences between HSV-1 and HSV-2 glycoprotein G have facilitated the identification of antibodies to either virus type (Lee *et al.*, 1985).

Serological studies provide information on the prevalence of HSV in the population and allow the examination of factors associated with susceptibility to exposure. Seroepidemiological surveys have identified socioeconomic status and geographical location as affecting the acquisition of antibodies to both HSV-1 and HSV-2 (Rawls and Campione-Piccardo, 1981; Whitley, 1985; Whitley, 1990). In developing countries, HSV-1 seroconversion occurs earlier in life than developed countries. In developed countries and higher socioeconomic groups, primary infection may be delayed until adolescence and adulthood, perhaps reflecting reduced person-to-person contact in these groups during childhood. Antibodies to HSV-2 are not usually detected until puberty and seroprevalence rates are associated with past sexual history (reviewed by Corey, 1985). As for HSV-1, socioeconomic group and

geographical location are factors that can influence the prevalence of HSV-2 infection.

(ii) Varicella-zoster virus

VZV infection can produce two distinct clinical syndromes, varicella (chickenpox) and herpes zoster (shingles). Varicella has been recognised for centuries as a relatively benign infection of childhood. Herpes zoster is usually found in older persons and the immunocompromised and is associated with reactivation of VZV from latency. VZV was first isolated in tissue culture by Weller and Stoddard (1952) from vesicle fluid of both varicella and zoster patients. Viral isolates obtained from varicella or zoster patients were found to be identical on the basis of antigenicity and DNA restriction enodonuclease digestion patterns (Richards *et al.*, 1979). DNA from an isolate of VZV has been completely sequenced and has a genome size of 124 kilobase pairs (Davison and Scott, 1986).

The transmission of VZV to a susceptible host requires close contact with an individual with varicella or herpes zoster (reviewed by Zaia, 1981; Gelb, 1990). The virus appears to enter through the mucosa of the upper respiratory tract and oropharynx or alternatively through the conjunctiva. Virus replicates and spreads from this site and disseminates via the bloodstream to the skin. The cutaneous lesions of VZV originate with the infection of capillary endothelial cells, then epithelial cells of the epidermis which leads to the formation of vesicles. These vesicles become sites of inflammation with the infiltration of polymorphonuclear cells and macrophages. Following primary infection, VZV remains latent in sensory ganglia. Upon reactivation, herpes zoster occurs. The stimuli for reactivation are unclear but may include immunosuppression (Hope-Simpson, 1965). The histopathology of the skin lesions of herpes zoster is identical to varicella, but also includes acute inflammation of the corresponding sensory nerve and ganglion.

The majority of persons living in industrialised societies within the temperate climate zone contract varicella during childhood (Preblud *et al.*, 1984). Primary VZV infections show a marked seasonality with peak incidence in early spring and late winter. In tropical regions, primary infection in adulthood is more common than in temperate climate zone. Herpes zoster usually occurs in

persons over the age of 45 years and there is no evidence that socioeconomic class affects the incidence of VZV infections (Gelb, 1990).

VZV infection usually runs a benign, self-limiting course, although primary infection in certain patient groups can lead to complications. Life-threatening illness in neonates can result from infection *in utero* especially if the mother is infected immediately prior to birth. In children with underlying illness such as leukaemia, more severe illness can result on infection with VZV. Increased prevalence and severity of herpes zoster is found in immunosuppressed individuals.

The diagnosis of VZV infections, varicella and herpes zoster, most often rests on the appearance and distribution of the characteristic skin lesions. The clinical presentation of primary infection can vary including inapparent infection in a minority of cases. Laboratory diagnosis of VZV infections include identification of herpesvirus particles by electron microscopy and isolation of virus from, or detection of VZV antigens in, vesicular fluid (Frey *et al.*, 1981). Serological diagnosis is based on the detection of a fourfold increase in VZV antibody titre or the detection of VZV antibody of the IgM subclass (Wiedbrauk and Johnston, 1992).

A variety of serological assays are used to determine the VZV humoral immune status of an individual more often than diagnosis of infection itself. Such testing identifies VZV antibody negative persons who are at risk from VZV infection. Susceptible persons such as cancer patients receiving immunosuppressive therapy or pregnant mothers who have had contact with a varicella or zoster patient may be targetted for passive immune therapy.

(iii) Cytomegalovirus

Cytomegaloviruses are ubiquitous agents that frequently infect animals, including humans. Humans are believed to be the only reservoir for human CMV, hereafter referred to as CMV, and transmission of the virus occurs by direct or indirect person to person contact.

The isolation of CMV was independently reported by three groups (Smith, 1956; Rowe *et al.*, 1956; Weller *et al.*, 1957). There are a large number of genetic

variants of CMV and some strains may be more virulent compared to others (Huang *et al.*, 1976).

CMV is a virus of low pathogenicity in humans, but primary infection and reactivation or reinfection have been associated with clinical disease (reviewed by Weller, 1981). CMV characteristically produces cell enlargement with intranuclear inclusions which are termed cytomegalic cells. Such cells can be found in a variety of organs such as the kidney, liver, lungs, and salivary glands, particularly parotid glands. CMV exhibits a tropism for ductal epithelial cells although polymorphonuclear leukocytes and monocytes may harbour CMV at various stages following infection (Saltzman *et al.*, 1988; Schrier *et al.*, 1985). In non-viraemic healthy CMV carriers the virus is not normally present in polymorphonuclear cells whereas adherent peripheral blood mononuclear cells, likely to be monocytes, harbour the virus and act as a site of persistence (Taylor-Wiedeman *et al.*, 1993).

Congenital or neonatal infections by CMV can result in illness (reviewed by Ho, 1991). Approximately 20% of congenitally infected babies are symptomatic at birth. The risk of intrauterine infection following primary infection of the mother is high, although intrauterine transmission can also follow CMV reactivation in the mother. Cytomegalic inclusion disease in the newborn most often involves reticuloendothelial and central nervous systems with or without eye and auditory damage (reviewed by Alford and Britt, 1990; Ho, 1991). Perinatal infection can be acquired either at delivery from exposure to virus in the maternal genital tract, post-delivery through infected breast milk, or by nursery spread. Infection is usually sub-clinical although symptomatic infection such as pneumonitis may occur as a relatively rare event (Ho, 1991). In young children aged under 7 years, CMV infection may cause hepatitis or respiratory infection (Alford and Britt, 1990). In adults CMV may also be transmitted by sexual contact, although other routes such as oral contact are more important.

CMV infection in older children and adults can result in a mononucleosis syndrome, most commonly following primary infection, but less frequently as a result of reactivation or reinfection. CMV mononucleosis can also occur following blood transfusion either as a primary or reactivated infection. Infectious mononucleosis (IM) can be defined as an acute febrile illness characterised haematologically by lymphocytosis of 50% or more, of which 10% at least are atypical, and serologically by the presence of heterophile antibody

determined by the Paul-Bunnell test (discussed 4.1). The clinical course of CMV mononucleosis is usually mild and a proportion of infectious mononucleosis (IM)-like cases are attributable to CMV infection.

Infection with CMV either primary, reactivated or by reinfection can result in more severe illness in immunocompromised persons. Populations at greatest risk are those receiving immunosuppressive therapy such as organ transplant patients or cancer patients, and persons with acquired immune deficiency syndrome (AIDS). Clinical illness can include a mononucleosis syndrome, pneumonia, and chorioretinitis, especially in AIDS patients (Alford and Britt, 1990).

The most specific method for establishing a diagnosis of CMV infection is virus isolation (Stagno *et al.*, 1981; Alford and Britt, 1990). A disadvantage with this technique is the time required to successfully isolate virus. However, the use of monoclonal antibodies to specific CMV proteins in the Detection of Early Antigen Fluorescent Foci (DEAFF) test has provided a more rapid detection system for CMV in short-term tissue culture or biopsy samples. The polymerase chain reaction assay can be used to detect CMV in different clinical samples such as peripheral blood and urine. The presence of CMV IgM antibodies is also good evidence of primary infection and a significant rise of CMV IgG antibody titres in sequential serum samples may imply recurrent CMV infection. Reinfection can be distinguished from reactivation by restriction enzyme analysis of viral DNA indicating involvement of different viral strains.

Serological surveys have proved useful as a means of studying the epidemiology of CMV (reviewed by Alford *et al.*, 1981). CMV is acquired commonly during childhood. Rates of infection can vary according to locale, and even between different age and socioeconomic groups within the same geographical location. Rates of infection are influenced by both hygeine and closeness of contacts within population groups. Climate and season do not appear to affect infection rates.

(iv) <u>Epstein-Barr virus</u>

EBV is an enveloped icosahedral virus that contains a 172 kilobase pair DNA genome (Baer *et al.*, 1984). EBV was originally discovered in 1964 by Epstein and colleagues who reported the ultrastructural demonstration of a herpes-like

virus in lymphoblastoid cell lines (LCLs) established from Burkitt's lymphoma (BL) explants (Epstein *et al.*, 1964). Burkitt suggested that a particular lymphoma , which took his name and was the most common tumour affecting children in certain parts of East Africa, might be caused by a virus (Burkitt, 1962). Since the initial visualisation of EBV in these cultures, the life cycle, biological properties, epidemiology, and disease associations of this virus have been extensively studied.

EBV, like other herpesviruses, can produce either silent or symptomatic primary infection. Primary infection usually occurs during childhood (reviewed by Evans and Niederman, 1989) and the virus persists in the host for life. The vast majority of childhood infections are either asymptomatic or associated with mild, non-specific upper respiratory tract symptoms. Reactivations, which are rarely symptomatic, may be reflected by increased virus in saliva and a rise in antibody titres to EBV antigens. The classical clinical syndrome associated with primary EBV infection is IM (Henle *et al.*, 1968) which is most commonly seen in adolescents and young adults. The mononucleosis is a polyclonal transformation of B cells (Brown *et al.*, 1984) and the majority of clinical manifestations of IM are attributable to immune responses to the proliferating B cells. Serial analysis of T and B cell populations in IM patients have shown that there is an increase in B cell numbers in the first week of illness which returns to normal after several weeks. The number of T cells reaches its peak after the second week and remains high for over a month (Mangi *et al.*, 1974). The increased B cell numbers are likely to represent EBV-induced proliferation and the increased T cells reflect an immunological response against the virus. Atypical lymphocytes, primarily T cells are found in the blood during IM (Sheldon *et al.*, 1973).

Viral transmission in adults requires intimate salivary contact. Transmission by blood transfusion has also been noted (Gerber *et al.*, 1969). The main site of entry of EBV following exposure to an infectious fluid such as saliva, is the oropharynx. The virus replicates in many epithelial compartments such as the salivary glands and buccal and pharyngeal epithelial cells (reviewed by Miller, 1990). A range of viral antigens consisting of latent, early and late gene products have been detected in oropharynx epithelia indicating a probable site of infectious virus production. Virus produced in the oropharynx can infect B cells that circulate through this anatomical site or alternatively be secreted in saliva. Around 15-20% of normal seropositive individuals will secrete EBV at

any one time (Strauch *et al.*, 1974), although if saliva is concentrated before testing for EBV, the majority of saliva samples are EBV positive (Yao *et al.*, 1985). This would suggest that the oropharynx is a site of chronic productive infection rather than a site of EBV latency. The virus has also been detected in vaginal secretions and cervical epithelial cells although the role of sexual contact or mother to infant transmission during delivery is not known (Sixbey *et al.*, 1986).

Recent studies favour the concept that the virus persists in the host through carriage in B cells, mainly in a non-productive, latent state. Two lines of evidence suggest this. The first is that treatments including irradiation and cytotoxic drugs that are used in patients receiving bone marrow transplants eradicates EBV from the host, most probably by destroying the cellular compartment where the virus is latent (Gratama *et al.*, 1988). Haemopoietic cells are most affected by such treatment. Secondly, shedding of EBV in saliva is eliminated in persons treated orally with the antiviral drug acyclovir. The virus however, remains detectable in the B cell population (Yao *et al.*, 1989).

A property of EBV is its ability to infect and immortalise resting B cells *in vitro* to produce LCLs. This process has been proposed to occur in two stages (Miller, 1990). The first is the triggering of B cell activation following binding of the virus to the cell via the C3d complement receptor (CD21). The second phase requires the expression of EBV latent genes and results in a permanent blastogenesis of the B cell. A number of EBV genes are expressed in LCLs. These are the latent genes encoding six EB nuclear antigens (EBNAs), latent membrane protein (LMP)-1, 2a and 2b, and two small EBV-encoded RNAs (EBER-1 and EBER-2).

The role of expression of each of these latent genes is an area of intensive study (reviewed by Knutson and Sugden, 1989; Kieff and Liebowitz, 1990). The *in vitro* infection of B cells by EBV results in the expression of latent gene products and continuous cell growth. Changes in the phenotype of the cell also occurs with the upregulation of cellular activation markers such as CD23 and adhesion molecules. The expression of the cellular oncogene, bcl-2, which protects cells from programmed cell death, is also induced. The transfection of individual genes into B cells has facilitated the identification of the gene products responsible for these growth and phenotypic changes. EBNA-2 and EBNA-3c have been shown to upregulate CD23 (Wang *et al.*, 1987; Wang *et*

al., 1990) which is thought to be critical in the production of LCLs. LMP-1 has been shown to upregulate bcl-2 and adhesion molecules as well as CD23 (Henderson *et al.*, 1991; Wang *et al.*, 1990). LMP-1 has also been reported to transform rat fibroblast cell lines (Wang *et al.*, 1985) and can protect B cells from apoptosis by induction of bcl-2 (Gregory *et al.*, 1991). Thus, EBNA-2 and LMP-1 appear to be essential for B cell immortalisation. In agreement with this is the fact that viral strains, such as P3HR-1, which have a deletion spanning the EBNA-2 gene, are non-transforming. LCLs often contain a small fraction of cells spontaneously entering the lytic cycle. A switch from latency to replication can be activated *in vitro* by chemicals such as 12-0-tetradecanoyl phorbol-13-acetate (TPA) (Zur Hausen *et al.*, 1978). TPA initiates the protein kinase C-mediated activation of transcription of the BZLF1 gene. The product of this gene is the ZEBRA protein which targets to the nucleus where it turns on the R protein encoding gene, BRLF1. Together ZEBRA and R proteins act coordinately to turn on the first EBV early genes (Kieff and Liebowitz, 1990).

Another pattern of gene expression during latency was identified in certain Burkitt's lymphoma cell lines which maintained a similar surface phenotype to BL tumour cells *in vivo*. In contrast to LCLs, which display the full range of latent gene products and spontaneous entry into the lytic cycle, these BL cell lines express only EBNA-1 (Rowe *et al.*, 1987) and low levels of B cell activation markers and adhesion molecules (Gregory *et al.*, 1988). Such BL cell lines do not enter the lytic cycle spontaneously, but this can be induced with TPA. It now appears that there exists a futher pattern of latency that is intermediate between that of BL cells and LCLs. A study by Rowe *et al.* (1992) examined the expression of latent genes in BL cell lines following stimulation with anti-immunoglobulin. BL cells could move directly to the expression of early lytic antigens without the expression of other latent genes. A second pathway in a population of cells was the activation of full latent gene expression and conversion to a cellular phenotype similar to LCLs. However, a third pathway of EBV gene activation was found which consisted of EBNA-1 and LMP expression with no induction of the lytic cycle. Rowe *et al.* (1992) have referred to the distinct forms of latent gene expression as latency (Lat) I, Lat II, and Lat III. Lat I corresponds to a BL-like cell, with expression limited to EBNA-1. Lat II refers to the pattern observed in cells which express EBNA-1 and LMPs. Lat III is used to describe cells expressing the full spectum of EBNAs and LMP such as LCLs. The circumstances under which Lat II expression occurs *in vivo* is unclear. The added expression of LMP may provide a

proliferative advantage to EBV-infected B cells, although as detailed below, LMP is immunogenic and the target of cytotoxic T cells.

Immunity to EBV includes both a cell-mediated and humoral response. The proliferation of EBV-infected B cells is controlled by cellular immune mechanisms which include human leukocyte antigen (HLA) non-restricted natural killer cells and HLA-restricted cytotoxic T cells (reviewed by Rickinson, 1986). The target antigens of a protective cytotoxic T cell response can vary amongst individuals based on the presence of MHC class I alleles (reviewed by Kurilla, 1993). Thus, EBNA-2 is recognised efficiently by cytotoxic T cells in association with a number of alleles including HLA-A2 and -B7 whereas LMP-1 peptides are presented by HLA-A24, -B8, -B40 and -B51. Most individuals recognise several, but not the complete set of EBV latent gene proteins. There is no evidence that EBNA-1 is recognised by cytotoxic T cells. Thus, long lived B cells *in vivo* with latent gene expression restricted to EBNA-1 would allow the persistence of the virus in the host. Such cells would subsequently be recognised by cytotoxic T cells were their pattern of latency to move to Lat II or Lat III.

Neutralising antibody may play a role in limiting the spread of the virus from a site of oropharyngeal replication. Together, the cell-mediated and humoral responses suppress EBV-induced B cell proliferation and most oropharyngeal spread of the virus. The development of an effective cell-mediated immune response restricts IM to a self-limiting illness. However, fatal IM can occur rarely and in males may be linked to a genetic X-linked lymphoproliferative syndrome (reviewed by Purtilo *et al.*, 1992). The host's immune control over EBV may also be affected by immunosuppressive treatment (Crawford *et al.*, 1986).

EBV-associated lymphoproliferative disease can occur in patients with either congenital or acquired immunodeficiency states. Acquired immunodeficiency may be caused by immunosuppressive drug therapy eg. Cyclosporin A in organ transplant patients or in persons with AIDS. Increased viral production in both epithelial and lymphoid compartments as a result of a deficiency in EBV immunosurveillance may lead to increased numbers of transformed cells and finally lymphoproliferative disease. In transplant patients, EBV-induced lymphoproliferation can result in oligoclonal B cell populations which may give rise to a clonal population with the features of frank lymphoma (Cleary *et al.*,

1986; Miller, 1990). In AIDS patients, EBV-associated lesions can include diffuse polyclonal lymphomas, lymphocytic interstitial pneumonitis, and oral hairy leukoplakia (Greenspan *et al*, 1985).

EBV is linked to a number of malignancies in addition to BL, including non-Hodgkin's lymphoma (NHL), Hodgkin's disease (HD), and nasopharyngeal carcinoma (NPC). The role of EBV in cancer is discussed later in this chapter (see 1.2.3.ii).

Serological investigations have provided much information on the epidemiology of EBV. The ability to detect antibodies to particular EBV antigens has allowed a more detailed interpretation of EBV serology. The specificities of EBV humoral immunity appear to alter in health and EBV-associated disease. EBV-viral capsid antigen (VCA) IgG antibodies usually persist for life following initial infection and their presence can be used as a marker for past exposure to the virus. Antibodies to EBV-early antigens (EA) are present transiently following primary infection and appear following episodes of viral reactivation. The presence of EBV-EA antibodies suggests ongoing viral replication (Ooka *et al.*, 1991). Patterns of EBV antibody reactivities have proved useful in demonstrating the association of EBV with disease and have been used to predict persons at high risk from the cancers BL and NPC (reviewed by Evans and De-Thé, 1989; De-Thé *et al.*, 1989).

The detection of IgG antibody to EBV-VCA by indirect immunofluorescence assay (IFA) has been the main technique used to examine EBV seroepidemiology (Evans and Niederman, 1989). EBV infection can occur very early in life, shortly after the disappearance of maternally-derived antibodies. The age at which primary infection occurs can vary with geographical locale and socioeconomic group within the same area. Primary infection later in life, delayed until the second or third decades often results in the development of IM. Primary infection can occur at any age including the elderly, where EBV-induced IM has been reported (Kirov *et al.*, 1989).

(v) <u>Human herpesvirus-6</u>

Salahuddin *et al.* (1986) at the National Cancer Institute, National Institutes of Health, USA, reported the isolation of HHV-6 in studies that were initiated to try and discover a virus or viruses involved in AIDS-associated lymphoma and

haematological malignancies. The authors described the identification of a new herpesvirus which they termed human B-lymphotropic virus (HBLV), after its apparent B cell tropism in cord blood lymphocyte cultures. Virus was isolated from the peripheral blood leukocytes of six individuals. Two of these individuals were infected with HIV, one with AIDS-related lymphoma and the other with dermatopathic lymphadenopathy. The other four isolates reported were derived from, a person with angioimmunoblastic lymphadenopathy, one with cutaneous T cell lymphoma, one with immunoblastic lymphoma, and one with acute lymphocytic leukaemia. Molecular analysis of the viral genome confirmed HBLV as a novel herpesvirus (Josephs *et al.*, 1986).

In 1987, Downing *et al.* and Tedder *et al.* independently reported the isolation of herpesviruses from AIDS patients, and antigenic and molecular analyses suggested these were closely related or identical to HBLV. However, these two studies suggested that T cells rather than B cells were the target cell population for HBLV infection of cord blood lymphocytes, a fact confirmed for the original isolates by Lusso *et al.* (1987). These findings prompted a renaming of HBLV to HHV-6 in accordance with the internationlal nomenclature of herpesviruses (Ablashi *et al.*, 1987). In 1988, further HHV-6 isolates were made from immunocompromised individuals (Agut *et al.*, 1988; Lopez *et al.*, 1988).

The ability to isolate HHV-6 from patients with lymphoproliferative disorders led to the proposition that HHV-6 could somehow be aetiologically linked to these disease groups. Studies to define more clearly the association of HHV-6 with haematological malignancies were therefore initiated, including the studies reported in Chapters 2 and 3.

1.2 VIRUSES AND MALIGNANCY

Viruses from 7 different families have been associated with malignancies occurring either in the natural host of the virus or in experimental animal models. These virus families and associated cancers are described in Table 1.2.1.

Table 1.2.1

TUMOUR VIRUS GROUPS AND ASSOCIATED CANCERS

TAXONOMIC GROUP	ASSOCIATED CANCERS
I. RNA VIRUSES	
1. Retroviruses	
Oncoviruses	Haematopoietic cancers involving lymphoid, myeloid, or erythroid cells; various sarcomas and carcinomas
2. Flaviviruses (Hepatitis C Virus)	Hepatocellular carcinoma
II. DNA VIRUSES	
2. Hepadnaviruses	Hepatocellular carcinoma
3. Polyomaviruses	Various solid tumours
4. Papillomaviruses	Papillomas and carcinomas
5. Adenoviruses	Various solid tumours
6. Herpesviruses	Lymphomas and carcinomas
7. Poxviruses	Myxomas and fibromas

Table of tumour virus groups and associated cancers (Benjamin and Vogt, 1990).

1.2.1 Action of viruses in tumour development

(i) RNA viruses

All RNA viruses that are oncogenic belong to the retrovirus subfamily, oncoviruses. The mechanisms however by which these viruses can promote cell transformation are varied. At present there are 3 proposed mechanisms by which retroviruses induce tumours and transform cells.

a. Transducing retroviruses

Transducing retroviruses contain within their genome a gene that codes for a viral oncogene. The product of these oncogenes (oncoproteins) can influence the control of cell growth and differentiation. The viral oncogenes are derived originally from cellular oncogenes that have become incorporated into the viral genome, usually with the subsequent loss of viral sequences. The normal cellular equivalent, proto-oncogenes, are not normally oncogenic, although structural or functional changes can reveal the transforming potential of these genes. In some instances, dysregulated expression of the oncogene is sufficient to induce transformation. Structural and functional changes in the viral oncogene are likely to have resulted from deletions and point mutations in its sequence. Many different animal retroviruses have been found to contain viral oncogenes and the cellular homologue of the oncoprotein identified (reviewed by Benjamin and Vogt, 1990). The cellular homologue of viral oncoproteins include a growth factor, growth factor receptors, molecules involved in signal transduction, and transcription factors, all proteins that are important in the regulation of cell growth. No transducing retroviruses are known to be human pathogens.

b. Cis-activating retroviruses

This class of RNA tumour viruses do not contain viral oncogenes, but induce tumours if insertion into the host genome as a provirus occurs at particular sites and subsequently deregulates the expression of a cellular proto-oncogene. Amongst cis-activating retroviruses are murine leukaemia virus and avian leukosis virus (ALV). Feline leukaemia virus (FeLV) can either be cis-activating or transducing as FeLV/myc recombinant viruses have been reported (Neil and Onions, 1985). ALV can cause a B cell tumour in young chickens that over

expresses the proto-oncogene c-myc. The provirus has been found to insert close to the c-myc proto-oncogene (Hayward *et al.*, 1981). Friend murine leukaemia virus is also classed as a cis-activating retrovirus. This virus causes erythroid malignancies which may occur in two stages. Virus stocks contain two agents, a replication-competent and a replication-defective virus called spleen focus forming virus (SFFV), with the former acting as a helper virus for the latter. SFFV encodes an altered env protein, gp55, which binds to the erythropoietin receptor and may induce proliferation (Li *et al.*, 1990). The presence of helper virus increases the probability of a second event whereby SFFV integrates into preferred sites of the host genome. One site has been termed Spi-1 and the virus has also been found to integrate into the cellular proto-oncogene p53 gene (David *et al.*, 1988) inactivating it. This cellular proto-oncogene functions as a tumour suppressor gene (Levine *et al.*, 1991). To date no cis-activating retroviruses have been found in humans.

c. Trans-activating retroviruses

In this model a non-structural virus-coded protein acts as a transcriptional regulator to alter the expression of one or more genes important in the control of cell growth and thus induce cell transformation. A virus that could function in this manner is human T-lymphotropic virus type 1 (HTLV-1). HTLV-1 is associated with adult T cell leukaemia/lymphoma (ATLL), a malignancy of T cell origin. Of the possible viral oncogenes encoded by HTLV-1, the best studied is tax. The tax protein is a transcription factor and has been shown to induce a high level of expression of both interleukin-2 (IL-2) and the IL-2 receptor alpha, granulocyte-monocyte colony-stimulating factor, and the proto-oncogene c-fos (reviewed by Hjelle, 1991). It is possible that tax is involved in the initiation of transformation by mediating the presumed autocrine stimulation of T cells. The majority of persons infected with HTLV-1 do not develop ATLL and the period from time of infection to malignancy is a number of years. Therefore, further and as yet ill-defined changes such as a chromosomal abnormality may result in the emergence of a monoclonal T cell blast.

(ii) <u>DNA viruses</u>

DNA viruses associated with malignancies are distributed among six family groups (Table 1.1.1). Polyomaviruses and adenoviruses do not cause tumours in their natural hosts, although the mechanisms by which they can induce

transformation have been extensively studied in tissue culture and experimental animals. Viruses from the other 4 groups have been linked to naturally-occurring cancers, both benign and malignant, in humans and animals.

a. DNA viral oncoproteins

Studies on adenovirus and polyomavirus have shown that these viruses code for oncoproteins that are directly associated with cell transformation. The middle T antigen of simian virus 40 (SV40) interacts with the cellular proto-oncogene Src. This interaction stimulates the protein kinase activity of Src and disrupts its normal regulatory role (Benjamin and Vogt, 1990). Polyomaviruses (SV40 large T antigen), adenoviruses (E1B and E1A proteins) and papillomaviruses (E6 and E7 gene products) code for proteins that stably bind and inactivate two cellular nuclear phosphoproteins, p53 and retinoblastoma gene product (Rb) which normally function as tumour suppressor genes (Levine *et al.*, 1991). Thus, in experimental systems, DNA viruses have been shown to carry viral oncogenes.

b. Viral-mediated evasion from immunosurveillance

Viruses could facilitate the development of malignancies by interfering with the recognition of the tumour cells by the immune system (reviewed by Maudsley and Pound, 1991). Recognition of virally-infected cells by the immune system, predominantly cytotoxic T cells, relies on the presentation of viral antigen in the form of short peptides in association with MHC class I molecules. Interference in this presentation process would lead to the evasion of the infected and potential tumour cell from immune-mediated lysis. Adenovirus type 12 E1A antigen acts at the level of transcription to decrease constitutive as well as cytokine-induced MHC class I expression. Adenovirus type 2 E3 protein decreases MHC class I expression by interfering in the transport of the molecule to the cell surface. Viruses may also alter the cell surface expression of adhesion molecules which are important in the recognition of infected cells by the immune system. EBV positive BL cell lines exhibited a down regulation af 2 adhesion molecules, leukocyte functional antigen-3 and intercellular adhesion molecule-1 (Gregory *et al.*, 1988). As a result tumour cells may escape virus-specific immunosurveillance.

c. Indirect action of viruses

Viruses such as HIV may be involved in tumourogenesis indirectly by creating an immunoincompetent state. The impairment of the immune system could result in the failure of immunosurveillance to check the growth of malignant cells. Alternatively, the destruction of cells of the immune system by a virus could lead to the compensatory recruitment of haemopoietic precursor cells. This increased proliferation would provide a greater chance of mutational changes leading to the emergence of a tumour cell. In this respect certain types of cancer such as lymphomas occur with increased frequency in persons with AIDS (Beral *et al.*, 1991). In the absence of a viral-mediated immunosuppression, immunosurveillance may be affected by other environmental factors such as chemical agents (Newcombe, 1992; Mueller *et al.*, 1992) or as a result of another disease process such as rheumatoid arthritis (Porter *et al.*, 1991) and subsequently increase the risk of lymphoma development.

1.2.2 Association Of Viruses With Cancer

One of the most difficult problems in studying the involvement of viruses in disease is encountered in the demonstration of a causal relationship between virus infection and malignancy. In humans, the time period between initial infection and the appearance of a tumour may be a number of years and only a small percentage of persons with the virus will develop the malignancy. This would seem to be the case for HTLV-1 and ATLL. Only a small percentage of HTLV-1 infected persons develop ATL which may take many years after infection to occur (Hjelle, 1991). Transducing retroviruses that do produce tumours rapidly following infection are not known to be human pathogens. Viral involvement is also likely to be one step in a multi-step process that leads to the emergence of a tumour cell. Other factors may include chromosomal abnormalities or environmental agents. Thus, the demonstration of a cause and effect relationship between the virus and malignancy becomes complicated. Amongst the different families of viruses, those with a capacity for persistence following primary infection such as herpesviruses, hepadnaviruses, retroviruses, papovaviruses, and adenoviruses are the most likely candidates for the causation of certain cancers and chronic diseases.

In animals, the oncogenic nature of a virus can be demonstrated experimentally by its ability to induce tumours and mechanisms of transformation can be studied in detail. However, investigations of a more observational nature are required for the study of viral involvement in human cancers. The design of studies to determine viral elements in the causation of human malignancies has been outlined by Evans (1989) and include different approaches.

One method involves the use of animal models to identify the oncogenic potential of human viruses. A drawback to this approach is that the results are not necessarily indicative of such capabilities in the natural human host.

A second method is to study the pattern of the immune response to the virus. Immune parameters can be compared between cancer patients and controls to highlight differences in the host/viral balance in health and disease. Such immune system markers include the presence and titre of antibody to viral antigens or the strength of the cell-mediated response to the virus.

A third, and perhaps the most important approach is the demonstration of the virus, either viral nucleic acid or viral proteins, in tumour cells. This has only become feasible over the last decade with the development of suitable viral-specific molecular and antibody probes.

All three of the above approaches have been applied in studies aimed at dissecting the role of human viruses in a range of malignancies. EBV, hepatitis B virus, papillomaviruses, and HTLV-1 have consistently been linked to specific malignancies (reviewed by Zur Hausen, 1991). The transforming capabilities of human herpesviruses have recently been reviewed (Knipe, 1982; MacNab, 1987). All six human herpesviruses including HHV-6 (Razzaque, 1990) have been shown to transform cell lines *in vitro* which induce tumours when injected into mice. EBV only has been shown to contain a transforming protein (LMP) (Wang *et al.*, 1985). However, herpesviruses that lack a viral oncoprotein or specific DNA sequence that transforms cells, may play a role in oncogenic transformation by activating cellular genes, as has been proposed for HSV and CMV (MacNab, 1987).

1.2.3 Association Of Human Herpesviruses With Malignancy

(i) HSV and human malignancies

HSVs have been associated with human cancers of several sites. The majority of investigations have examined the role of HSV-2 in squamous cell carcinoma of the cervix (reviewed by Rawls, 1985). An early observation in the epidemiology of cervical carcinoma was the demonstration that the tumour occurred very rarely in nuns and with a greater frequency in married as compared to unmarried women. This observation led to the proposition that a sexually transmitted disease was somehow involved in the development of cervical cancer (reviewed by Melnick *et al.*, 1989). The association of viruses as aetiological agents was supported by the detection of preinvasive cervical cancer cytological abnormalities in women with a past history of genital herpes infection which is mainly attributable to HSV-2.

A large number of serological studies found statistically significant associations between antibody to HSV-2 and cervical carcinoma (Rawls, 1985; Whitley, 1990). Women with cervical cancer as a group have consistently shown either higher seroprevalence or antibody titres to HSV-2 compared to control populations. The demonstration of a direct association of HSV-2 with cervical cancer was difficult. HSV-2 infection could occur in a similar population and at a similar frequency as another agent because of certain lifestyle factors which is involved in the genesis of cervical carcinoma.

The ability to detect HSV-1 or HSV-2 nucleic acid sequences or HSV-encoded proteins inconsistently in cervical cancer using a number of methodological approaches questions the role of HSVs in this malignancy (MacNab, 1987; Whitley, 1990). More recently the detection of particular genotypes of HPV in cervical cancer indicates a more important role for this group of viruses (Zur Hausen, 1991). The fact that HPVs can also be sexually transmitted (Ho *et al.*, 1993) helps explain the epidemiological link between HSVs and cervical cancer. The potential role of HSV-2, which has transforming capabilities *in vitro* (Tevethia, 1985), as a cofactor in cervical cancer remains to be clarified.

(ii) EBV and human malignancies

EBV, the primary aetiological agent of IM, has also been linked to a range of neoplastic conditions including BL, NPC, HD, and NHL.

a. Burkitt's lymphoma

The initial isolation of EBV from a BL-derived lymphoblastoid cell line suggested an association between the virus and the malignancy (Epstein *et al.*, 1964). BL is a malignant lymphoma that belongs to the high-grade malignant lymphoma, small noncleaved cell group (Rosenberg, 1982). BL tumour cells are a monomorphic outgrowth of undifferentiated B cells, all of which are positive for immunoglobulin heavy chain expression and commonly display light-chain expression (Evans and De-Thé, 1989).

BL is described as endemic or non-endemic based on its geographical distribution. Endemic BL (eBL) or African BL occurs with high incidence in equatorial Africa and Papua New Guinea where it is the commonest childhood cancer (Burkitt, 1983). The distribution of eBL is identical to the geographical distribution of holoendemic *Plasmodium falciparum* malaria suggesting a role for malarial infection in BL development. Sporadic BL (sBL) or non-African BL is seen in older children and adults in other areas of the world. Sporadic BL presents predominantly with intra-abdominal tumours whereas eBL classically involves the jaw and abdomen. Sporadic BL and eBL are histologically and cytologically identical. BL is considered to be a model for the multi-step development of a cancer and, as such, cofactors have been recognised that are associated with the development of the tumour (Klein, 1983). Epidemiological studies have highlighted the involvement of three factors that are important in the development of the cancer.

Malaria: A factor that predisposes an individual to the development of eBL is malarial infection. Malaria is known to have a profound immunosuppressive effect and disrupt the efficacy of cell-mediated immunosurveillance (Weidanz, 1982). In addition, antigens of *Plasmodium falciparum* may act as mitogens to B cells resulting in chronic antigenic stimulation. Such disruption could facilitate the emergence of the tumour cell of BL.

Chromosomal translocations: A common cofactor in both eBL and sBL is the presence of a chromosomal translocation in the tumour cell population (Shiramizu *et al.*, 1991). The translocation commonly results in the rearrangement of the c-myc proto-oncogene, a gene important in the regulation of cellular growth, from chromosome 8 to within the immunoglobulin heavy chain genes of chromosome 14. Other less common translocations involve the rearrangement of c-myc to either of the immunoglobulin light-chain gene sites. Each of the 3 translocations results in the activation and deregulation of the c-myc oncogene which is believed to be a crucial and necessary event in the pathogenesis of BL (Cory, 1986).

Role of EBV in BL: Similar to a chromosomal translocation, the involvement of EBV is associated with both eBL and sBL, although not in all cases. EBV involvement is almost always found in eBL and less frequently in sBL (Evans and De-Thé, 1989). The association of EBV with BL has been demonstrated by both seroepidemiological and molecular approaches.

BL patients were found to have higher antibody titres to EBV-VCA compared to control populations. This is particularly a feature of eBL groups (Henle *et al.*, 1969). In American (sporadic) BL one study found 20% of cases lacked demonstrable EBV antibody and fewer cases displayed higher EBV antibody titres as compared to eBL cases (Evans and De-Thé, 1989). The elevated antibody titres in BL appear to be restricted to EBV. The serology of other viruses in BL has not been researched in depth although one such study described antibody titres to HSV, CMV and measles virus as being similar either before or after the development of BL compared to controls (De-Thé *et al.*, 1978). Children with EBV-VCA antibody titres at least fourfold above the geometric mean titre (GMT) of the normal population group have an increased risk of developing BL estimated to be approximately 30 times (De-Thé *et al.*, 1978). Thus, there is strong serological evidence for a role for EBV in BL, particularly eBL.

Molecular studies provide further evidence for an association between EBV and the development of BL. Biopsies of BL from endemic cases commonly contain EBV detectable by hybridisation with EBV-specific DNA probes (Zur Hausen *et al.*, 1970). For sBL, EBV DNA has been found in approximately 20% of cases (Ziegler *et al.*, 1976). Tumour cells contain the complete EBV genome which is

mainly in episomal form although integrated EBV DNA may also be present (Evans and De-The, 1989).

Relationship of cofactors in the pathogenesis of BL: The identification of 3 cofactors closely associated with BL has led to predictions as to the pathogenesis of BL (Evans and De-The, 1989).

For eBL, it is thought necessary for EBV infection to occur early in life. During episodes of acute malaria, immunosurveillance including any EBV specific cytotoxic T cell response may be suppressed. EBV-induced B cell proliferation could result, aided by the mitogenic stimulation of B cells by antigens of *Plasmodium falciparum*. The resulting proliferation of B cells provides a greater opportunity for an abnormal cell to emerge following a specific chromosomal translocation and oncogene activation. In sBL, in the absence of malarial infection and possibly EBV involvement, the process is dependent on other factors that lead to B cell proliferation, chromosomal translocation and the development of BL.

b. Nasopharyngeal carcinoma

The most common nasopharyngeal cancer is NPC, a tumour of epithelial origin. NPC accounts for up to 90% of nasopharyngeal neoplasms in the USA and up to 98% in some areas of Asia (reviewed by Gaffey and Weiss, 1990). Histologically undifferentiated NPC is characterised cytologically by malignant cells with indistinct cytoplasmic borders resulting in a syncytial pattern of growth and a reactive inflammatory infiltrate consisting predominantly of lymphocytes. As has been determined for BL, there is good evidence of a viral aetiology for NPC.

The association of EBV with NPC is strong. EBV DNA is detectable in the majority of cases of undifferentiated NPC (Zur Hausen *et al.*, 1970) and has been observed in differentiated forms of the carcinoma as well (Raab-Traub *et al.*, 1987). On the basis that the episomal form of EBV can vary in size depending on the number of terminal repeats, it has been possible to demonstrate that clonal EBV is present in each NPC tumour. The presence of clonal EBV suggests that the virus was present in the progenitor tumour cell and argues for a direct role for the virus in the development of the malignancy (Raab-Traub and Flynn, 1986). The role of EBV in NPC is unclear.

Reactivation of EBV from latency in the nasopharynx may represent the ultimate or penultimate step prior to the clinical onset of disease (De-The and Zeng, 1986).

There is a characteristic EBV antibody pattern associated with NPC. Patients with undifferentiated NPC have high EBV-VCA, EBV-EA and EBNA antibody titres. The presence of EBV specific antibodies of the IgA subclass in both serum and saliva may also identify persons at high risk from NPC and a rise in EBV specific IgA antibody titre is associated with advancing stages of the disease (De-The *et al*, 1989). The presence of elevated antibody titres to EBV-EA and of the IgA subclass may represent the immune response to reactivation of EBV and its replication in the nasopharynx prior to the development of the tumour. Recently, using a molecular serological approach, Littler *et al.* (1991) developed assays that detect antibodies to particular EBV-EA such as EBV thymidine kinase. Preliminary analysis found that antibodies to such antigens were useful predictors of NPC. As for BL, molecular and serological investigations have proved invaluable in elucidating the involvement of EBV in NPC.

Other factors that are associated with NPC development are less well defined. NPC has a worldwide occurrence although it is more common in regions such as the southernmost provinces of China. Influences such as genetic factors (De-The *et al.*, 1989) or environmental factors may be important. Exposure to nitrosamines in salted fish or chemical compounds contained in certain plants which can affect EBV latency and replication (Ito, 1986) have been linked to increased NPC cases in particular geographical locations.

c. Non-Hodgkin's lymphoma

NHLs, predominantly of B cell origin, are common in immunodeficient patients and have been associated with EBV. A number of causes of immunodeficiency are possible including acquired immunosuppression of which the best example is AIDS. NHLs are found with increased frequency in persons infected with HIV. EBV has been detected in up to 50% of HIV-associated NHLs (Hamilton-Dutoit *et al.*, 1991) and is found in the brain tissue of virtually all cases of AIDS-related primary lymphoma of the central nervous system (MacMahon *et al.*, 1991). The majority of HIV-associated NHLs also show evidence of c-myc

gene rearrangements similar to the translocations present in BL (Groopman *et al.*, 1986; Subar *et al.*, 1988).

Induced immunosuppression is most commonly encountered in allograft transplant recipients. In this group of patients the incidence of NHL can range from 1% to 15% (Cleary *et al*, 1986) and disease may regress with a reduction in immunosuppressive treatment. EBV has been detected in the majority of NHLs arising in post transplant patients. At present the exact nature of the role of EBV in such malignancies is unclear although EBV-induced B cell clonal expansion with concomitant genetic alterations has been proposed (Gaffey and Weiss, 1990).

A third immunosuppressed group are patients with congenital immunodeficiencies. Such persons have an extremely high incidence of NHL compared to control populations. Congenital disorders include X-linked lymphoproliferative disease, Chediak-Higashi syndrome and severe combined immunodeficiency (Purtilo *et al.*, 1992). Again, NHLs in these groups of patients are usually of the B cell lineage and contain EBV genomic material.

The majority of EBV-associated NHLs are B cell lymphomas. However, EBV has been detected rarely in T cell malignant lymphomas such as angioimmunoblastic lymphadenopathy (AILD) and peripheral pleiomorphic T cell lymphomas (Korbjuhn *et al.*, 1993). However, EBV is not clonal in AILD and not all of the tumour cells contain the virus (R. Jarrett, personal communication). EBV has also been detected in Ki-1 (CD30)-positive anaplastic large cell lymphomas (Herbst *et al*, 1991a).

d. Hodgkin's disease

For over two decades seroepidemiological and more recently molecular evidence has suggested an association between EBV and HD (discussed Chapter 3).

Serological studies have been used to investigate the potential role of a number of viruses in HD, from which a consistent association of EBV with the disease has emerged. As a group, patients with HD have elevated antibody levels to EBV antigens including both EBV-VCA and EBV-EA (Levine *et al.*, 1971a; Henderson *et al.*, 1973; Henle and Henle, 1973; Evans *et al.*, 1978). Raised

antibody titres to EBV have been found to precede the diagnosis of HD (Mueller *et al.*, 1989). These seroepidemiological studies were instrumental in highlighting the possible involvement of EBV in HD. However, only 30-40% of HD cases have been reported to exhibit elevated antibody titres to EBV (Evans and Gutensohn, 1984) which could signify a separate non-EBV associated subset. In such a subset of HD, the role of other viruses has to be considered.

Early molecular studies failed to detect EBV genomes in HD tumour biopsies (Pagano *et al.*, 1973; Lindahl *et al.*, 1974). In 1987, Weiss *et al.* reported the detection of EBV genomes in 4 of 21 cases of HD by Southern blot hybridisation. More recently other studies have confirmed the detection of EBV nucleic acid sequences in HD biopsy material (Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989; Gledhill *et al.*, 1991). In combination with the serological studies the case for EBV involvement in at least a subset HD grows more convincing.

(iii) HHV-6 and human malignancies

Following the initial isolation of HHV-6 from patients with immunosuppressive and lymphoproliferative disorders (see 1.1.4.5) two molecular studies were initiated to look for the presence of HHV-6 DNA in biopsy tissues from patients with a variety of lymphomas and leukaemias. Josephs *et al.* (1988) detected viral sequences in 3 lymphomas of B cell derivation. These lymphomas were an EBV positive eBL, a follicular large cell lymphoma, and two EBV negative tumours from a patient with Sjögren's syndrome (SS). Jarrett *et al.* (1988) detected HHV-6 specific DNA sequences in two cases of NHL from 117 tissue samples examined. One was a T cell lymphoma from a person with a preceeding history of angioimmunoblastic lymphadenopathy and the other was a B cell lymphoma that occurred in a patient with SS.

The molecular detection of HHV-6 in a series of lymphoma patients provided further evidence for the involvement of HHV-6 in lymphoid malignancies. It is interesting that both studies independently reported the detection of HHV-6 in NHLs that occurred in the context of SS.

SS is an autoimmune disease first recognised by Henrik Sjögren that can occur either on its own or in association with another connective tissue disease. SS is characterised by the symptoms of dry eyes and mouth which result from the

autoimmune-mediated destruction of lacrimal and salivary glands. A recognised consequence of SS is the increased risk of developing lymphoma, particularly B cell NHL (Kassan *et al.*, 1978). The aetiology of this disease is unknown although factors such as viral infection have been proposed (Flescher and Talal, 1991). EBV has been linked to the pathogenesis of SS (Fox *et al.*, 1986b) and the association of EBV with NHL is well documented. The detection of HHV-6 positive NHLs in SS patients could reflect the involvement of HHV-6 not only in tumour development, but in the actual autoimmune disease itself.

1.3 OBJECTIVE OF THE PROJECT

The objective of this project was to further characterise the relationship of HHV-6 with disease. Seroepidemiological investigations were adopted in order to investigate:

(i) The association of HHV-6 with a range of human leukaemias and lymphomas. Such an experimental approach has successfully been used previously to highlight the association of EBV with BL, NPC and HD.

(ii) The role of HHV-6 in IM-like illness. The majority of IM illness is attributable to primary EBV infection although less frequently other herpesvirus infections can result in this clinical outcome. Therefore, the association of HHV-6 infection with IM-like disease was investigated.

During the course of this project the isolation of a novel herpesvirus, HHV-7, was reported (Frenkel *et al.*, 1990). The studies were extended to examine the seroepidemiology of HHV-7 in children and adults.

A final aim of the project was to examine the role of viruses in Sjogren's syndrome. Viruses have been associated with the pathogenesis of SS as well as the subsequent development of NHL. A novel *in vitro* model was designed with which to study viral involvement in SS.

CHAPTER 2

THE SEROEPIDEMIOLOGY OF HHV-6 FROM A CASE-CONTROL STUDY OF LEUKAEMIA AND LYMPHOMA

2.1 INTRODUCTION

HHV-6 was first isolated from 6 patients with lymphoproliferative disease and immunosuppressive disorders (Salahuddin *et al.*, 1986). The virus was initially termed human B lymphotropic virus as it was thought to show a tropism for freshly isolated B cells. Initial serological studies suggested that the virus was of low prevalence in the population. Sera from the six persons from whom the viral isolates had been made reacted immunologically with each of the six isolates (Salahuddin *et al.*, 1986). However, only 4 of 220 randomly selected healthy donors were seropositive.

Shortly afterwards Downing *et al.* (1987) and Tedder *et al.* (1987) reported further isolates of HHV-6 which were derived from patients with AIDS. Tedder *et al.* (1987) found 18% of British blood donors were HHV-6 seropositive by an indirect immunofluorescence assay when restricting positive scoring of sera to those with unambiguous staining. These original findings suggested a possible association between HHV-6 and lymphoproliferative disease or immunosuppression. HHV-6 appeared to have a low seroprevalence in the general population.

Since that time a number of laboratories have used a variety of techniques to investigate the epidemiology of HHV-6 and determine its role in human disease. Seroepidemiological studies have been the most common approach.

Briggs *et al.* (1988) reported the prevalence of antibody to HHV-6 by age. That study found the acquisition of HHV-6 antibodies occurred early in life and that 60% of children aged 2 years were seropositive. Antibodies, most probably maternally-derived, were usually detected at birth and declined in the first few months of life. Rates of seropositivity were at their lowest around 6 months of age and rose from this time, presumably as infants experienced primary infection. Fifty-two percent of healthy blood donors were seropositive. The authors also suggested that the serological response to HHV-6 in females may differ from that in males.

Further serological studies confirmed a more widespread nature of HHV-6 infection than was initially proposed (Brown *et al.*, 1988; Pietroboni *et al.*, 1988b; Krueger *et al.*, 1988; Linde *et al.*, 1988; Saxinger *et al.*, 1988) with levels of HHV-6 seropositivity in adult populations ranging from 63% to 97%.

HHV-6 has been detected by the polymerase chain reaction (PCR) in the saliva and peripheral blood in the majority of healthy persons (Gopal *et al.*, 1990; Jarrett *et al.*, 1990). Furthermore, HHV-6-specific DNA and protein have been localised in major (Fox *et al.*, 1990a) and minor salivary glands (Krueger *et al.*, 1990) using *in situ* hybridisation and immunohistological techniques. There is, therefore, good evidence that HHV-6 persists following primary infection and that the salivary glands are a site of replication. Saliva is likely to be an important vehicle for transmission of HHV-6. Two groups have reported the isolation of HHV-6 from the saliva of healthy persons (Harnett *et al.*, 1990; Levy *et al.*, 1990a).

Virus isolation from the peripheral blood of healthy persons has proved difficult. It is likely that the virus can only be isolated from this source during primary infection or episodes of virus reactivation. A number of isolates have been obtained from the blood of HIV-1 infected individuals (Lopez *et al.*, 1988; Pietroboni *et al.*, 1988b). Yamanishi *et al.* (1988) demonstrated the frequent isolation of HHV-6 from the peripheral blood of infants with exanthem subitum (roseola infantum) or sixth disease. The authors identified HHV-6 seroconversion in the sera of this group of children providing strong evidence for an aetiological role for HHV-6 in this disease. This aetiological link has subsequently been supported by further studies (Takahashi *et al.*, 1988; Asano *et al.*, 1989; Enders *et al.*, 1990; Kondo *et al.*, 1990). Exanthem subitum is most commonly seen in infants under the age of 2, the age group where HHV-6 primary infection is most likely to occur. More recently, HHV-6 has been proposed as a major cause of acute febrile illness in young children (Pruksananonda *et al.*, 1992).

Virus isolation, seroconversion, the presence of HHV-6-specific IgM class antibodies and a rise in IgG antibody titres have been used to study the association between HHV-6 and a range of clinical presentations in adults and children. In adults, reports suggest that HHV-6 related disease may range from unapparent infection or mild afebrile illness with non-specific symptoms (Niederman *et al.*, 1988) to IM-like symptoms and fatigue (Kirchesch *et al.*, 1988). HHV-6 infection has also been associated with hepatitis (Dubedat and Kappagoda, 1989; Ward *et al.*, 1989; Irving and Cunningham, 1990; Steeper *et al.*, 1990; Asano *et al.*, 1990; Tajiri *et al.*, 1990) and chronic fatigue syndrome (Ablashi *et al.*, 1988).

The original association of HHV-6 with lymphoproliferative disease was supported by later studies identifying HHV-6-specific DNA sequences by Southern blot hybridisation in tissue samples from patients with lymphoid malignancies (Josephs *et al.*, 1988; Jarrett *et al.*, 1988). These studies however, could provide no information as to the cellular source of the virus in the tissue biopsy. In addition, Ablashi *et al.* (1988) reported higher HHV-6 antibody titres in African BL and HD, but not in NPC and chronic lymphocytic leukaemia (CLL). Biberfeld *et al.* (1988) also found higher HHV-6 seroprevalence in HD.

HHV-6 is not the first virus to be associated with HD. Since 1970, serological studies have linked EBV with HD (Johansson *et al.*, 1970; Levine *et al.*, 1971a) and molecular studies have recently provided stronger evidence for a role of EBV in at least some cases of HD (Weiss *et al.*, 1987). Epidemiological evidence supports the hypothesis that HD has a viral aetiology particularly in young adults (Gutensohn and Cole, 1980). It is possible that more than one virus could be involved in HD and based on the epidemiology of the malignancy, MacMahon (1966) proposed that multiple aetiologies may be present.

One of the drawbacks in the use of serology when investigating the association of HHV-6 with disease is the fact that over 50% of the healthy population of the UK is seropositive (Briggs *et al.*, 1988). Therefore, the use of case-control seroepidemiological studies is necessary.

In order to investigate any association of HHV-6 seropositivity and antibody titre with haematological malignancies, I have screened serum samples from appropriate groups (Clark *et al.*, 1990). An epidemiological case-control study of leukaemias and lymphomas was conducted in Yorkshire between 1980 and 1986 to examine factors involved in the aetiology of these malignancies (Bernard *et al.*, 1987). Sera were stored from the majority of cases and controls to allow epidemiological data to be combined with serological investigations.

2.2 MATERIALS AND METHODS

2.2.1 Source Of Sera

Sera from 477 cases of leukaemia/lymphoma and 463 controls were available for analysis. The sera were coded prior to dispatch to the LRF Virus Centre in Glasgow from the LRF Clinical Epidemiology Centre in Leeds. Staff in Glasgow were blind to the case-control status of each sample. The cases were a cross-section of adults aged over 14 years diagnosed while resident in the Yorkshire Health Region between 1980 and 1986. They were interviewed and blood taken as soon as possible after diagnosis. Controls were selected during the same time period from hospital in-patients with a wide variety of non-malignant conditions. The original epidemiological study from which these case and controls were derived has been analysed by disease subtype: HD (Bernard *et al.*, 1987), NHL (Cartwright *et al.*, 1988b), chronic lymphocytic leukaemia (CLL) and malignant lymphocytic leukaemia (MLL) (Cartwright *et al.*, 1987), acute myeloid leukaemia (AML) (Cartwright *et al.*, 1988a), chronic myeloid leukaemia (CML) (McKinney *et al.*, 1990) and acute lymphoblastic leukaemia (ALL).

After the initial survey, a further group of HD sera and controls from the original epidemiological study were analysed. The second group of sera were derived to augment the original data and involved sampling which favoured persons without siblings in both the HD cases and controls.

2.2.2 Tissue Culture

A sub-clone of the Jurkat cell line, J JHAN, was propagated in RPMI 1640 supplemented with 400U/ml penicillin, 400µg/ml streptomycin, 2mM L-glutamine (complete medium) and 10% heat-inactivated fetal calf serum (FCS) (heat-inactivated at 56°C for 30 minutes) (all Life Technologies). Cells were split twice a week at a ratio of 1:5 or resuspended at $5x10^5$ cells/ml by total medium replacement, gassed with 5% CO_2 and incubated in sealed tissue culture flasks (Costar) at 37°C.

The AJ strain of HHV-6 (Tedder *et al.*, 1987) was grown in J JHAN cells. Virus-infected cells were passaged onto uninfected J JHAN cells at a ratio of 1:10. Viral infection was passaged when between 50-60% of the cells appeared infected. Infection was judged by the number of balloon-like and large cells in

the culture compared to uninfected J JHAN cells. All tissue culture work was carried out in a Class II Microbiological Safety Cabinet (Envair or Medical Air Technology).

2.2.3 Cell Stocks

Uninfected and HHV-6 infected J JHAN cells were pelleted by centrifugation at 250*g* for 5min in a Beckman GPR benchtop centrifuge. Cells were resuspended at 3-5x10^6 cells/ml in freeze-down medium (complete medium containing 20% FCS plus 10% dimethyl sulphoxide-DMSO)(Sigma). One millilitre aliquots in Nunc liquid nitrogen vials were placed in the vapour phase of a liquid nitrogen storage tank. After 24 hours the vials were immersed in liquid nitrogen and stored until required. Cell lines were recovered by retrieval of vials from the liquid nitrogen store and rapid thawing in warm water. Thawed cells were added slowly to 5ml complete medium plus 10%FCS, centrifuged, washed once, and resuspended in fresh medium.

2.2.4 HHV-6 Indirect Immunofluorescence Assay

The assay was a modification of the indirect immunofluorescence assay developed by Henle and Henle (1966) and similar to methods used in HHV-6 serological studies by Salahuddin *et al.* (1986) and Briggs *et al.* (1988).

(i) Preparation of antigen slides

Infected cells were harvested when approximately 50-60% of cells appeared infected. Cell debris was removed by density centrifugation. Twenty-five millilitres of cells in culture medium were layered onto 15ml Ficoll-Hypaque (Pharmacia) in a 50ml conical tube. The gradient was centrifuged at 900*g* for 15min. The interface of the tissue culture medium and Ficoll-Hypaque was collected in as small a volume as possible, approximately 5ml, and washed twice in 30ml Hank's balanced salts solution (HBSS) (Life Technologies). Cells were resuspended at 2.5x10^6 cells/ml in either HBSS or phosphate buffered saline (PBS) (PBS without Calcium or Magnesium, Flow Laboratories or Life Technologies). Fifteen microlitres of cell suspension were spotted onto each well of a Teflon-coated 8-well Multiwell slide (Flow Laboratories) and allowed to air-dry. Slides were fixed for 10min with ice-cold acetone (May and Baker) and

stored at -20°C in air-tight slide storage containers until use. Slides were not stored for longer than 4 weeks.

(ii) Preparation of sera

Sera were pre-adsorbed with control J JHAN cells to remove non-specific antibody binding to J JHAN cellular antigens.

a. Preparation of J JHAN for pre-adsorption

Uninfected J JHAN cells were pelleted and resuspended in PBS or HBSS and washed once. The cell pellet, approximately 10^8 cells, was resuspended in 1ml of PBS or HBSS in a 50ml polypropylene tube. Twenty millilitres of ice-cold acetone were added and incubated at 4°C for 5-10min. PBS or HBSS was added to a volume of 50ml and centrifuged. The cells were washed twice more in PBS or HBSS, resuspended at a final concentration of 10^7 cells/ml and stored at 4°C until use.

b. Adsorption of sera

A diluent consisting of HBSS plus 5% 0.45µm filtered (Gelman Sciences) horse serum (Flow Laboratories) and 0.1% sodium azide (Sigma) was used for the adsorption and dilution of sera. One hundred microlitres of acetone-fixed J JHAN cells were added to a 0.75ml microcentrifuge tube and pelleted at maximum speed in a MSE Microcentrifuge for 30sec. The supernatant was discarded. Thirteen microlitres of diluent and 2µl of test serum were added. Allowing for the volume of the pellet, the total volume was estimated to be 20µl. The pellet was resuspended by vortexing and incubated at 37°C for 40min with re-vortexing every 10min. The cells were pelleted as above and the supernatant (serum at a dilution of 1:10) stored at 4°C.

Positive and negative control serum samples were used in every assay and were adsorbed in a similar manner to test sera. The positive control was a serum sample from a member of the Department. The negative control was kindly provided by Dr. J. Fox, University College and Middlesex School of Medicine, London.

(iii) Indirect immunofluorescence assay

Sera were initially screened at a dilution of 1:40. Ten microlitres were spotted onto wells of HHV-6 antigen slides. Slides were incubated at 37°C for 40min in a moisture chamber then washed 4 times by immersion in PBS over 30min at room temperature. As much of the PBS was removed as possible without allowing the wells to dry out completely. The conjugate used was a mixed solution of purified fluorescein isothiocyanate (FITC)-labelled rabbit anti-human IgG heavy chain-specific and FITC-labelled rabbit anti-human IgM heavy chain-specific sera (Dako), at a final dilution of 1:25 in diluent. Ten microlitres of conjugate were added to each well and slides incubated at 37°C for 40min. The slides were washed as above. Slides were read with a Leitz Laborlux Ultra-Violet (UV) microscope using a x25 water immersion lens. Sera that exhibited any combination of granular, nuclear or cytoplasmic fluorescence were scored as positive. Sera scored positive at a dilution of 1:40 were diluted two-fold until the antibody end titre was reached. This was defined as the reciprocal of the serum dilution at which positive fluorescence was last seen.

2.2.5 Statistical Methods

The serology results were sent to the LRF Centre in Leeds where they were linked to the case-control epidemiological data set using the statistical package SPSSX (1983) and analysed by Dr. Freda Alexander. The percentage seropositive at each of the dilutions 1:40, 1:80, and 1:160 was compared in cases and controls, with cases split into leukaemias and lymphomas, and finally sub-divided into the 7 disease groups (NHL, HD, MLL, CLL, CML, ALL, and AML). Low-grade NHL (LG-NHL) and high-grade NHL (HG-NHL) were also distinguished. The analyses were restricted to these three dilutions as beyond this the numbers involved were too small for statistical testing. These analyses used logistic regression (McCullagh and Nelder, 1983; Payne *et al.*, 1987); in each case indicator variables for age in 7 strata and sex were first forced into the model. The design of this study was case-control but not matched case-control. The original epidemiological survey utilised matching by general characteristics (age plus or minus 5 years and sex) to achieve an age-sex distribution comparable for cases and controls. The selection of sera for this study was based on availability of sera and no attempt was made to match sera on retrieval. The analytical method of choice was therefore stratification by age and sex. Statistical significance of the differences in proportions has been

calculated using analysis of deviance with the asymptotic Chi-square distribution for the change in deviance. The regression co-efficients yield estimates of the odds ratio (OR) from which test-based confidence intervals are derived.

In addition, results at different dilutions have been combined by comparing GMTs for the groups. Analysis of variance was applied to log(GMT), and sex and age adjustments made as before. In each case the ratio of GMT in the case group to that in the controls was estimated and its statistical significance computed using the F-test (Snedecor and Cochran, 1980). In calculating GMT, sera negative at the lowest dilution can be omitted from the calculation (Evans and Gutensohn, 1984; Sohier *et al.*, 1974) or allocated an arbitrary non-zero origin (Henderson *et al.*, 1973; Bray *et al.*, 1983). Since this study contained substantial numbers of sera negative at the 1:40 dilution, the second solution was preferable. Results reported here use 1:12 as an origin which normalised the distribution of log(GMT) in the controls. Other alternatives between 1:1 and 1:16 and omitting all negative sera were also analysed and it was confirmed that the results for ratios of GMT were not sensitive to the choice.

The data on the two HD sera and control groups were combined for analysis to investigate the effect of childhood social contact using sibship size on young and old age groups, with HHV-6 antibody levels as an indication of exposure to common viruses.

2.3 RESULTS

2.3.1 Reproducibility Of The HHV-6 IFA

Assay controls consisted of positive and negative serum samples included in each assay run. The IFA was scored by comparing the staining pattern of the positive control sera with test sera. Figure 2.3.1 displays the fluorescent staining patterns of HHV-6 infected cells with the positive and negative control sera.

The antibody end titres of a number of sera did not vary when tested at different times with various batches of slides highlighting the reproducibility of the IFA. The IFA was reproducible between laboratories. Twenty sera were screened by Glasgow and Dr. J. Fox, University College and Middlesex School of Medicine, London, using HHV-6 (AJ-strain) infected J JHAN cells. The results reported by the two laboratories were similar (data not shown).

2.3.2 Results Of The Statistical Analyses

Initially sera from 940 individuals were sent to the LRF Virus Centre in Glasgow and screened for HHV-6 specific antibodies by an IFA. The sera tested were a subset of those obtained in the complete epidemiological study. Details of the cases and controls in the original study and the subset from which samples were analysed are shown in Table 2.3.1. Overall, cases from which serum samples were sent to Glasgow represented the age, sex and geographical distribution of disease groups in the original study and consequently the incidence of the disease in Yorkshire.

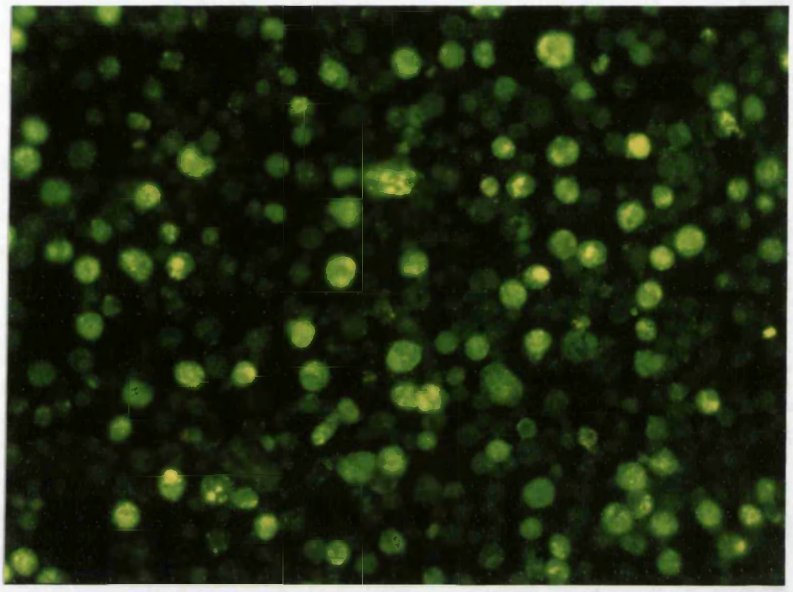
In the control group alone (total of 463 sera), seropositivity at a 1:40 serum dilution was placed at 55% (Table 2.3.2). Female controls displayed higher seropositivity than males (Fig. 2.3.2). Sixty-three percent of females were seropositive at 1:40 serum dilution compared with only 49% of males.

The age distribution of the cases and controls was structured into 7 categories. The age categories and the percentage of the study population within these age groups are shown in Figure 2.3.3. There was a bias towards older age groups. Of the cases and controls 26.5% were over the age of 70 years.

Figure 2.3.1

HHV-6 IFA: STAINING PATTERN USING POSITIVE AND NEGATIVE CONTROL SERA

A.



B.

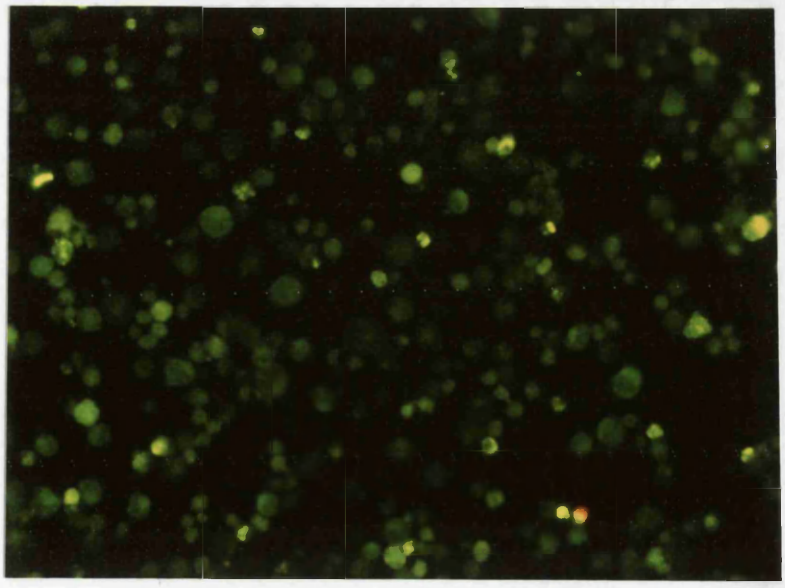


Figure 2.3.1 Immunofluorescent microscopy of HHV-6 infected J JHAN stained with: A. positive control serum; B. negative control serum (x250). Sera used at a 1:10 dilution.

Table 2.3.1

DISTRIBUTION OF ANALYSED CASE CONTROL SERA BY DIAGNOSTIC SUBGROUP

Category	Serum analysed (row%)	Total No. interviewed*
CASES:		
NHL	103 (24)	437
HD	98 (40)	248
MLL	38 (45)	85
CLL	101 (41)	245
CML	40 (33)	122
ALL	32 (50)	64
AML	65 (40)	161
CONTROLS:	463 (20)	2442
TOTAL	940 (25)	3804

* Serum samples were not available for all interviewed subjects in the Yorkshire case control study

Table 2.3.2

HHV-6 SEROPOSITIVITY AS A PERCENTAGE AT DIFFERENT SERUM DILUTIONS IN CASES AND CONTROLS

Serum dilution:	1:40	1:80	1:160	1:320	1:640	1:1280
Controls	255 (55%)	186 (40%)	85 (18%)	34 (7%)	11 (2%)	7 (1.5%)
All cases	321 (67%)	272 (57%)	181 (38%)	110 (23%)	44 (9%)	19 (4%)
Leukaemia cases[1]	149 (63%)	124 (52%)	83 (35%)	47 (20%)	19 (8%)	8 (3%)
Lymphoma cases[2]	172 (72%)	148 (62%)	98 (41%)	63 (26%)	25 (10%)	11 (5%)
AML cases	50 (77%)	45 (69%)	34 (52%)	20 (31%)	8 (12%)	5 (8%)
ALL cases	19 (60%)	16 (50%)	7 (22%)	3 (9%)	0 (0%)	0 (0%)
CML cases	26 (65%)	20 (50%)	14 (35%)	6 (15%)	2 (5%)	0 (0%)
CLL cases	54 (53%)	43 (43%)	28 (28%)	18 (18%)	9 (9%)	3 (3%)
MLL cases	24 (63%)	20 (53%)	12 (32%)	9 (24%)	5 (13%)	3 (8%)
HD cases	75 (77%)	65 (66%)	45 (46%)	30 (31%)	11 (11%)	4 (4%)
NHL cases:	73 (71%)	63 (61%)	41 (40%)	24 (23%)	9 (9%)	4 (4%)
HG-NHL	29 (63%)	23 (50%)	10 (22%)	7 (15%)	3 (6.5%)	2 (4%)
LG-NHL	39 (80%)	35 (71%)	28 (57%)	16 (33%)	6 (12%)	2 (4%)

[1] Leukaemia cases include ALL, AML, CLL, CML.
[2] Lymphoma cases include NHL, HD, MLL.

Figure 2.3.2

HHV-6 SEROPOSITIVITY BY SEX STATUS
(controls only)

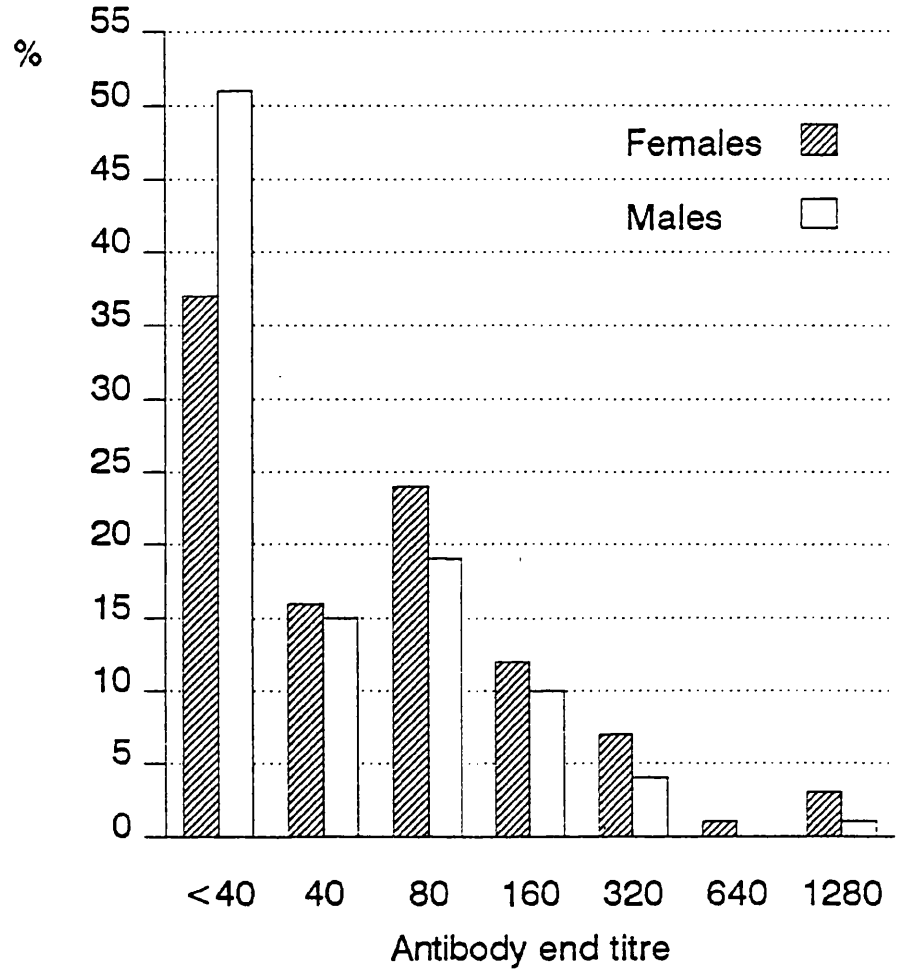


Figure 2.3.2 Percentages of sera with antibody end titres from <40 to 1280, separated by sex. Slightly higher HHV-6 seropositivity was observed in females.

Figure 2.3.3

AGE DISTRIBUTION OF CASES AND CONTROLS IN THE STUDY POPULATION

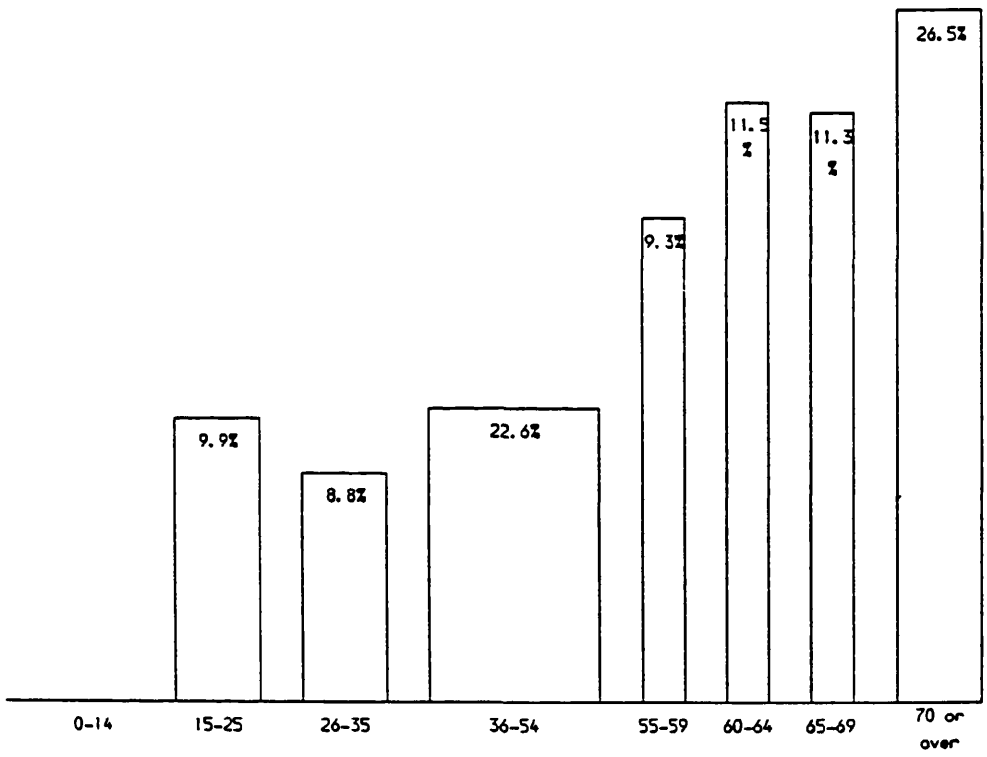


Figure 2.3.3 Percentages of cases and controls in age categories. There was a bias towards older age groups. Over half of the study population was aged 55 years or over.

In the formal statistical analyses employing ORs, the lymphomas showed more evidence of seropositivity than the leukaemias, although the difference was not significant. Seropositivity in cases was significantly in excess of that in the controls at all three serum dilutions (1:40, 1:80, and 1:160). Amongst the 7 disease groups, HD, NHL, and AML showed significantly increased HHV-6 seropositivity compared to controls at each serum dilution. When the analyses were restricted to NHL and a comparison made between low- and high- grade disease, only LG-NHL showed evidence of increased seropositivity compared with the controls. The results of the analyses are shown in Table 2.3.3.

When GMT ratios were analysed, similar results to the OR analyses were obtained (Table 2.3.3). Thus, lymphoma cases showed a higher GMT ratio than leukaemia cases, the difference between the two groups now attaining significance. Analysing by disease subtype AML, MLL, HD, and NHL had GMT ratios significantly greater than 1. For the latter group the effect was concentrated in LG-NHL. These values suggest HHV-6 antibody titres are significantly elevated in these disease groups.

2.3.3 Analysis Of HHV-6 Serology In HD Based On Sibship Size

The second batch of sera sent to Glasgow included a further 91 samples from HD cases. The seropositivity rate in this second group was 83.5% at a 1:40 serum dilution compared to 77% for the first batch of HD sera, confirming increased HHV-6 seropositivity in HD.

The two batches of HD sera and controls were combined for the analysis of the modifying effect of childhood social contact measured by sibship size. The risk factor of sibship size used either "no siblings" or "some siblings" as separate categories. In addition, the HD cases and controls were split into a younger (15-34yr) and older (34-84yr) age groups. Results from 189 HD cases and 195 controls were available for analysis. Table 2.3.4 gives details of the results. There is some evidence from the GMT ratios for increased HHV-6 antibody titres in younger HD cases, particulary those whose early life is likely to have lacked social contact.

Table 2.3.3

ASSOCIATION OF HHV-6 SEROPOSITIVITY WITH DISEASE GROUP STATUS

	Odds Ratio[3] (95% Confidence Interval, CI)			
Serum dilution:	1:40	1:80	1:160	Case:Control GMT ratios (95% CI)
Controls	1.00[4]	1.00[4]	1.00[4]	
All cases	1.72 (1.31-2.25)	2.00 (1.54-2.60)	2.78 (2.05-3.76)	1.78 (1.51-2.11)
Leukaemia cases[1]	1.43 (1.04-1.99)	1.67 (1.21-2.30)	2.48 (1.73-3.56)	1.57 (1.28-1.93)
Lymphoma cases[2]	2.07 (1.47-2.91)	2.39 (1.73-3.31)	3.09 (2.18-4.39)	2.02 (1.64-2.48)
AML cases	2.84 (1.54-5.25)	3.40 (1.94-5.97)	4.95 (2.86-8.55)	2.66 (1.90-3.74)
ALL cases	1.09 (0.51-2.02)	1.40 (0.67-2.92)	1.32 (0.54-3.22)	1.10 (0.68-1.78)
CML cases	1.52 (0.76-3.01)	1.48 (0.77-2.85)	2.43 (1.21-4.85)	1.45 (0.95-2.20)
CLL cases	1.05 (0.67-1.63)	1.18 (0.76-1.85)	1.78 (1.07-2.95)	1.29 (0.97-1.71)
MLL cases	1.51 (0.75-3.04)	1.70 (0.86-3.33)	2.11 (1.01-4.41)	1.74 (1.12-2.69)
HD cases	2.26 (1.35-3.79)	2.65 (1.66-4.25)	3.56 (2.21-5.74)	2.16 (1.61-2.90)
NHL cases	2.18 (1.36-3.48)	2.49 (1.60-3.88)	3.08 (1.93-4.92)	2.01 (1.52-2.66)
HG-NHL cases	1.37 (0.73-2.58)	1.42 (0.77-2.64)	1.17 (0.55-2.49)	1.31 (0.90-1.89)
LG-NHL cases	3.73 (1.79-7.77)	4.41 (2.26-8.54)	6.81 (3.60-12.87)	3.05 (2.13-4.38)

1 Leukaemia cases include ALL, AML, CLL, CML.
2 Lymphoma cases include NHL, HD, MLL.
3 Ratios computed adjusting for age in 7 strata and sex.
4 Reference category.
5 Sera negative at 1:40 have been allocated a titre of 12.

Table 2.3.4

HHV-6 SEROPOSITIVITY FOR HD CASES AND CONTROLS BY AGE GROUP AND SIBLING STATUS

		Number seropositive (% seropositive) at 1:40 dilution		GMT Ratios[1,2]
		Cases	Controls	Cases : Controls (95%CI)
Younger (15-34yr)	No siblings	9 (100%)	3 (38%)	8.0 (3.0-21.2)
	Some siblings	73 (88%)	55 (59%)	2.7 (1.9-3.9)
Older (35-84yr)	No siblings	5 (50%)	5 (50%)	2.3 (0.6-8.9)
	Some siblings	64 (74%)	55 (53%)	2.0 (1.5-2.8)

[1] Ratios computed after adjusting for age in 7 strata and sex.
[2] Sera negative at 1:40 dilution have been allocated a titre of 12.

2.4 DISCUSSION

Utilising selected sera from an epidemiological case-control study of leukaemias and lymphomas conducted in Yorkshire between 1980 and 1986 (Bernard *et al.*, 1987), we tested for an association of HHV-6 seropositivity and antibody titre with haematological malignancies.

In this study, 55% of the control population (total of 463) were seropositive at a 1:40 serum dilution. We found higher antibody titres in females compared to males, a phenomenon that has previously been reported for other viruses including EBV (Biggar *et al.*, 1981).

The large number of sera in the control group make this study one of the most extensive HHV-6 seroepidemiology studies of the general population. The results of a number of other HHV-6 serological surveys are summarised in Table 2.4.1. Most of the studies, including this one, utilised an HHV-6 IFA, although HHV-6 enzyme linked immunosorbent assays (ELISA) and anti-complement immunofluorescence (ACIF) assays have been used.

HHV-6 infection is common. More than half of every population tested has been seropositive. The different rates of HHV-6 seropositivity existing among the study populations may be attributable to variations in methodology. The starting serum dilution used is the most important factor affecting rates of seropositivity. As would be expected, workers using 1:10 as the starting serum dilution reported higher seroprevalence rates than those using 1:40 (Table 2.4.1). The HHV-6 IFA may be more sensitive than an ACIF assay (Couillard *et al.*, 1992) although it is not as sensitive as ELISA (Halprin *et al.*, 1986; Saxinger *et al.*, 1988). However, defining true negative sera has posed problems in the ELISA. IFA using lytically infected cell lines as a source of antigen generally appear to be more sensitive than assays utilising HHV-6 infected umbilical cord blood mononuclear cells (CBMCs) (Table 2.4.1). This may be because IL-2, which has been shown to have an inhibitory effect on the replication of HHV-6 in thymocytes (Roffman and Frenkel, 1990), is used in maintaining CBMCs in culture. Different viral strains may replicate more efficiently in different cell culture systems (Levy *et al.*, 1990b) and the amount of viral antigen may vary with different virus/cell line combinations (Enders *et al.*, 1990; Table 2.4.1). Greater sensitivity of the IFA has been reported using infected cells fixed in methanol as an alternative to acetone (Couillard *et al.*, 1992).

Table 2.4.1

SUMMARY OF HHV-6 SEROEPIDEMIOLOGICAL STUDIES

Study	Assay	Viral Strain	Propagation	Initial Serum Dilution	Age[a]	Geographical Locale	Percent Seropositive
Briggs *et al.* (1988)	IFA	AJ	J JHAN	1:50	Adults[a]	UK	52
Brown *et al.* (1988)	IFA	DV	HSB-2	1:10	>12yr[a]	USA	67
Pietroboni *et al.* (1988b)	IFA	nk*	nk*	1:10	>15yr[a]	Australia	94
Krueger *et al.* (1988)	IFA	GS	HSB-2	1:20	18-52yr[a]	Germany	63
				1:40			26
Linde *et al.* (1988)	IFA	GS	HSB-2	1:10	>1yr[a]	Sweden	85
Yoshikawa *et al.* (1989)	IFA	FG-1	CBMCs	1:10	15-27yr[a]	Japan	76
Levy *et al.* (1990a)	IFA	SF	MT-4 or PBMCs	1:8	Adults[a]	California	97
						China (PRC)	100
						England	67
						Scandinavia	88
						Dominican Rep.	91
						Haiti	100
Rodier *et al.* (1990)	IFA	GS	HSB-2	1:20	Adults	Djibouti, Africa	71
				1:40[b]			44
Enders *et al.* (1990)	IFA	St.W	J JHAN	1:16	>10yr[a]	Germany	70
		U1102	CBMCs	1:16			44
Levine *et al.* (1992d)	IFA	nk*	HSB-2	1:10	20-49yr	Ghana	100
						Malaysia	48
						USA	96
Okuno *et al.* (1989)	ACIF	Z-29	MT-4	1:10	>10yr[a]	Japan	81
Yanagi*et al.* (1990)	ACIF	Z-29	CBMCs	1:10	>14yr[a]	Japan	92
				1:40[b]			58
Saxinger *et al.* (1988)	ELISA	GS	HSB-2	1:400	6-74yr	USA	97

[a]Denotes that only part of the age distribution of the study is included here. [b]Signifies that the results at this serum dilution have been calculated from results given in the text. PBMCs=peripheral blood mononuclear cells; yr=years. *nk= not known. Table taken from Levine *et al.* (1992c)

HHV-6 seroprevalence may decline in older groups (Enders *et al.*, 1990; Levy *et al.*, 1990a), a finding reflected as declining antibody titres in one study (Yanagi *et al.*, 1990). In this study there was a bias towards older age groups. For that reason all of the statistical analyses have included adjustments for age and also sex to counteract the possible confounding effects of these factors.

In our study, lymphoma and leukaemia groups had both higher seropositivity at a serum dilution of 1:40 and higher HHV-6 antibody titres than the controls. Analysing the data both by ORs and by GMT ratios on individual disease subtypes, we identified strong differences between HHV-6 seropositivity in AML, HD, and LG-NHL and the controls. Higher antibody prevalence to HHV-6 has also been found in malignant lymphoma patients in two other studies, particularly HD and HG-NHL, although their design involved smaller sample numbers and they were not case-controlled (Ablashi *et al.*, 1988; Biberfeld *et al.*, 1988). More recently Torelli *et al.* (1991) reported increased antibody titres to HHV-6 in HD.

Similar case-controlled serological studies on other herpesviruses in leukaemia/lymphoma have identified higher antibody titres against EBV antigens in HD (Evans and Gutensohn, 1984), NHL (Dumont *et al.*, 1976) and CLL (Levine *et al.*, 1971b). Higher CMV antibody titres have been reported in HD (Langenhuysen *et al.*, 1974), although this finding is not consistent (Evans *et al.*, 1978; Henderson *et al.*, 1973). Contradictory evidence exists for increased seroprevalence of HSV-2 in HD (Catalano and Goldman, 1972; Hesse *et al.*, 1977; Evans *et al.*, 1978).

In lymphoproliferative disease, polyclonal B cell proliferation could lead to increased antibody levels to a whole range of antigens. The restriction of elevated titres to only some herpesviruses indicates a specific antibody response. Either *de novo* infection or increased viral replication caused by virus reactivation or altered chronic infection could be reflected by the specific humoral response. The identification of HHV-6 DNA and protein in salivary glands (Fox *et al.*, 1990a; Krueger *et al.*, 1990) and the detection of HHV-6 in saliva (Gopal *et al.*, 1990; Jarrett *et al.*, 1990) points towards the oropharynx as one site of viral replication and persistence. Although HHV-6 is detectable in the blood of the majority of healthy persons by PCR (Gopal *et al.*, 1990; Jarrett *et al.*, 1990), the virus has been difficult to isolate from this source. The majority of isolates have been made from the blood of exanthem subitum

patients or immunosuppressed individuals suggesting HHV-6 is normally in a latent state in blood. Recently Kondo *et al.* (1991) reported that monocytes are a possible site of latency.

In haematological malignancies, reactivation of HHV-6 might occur with increased frequency. Disease- or therapy- induced immunosuppression could promote reactivation. The majority of cases included in our study would have started treatment by the time a serum sample was taken. Sera were collected after a period of recovery and prior to the next round of therapy. The absence of elevated HHV-6 antibody titres in all patients receiving treatment and no association with any particular regimen suggests that treatment-induced immunosuppression has a limited effect on antibody titre.

Despite the observations of elevated HHV-6 antibody titres in AML, HD, and LG-NHL, HHV-6-specific DNA sequences have been detected only rarely by Southern blot hybridisation in these conditions. Combining the studies of Josephs *et al.* (1988) and Jarrett *et al.* (1988) 5 NHL samples were positive from 157 NHL, 37 HD and 10 AML patients. These data indicate that HHV-6 is unlikely to be the aetiological agent of AML and the majority of NHL cases.

In HD, *de novo* infection may account for the data presented here. Our analysis reveals strong evidence for increased HHV-6 seropositivity and GMT ratio in HD occurring in young adults without siblings. A number of studies have noted an association between HD in children and young adults and both personal and community socio-economic status (MacMahon, 1966; Gutensohn, 1982; Kirchoff *et al.*, 1980; Glaser, 1987). One lifestyle characteristic that is positively associated with HD is sibship size (Gutensohn and Cole, 1981). A person with no siblings is more likely to develop HD than someone with siblings. This feature may indicate a lack of social contact in the family group resulting in late exposure to infectious agents.

For HD there is accumulating epidemiological evidence that HD occurring among young adults is an unusual consequence of a common viral infection (Gutensohn and Cole, 1980), possibly linked to late age at first exposure to the virus (Mueller, 1987). A prior history of IM-like disease is a recognised risk factor for HD (Munoz *et al.*, 1978), and elevated antibody titres to EBV have been documented before the development of HD (Evans and Comstock, 1981;

Mueller *et al.*, 1989). Such findings could signify that HD is an abnormal response to herpesvirus infection, including EBV and HHV-6.

There is increasing molecular evidence to suggest a role for EBV in at least a subset of HD patients (Weiss *et al.*, 1987; Weiss *et al.*, 1989; discussed in Chapter 3). This is not the case for HHV-6. Josephs *et al.* (1988) and Jarrett *et al.* (1988) did not detect HHV-6-specific DNA in tumour biopsy tissue from a large series of HD patients by Southern blot hybridisation. Detection of virus in the putative tumour population of HD is problematic, since the Reed-Sternberg (RS) cells often only constitute a small percentage of the total population.

Torelli *et al.* (1991), using PCR, identified HHV-6 DNA sequences in 3 of 25 HD cases, but not in any of 41 cases of NHL. These findings may reflect the different sensitivities of the two detection assays, although 2 of the 3 PCR positive HD cases were confirmed by Southern blot hybridisation. The added sensitivity of PCR may allow the detection of HHV-6 in RS cells. Alternatively PCR may be amplifying HHV-6 DNA present within a small number of infected monocytes or lymphocytes. The identification of HHV-6 by *in situ* hybridisation within RS-cells may confirm a role in HD. Krueger *et al.* (1989) described HHV-6 positive cells by *in situ* hybridisation studies in a number of lymphoproliferative conditions including HD. However the authors did not report the presence of HHV-6 within the RS cell population.

Interaction between viruses may affect HHV-6 antibody titres. HHV-6 has frequently been isolated from persons infected with retroviruses (Salahuddin *et al.*, 1986; Downing *et al.*, 1987; Tedder *et al.*, 1987; Lopez *et al.*, 1988; Levy *et al.*, 1990b). Co-infection of CD4+ve lymphocytes by HIV-1 and HHV-6 *in vitro* produces accelerated HIV-1 expression and cellular death (Lusso *et al.*, 1989). This may occur owing to the ability of HHV-6 to transactivate the HIV promoter (Lusso *et al.*, 1989; Horvat *et al.*, 1989). It is possible a retrovirus may be stimulating herpesvirus replication. However, there is no serological evidence that a known retrovirus is involved in HD (Chorba *et al.*, 1988) and in males infected with HIV-1, HHV-6 antibody titres were similar to HIV-1 negative male blood donors (Fox *et al.*, 1988). Spira *et al.* (1990) found that HHV-6 antibody levels were not elevated in patients with AIDS. Linde *et al.* (1988,1990) have shown HHV-6 IgG antibody levels can be elevated following primary EBV and CMV infection. The involvement of another herpesvirus in particular

malignancies may promote HHV-6 reactivation that is reflected by increased HHV-6 antibody levels.

Cross-reactive antibodies between HHV-6 and an antigenically related herpesvirus involved in certain malignancies could lead to an apparent elevation of HHV-6 antibody titres. However, cross-reactive antibodies to the 5 previously identified human herpesviruses do not appear to interfere with the HHV-6 IFA including CMV (Morris *et al.*, 1988; Buchbinder *et al.*, 1989) which is most closely related to HHV-6 at the molecular level (Josephs *et al.*, 1986; Efstathiou *et al.*, 1988; Lawrence *et al.*, 1990).

This seroepidemiological study has identified elevated HHV-6 antibody titres in 3 conditions, LG-NHL, AML, and HD. To date HHV-6-specific DNA has been detected in tumour tissue of only a limited number of NHL cases. Conclusive molecular evidence is lacking for a role of HHV-6 in HD, although this study has provided serological evidence that HHV-6 antibody titres are elevated in younger cases of HD particularly those whose early life may have lacked social contact. It remains a possibility that HHV-6 may be acting as a marker for delayed infection to common viruses in young adult cases of HD.

CHAPTER 3

THE SEROEPIDEMIOLOGY OF HHV-6 AND EBV IN HODGKIN'S DISEASE

3.1 INTRODUCTION

The aetiology of Hodgkin's disease (HD) is poorly understood. The malignancy is unusual in that the bulk of the tumour is composed of reactive cells within which the malignant Reed-Sternberg (RS) cells are found. The cellular origin of these neoplastic cells remains controversial, but current opinion favours a lymphoid origin (reviewed by Drexler, 1992).

Sternberg (1898) and Reed (1902) originally described the typical multinucleated giant cells bearing their names and recognised the unique relationship of these cells to HD. Both authors believed the process to be infectious in nature. Sternberg proposed Mycobacterium tuberculosis, and Reed some other unidentified agent. Early in disease there is a functional impairment in cell-mediated immunity (Levy *et al.*, 1974) and circulating immune complexes are also present, indicating an active immune response (Long and Kaplan, 1977).

The diagnosis of HD rests on the identification of the RS cell in an appropriate histological setting. HD is divided histologically into four types based on the presence of RS cells and variants and the surrounding reactive cell population: lymphocyte predominant (LP); nodular sclerosing (NS); mixed cellularity (MC); and lymphocyte depleted. Recent data suggest that HDLP is a distinct entity which should be classified separately from the other forms of HD (Nicholas *et al.*, 1990).

The overall incidence of HD in the UK is 2.4 per 10^5 person years (McKinney *et al.*, 1989). HD can occur at any age although it is rare in children 4 years of age or younger (Kung, 1991). It is one of the most common forms of malignancy in young people and has an unusual age distribution which is quite distinct from that of the NHLs.

HD shows a bimodal age incidence curve (MacMahon, 1966), although the shape of the curve varies in different communities. Correa and O'Conor (1971) suggested that there are at least three epidemiological patterns of HD. In type I, the first peak of the bimodal age incidence occurs in childhood, there is a low incidence in the third decade and a second peak in older age groups. This pattern is found in developing countries. The type III pattern, which is seen in developed countries, is characterised by low rates in childhood and a

pronounced first peak in young adults. A second peak is observed in older adults. In both developed and developing countries the incidence of HD increases or reaches a plateau from 45 years of age (McKinney *et al.*, 1989; Correa and O'Conor, 1971). An intermediate pattern, type II, is found in rural areas of developed countries and is thought to represent a transition state between type I and type III patterns.

The age incidence curves for HDNS and the other subtypes are quite distinct (McKinney *et al.*, 1989; Glaser and Swartz, 1990). HDNS has a unimodal age incidence curve and accounts for the young adult peak observed in developed countries. The other subtypes show a gradual increasing incidence with increasing age. HDMC is relatively more common in children and older adults.

Vianna *et al.* (1971) first reported the aggregation of HD cases suggesting the possibility that the disease might occur in clusters. A recent survey in the UK (Alexander *et al.*, 1989; Cartwright *et al.*, 1990) found significant evidence of spatial clustering with 13% (0-34 years) of cases identified as clusters. The pattern of clustering would be consistent with an aetiology involving either delayed exposure to a common, but not ubiquitous, infectious agent or infection with a virus with a long latent period.

Gutensohn and Cole (1980) hypothesised that HD, particularly in young adults, is a consequence of delayed exposure to a common infectious agent. An increased risk of developing HD in young adults is associated with a high socioeconomic status such as a high standard of living in childhood (Gutensohn and Cole, 1980) as well as family and sibship size (Gutensohn and Cole, 1981). A child with no siblings is more likely to develop HD than a child with siblings. Such factors may influence the age at which common pathogens are first encountered. MacMahon (1966) hypothesised that HD is a grouping of at least three entities which may have distinct aetiologies. He proposed that these groups could be distinguished on the basis of age at onset of disease: 0-14 years; 15-34 years and 50 years and over. He suggested that HD in young adults may be caused by an infectious agent.

Serological studies have investigated the role of many viruses in HD, mainly focusing on the herpesviruses. The most consistent findings have been reported for EBV. As a group, patients with HD have repeatedly been shown to have elevated antibody titres to EBV antigens, including VCA and EA and these

increased antibody levels have been shown to precede diagnosis (Henle and Henle, 1973; Evans *et al.*, 1978; Evans and Gutensohn, 1984; Evans and Comstock, 1981; Mueller *et al.*, 1989). Studies that have examined EBV serology by HD histological subtype have produced inconsistent results (Levine *et al.*, 1971a; Hesse *et al.*, 1977; Evans *et al.*, 1978; Evans and Gutensohn, 1984). A past history of IM determined by a positive Paul Bunnell test, which is linked in the majority of cases to primary EBV infection in adolescence or early adulthood, is a recognised risk factor for HD (Munoz *et al.*, 1978).

Weiss *et al.* (1987) reported the detection of EBV genomes in tumour samples from 4 out of 21 cases of HD using Southern blot analysis. Other studies have produced similar findings (Anagnostopoulos *et al.*, 1989; Staal *et al.*, 1989; Gledhill *et al.*, 1991). However, EBV is not always detectable in tumour tissue and in serological studies a subset of HD patients are EBV seronegative suggesting no prior infection with this virus (Henle and Henle *et al.*, 1973; Evans and Gutensohn, 1984). Therefore, EBV cannot be implicated in all cases of HD.

A second herpesvirus linked to haematological malignancies is HHV-6. We found elevated antibody titres to HHV-6 present in three malignancies including HD (Clark *et al.*, 1990; Chapter 2). At present molecular evidence linking HHV-6 to HD is weak (Josephs *et al.*, 1988; Jarrett *et al.*, 1988; Torelli *et al.*, 1991).

The identification of elevated antibody titres to both EBV and HHV-6 could signify the involvement of each virus with distinct subsets of HD. Alternatively, an undefined interaction between these two viruses in HD could affect antibody titres to both of them. In support of the latter idea, Linde *et al.* (1988,1990) demonstrated that HHV-6 antibody levels are raised following primary EBV or CMV infection, perhaps as a result of viral reactivation. In HD, where there is increasing molecular evidence for a direct role of EBV, elevated antibody titres to HHV-6 may be a consequence of an interaction with EBV. If such an interaction in HD occurs, it would be predicted that elevated HHV-6 antibody levels would tend to concur with elevated EBV antibody levels. However, an independent association of HHV-6 antibody titres with HD might indicate a possible role for HHV-6 in a separate subset of HD cases.

This study was designed to investigate the association of antibody titres to HHV-6 and EBV in HD. The HD case and control sera from the HHV-6

seroepidemiology survey were re-screened for antibody titres to two EBV antigens, the EBV-VCA and EBV-EA. Antibodies to EBV-VCA are found in all individuals who have been infected with the virus, whereas antibodies to EBV-EA are detected during primary infection and during viral reactivation. EBV-EA antibodies are detectable only occasionally in healthy persons normally in association with high EBV-VCA titres.

The results were statistically analysed to determine the degree of correlation between antibody titres to the two viruses in HD cases and controls. Further, the serological differences between cases and controls for HHV-6 and EBV were examined in age groups corresponding to the peaks of HD age incidence curve found in developed countries. The statistical analyses included investigating the effect of sibship size, a recognised risk factor for developing HD, using the HHV-6 and EBV serological data.

HD cases can now be categorised as either EBV-associated or non-associated based on the presence of EBV within RS cells. This development stemmed from the availability of EBV-specific reagents that allow the *in situ* detection of EBV gene products within the RS cells of HD tissue sections (Pallesen *et al.*, 1991a; Weiss *et al.*, 1991; Armstrong *et al*, 1992a,1992b). One such assay involves the detection of the EBER transcripts in EBV-infected cells (Weiss *et al.*, 1991). By virtue of their abundance, estimated to be between 10^5 and 10^6 copies per viral genome (Lerner *et al.*, 1981), the EBERs provide an excellent target for *in situ* detection in routinely formalin-fixed and paraffin-embedded tissues. In order to examine the association of EBV and HHV-6 antibody titres with EBV-associated and non-associated HD cases, a group of HD patients were selected from whom sera and tissue sections were available. The sera were tested for antibodies to HHV-6, EBV-VCA and EBV-EA and the presence of EBV in RS cells determined by an EBER RNA *in situ* hybridisation technique as described by Weiss *et al.* (1991) and Armstrong *et al.* (1992b) using paraffin-embedded biopsy material.

3.2 MATERIALS AND METHODS

3.2.1 Source Of Sera

Sera from 189 HD cases and 215 controls were available for analysis. The sera had been collected as part of the epidemiological case-control study of leukaemias and lymphomas conducted in Yorkshire between 1980 and 1986. Staff at Glasgow were blind to the case/control status of each serum sample. Further details of the sample populations and source of controls are described in 2.2.1.

Sera and paraffin-embedded formalin-fixed biopsy tissue from 21 HD patients were kindly supplied by Drs. S. Proctor and B. Angus, Royal Victoria Infirmary, Newcastle.

3.2.2 Tissue Culture

The EBV-producer B95.8 marmoset cell line and EBV infected non-producer Raji cell line were maintained in complete medium (see 2.2.2) containing 10%FCS. Cells were split twice a week at a ratio of 1:5, gassed with CO_2 and incubated in sealed Costar tissue culture flasks at 37°C. Cell stocks were prepared and kept in liquid nitrogen as described in 2.2.3. B95.8 cells were stored in liquid nitrogen in a mixture of 10%DMSO and 90% FCS.

3.2.3 Indirect Immunofluorescence Assays

(i) HHV-6 IFA

All sera, except for those obtained from Newcastle, had been screened for HHV-6-specific antibodies. The latter were tested using the protocol described in 2.2.4 except that the conjugate consisted of the FITC-labelled rabbit anti-human IgG heavy chain specific sera only.

(ii) EBV-VCA IFA

Antibodies against EBV-VCA were detected using the IFA of Henle and Henle (1966). The EBV-producing cell line B95.8 was stimulated with 20ng/ml TPA (Sigma) for 3 days. At any time between 1-5% of B95.8 cells undergo

spontaneous virus reactivation to produce infectious virus and subsequently express the VCA. By stimulating the cells with TPA the number of cells entering a productive cycle of infection is increased (Zur Hausen *et al.*, 1978). Following TPA stimulation, the cells were harvested and used as the source of antigen on slides in an IFA identical to that detailed in 2.2.4 except the conjugate described in 3.2.3.(i) was used. No adsorption step of sera was carried out. All sera were initially screened at a dilution of 1:10 and those scored positive were diluted two-fold until the end titre was reached. This was defined as the reciprocal of the serum dilution at which VCA specific fluorescence was last seen.

(iii) EBV-EA IFA

Antibodies against EBV-EA were detected using the IFA described by Long *et al.* (1974). Raji cells were treated with 50µg/ml of 5-Iododeoxyuridine (IdU) (Sigma) for 3 days and used as the source of antigen in an IFA similar to the HHV-6 IFA described in 2.2.4 except that the conjugate described in 3.2.3.(i) was used. Virus activation in either producer or non-producer LCLs can be induced by thymidine analogues (Hampar *et al.*, 1972). In non-producing cell lines such as Raji, treatment with IdU results in the synthesis of EA but not VCA following short-term exposure. No serum adsorption step was carried out. Raji cells were air-dried and fixed on slides with ice-cold acetone. Such a method of fixation allows antibodies specific to both the diffuse- and restricted-components of the early antigen complex to be detected. As for EBV-VCA, sera were initially screened at a 1:10 dilution and an endpoint titration determined for EBV-EA positive sera.

(iv) Control sera

An EBV-VCA positive serum sample obtained from a person within the department was included in all EBV-VCA IFAs. An EBV-EA positive serum sample, kindly donated by Dr. S. Cameron, Glasgow Royal Infirmary, was included as an inter-assay control in the EBV-EA IFA.

3.2.4 Statistical Methods

Th statistical analysis was carried out by Dr. Freda Alexander. Antibody end titres were used to calculate GMTs. Pearson's correlation analysis was applied

to the natural logarithm of the antibody end titre (log(end titre)). Multiple regression and analysis of variance were applied to log(end titre) with negative sera arbitrarily allocated values of 2 for EBV-EA and 4 for EBV-VCA and HHV-6. The reasons for giving negative sera an arbitrary antibody titre are set out in 2.2.5. Case-control differences of log(GMT) were derived with and without including the correlation analysis results to adjust for antibody levels to the other viral antigens. These have been converted into ratios of GMTs. Adjustment for age in 7 strata and sex were included in all analyses.

The second group of HD case and control sera sent to Glasgow (see 2.2.1) were derived to augment the original HD cases and controls and involved sampling which favoured persons without siblings. Therefore, in addition, duplicate analyses have included adjustment for the presence/absence of siblings to confirm the results were not substantially altered by the sampling criteria.

The serology and EBER *in situ* results from the Newcastle group of patients were analysed by a Mann-Whitney test, using the statistical package Minitab.

3.2.5 EBER *In Situ* Hybridisation Assay

Thin sections from paraffin-embedded biopsy material were examined for the presence of EBV EBER RNA, which is abundantly transcribed in cells latently infected by EBV. Section material was hybridised with a biotinylated oligonucleotide probe complementary to EBER-1 RNA: 5' AGA CAC CGT CCT CAC CAC CCG GGA CTT GTA 3' using methodology described previously (Weiss *et al.*, 1991; Armstrong *et al.*, 1992b). Hybridisation was detected using an ABC method (Dako) utilising nitro blue tetrozolium as the chromogenic substrate. A distinct blue-black colour within the nucleus was considered a positive reaction. A known positive case was included in each assay and sections were hybridised with a nonsense probe with a base composition identical to that of the EBER-1 probe as a negative control. This assay was kindly performed by Alison Armstrong.

3.3 RESULTS

Antibody endpoint titrations to two EBV antigens, EBV-VCA and EBV-EA were determined in sera from 189 HD cases and 215 suitable controls. The sera had previously been screened for antibodies to the AJ strain of HHV-6 (Clark *et al.*, 1990; Chapter 2). GMTs for EBV-VCA and EBV-EA were calculated by case/control and either age (Table 3.3.1) or sex status (Table 3.3.2). There was evidence for slightly higher GMTs in females compared to males in both the case and control groups. Such a trend has previously been reported (Biggar *et al.*, 1981).

3.3.1 Correlation Analysis

The serological data were statistically analysed to determine the degree of correlation amongst antibody titres to HHV-6, EBV-VCA, and EBV-EA. The case and control groups were analysed separately and the correlation analysis results are shown in Table 3.3.3. There was a highly significant correlation between EBV-VCA and EBV-EA antibody titres in both the case and control groups. This was more evident in the cases. There was also a statistically significant correlation between HHV-6 antibody titres and EBV-EA in both the cases and the controls. The correlation coefficient of HHV-6 with EBV-VCA was statistically significant in only the control group ($p<0.1$).

A problem with correlation coefficients is that they can often be misleading when the data are extensive but the results of no practical importance. This can be true when two measurements such as antibody titres to two antigens are taken as proxies for each other. As an alternative analyses, multiple regression modelling confirmed significant associations between HHV-6 and EBV-EA antibody titres in both the cases and controls after adjusting for age and sex. In the controls, only a weak correlation between HHV-6 and EBV-VCA was found (F. Alexander, personal communication).

3.3.2 GMT Ratio Analysis

When GMT ratios were calculated, there was a statistically significant difference between the cases and controls for EBV-VCA, EBV-EA and HHV-6 (Table 3.3.4, unadjusted column). Thus, cases had significantly higher antibody titres to all 3 antigens compared to the controls.

Table 3.3.1

GMT OF EBV SEROLOGY BY AGE AND CASE CONTROL STATUS

	Cases		Controls	
Age (years)	EBV-VCA	EBV-EA	EBV-VCA	EBV-EA
15-25	697.0	19.7	195.8	7.9
26-35	449.2	12.1	410.4	6.4
36-54	1135.1	22.1	465.3	7.5
55-59	1248.7	21.7	604.1	6.8
60-64	1043.9	20.1	784.7	9.3
65-69	1689.0	31.9	681.6	17.3
70-	608.2	11.1	515.0	8.3

Table 3.3.2

GMT OF EBV SEROLOGY BY SEX AND CASE CONTROL STATUS

	Cases		Controls	
	EBV-VCA	EBV-EA	EBV-VCA	EBV-EA
Male	718.0	14.9	349.8	6.9
Female	851.9	22.3	436.7	9.2

Table 3.3.3

PEARSON CORRELATION COEFFICIENTS AMONGST HHV-6, EBV-EA, AND EBV-VCA ANTIBODY TITRES

	EBV-EA	EBV-VCA	HHV-6
Cases only:			
EBV-EA	1.00	0.58	0.17
EBV-VCA		1.00	0.05
HHV-6			1.00
Controls only:			
EBV-EA	1.00	0.37	0.17
EBV-VCA		1.00	0.10
HHV-6			1.00

EBV-EA and EBV-VCA correlations highly significant ($p<0.001$).

EBV-EA and HHV-6 correlations also significant ($p<0.05$).

Table 3.3.4

EBV AND HHV-6 GMT HD CASE : CONTROL RATIOS (95% CI)

	Unadjusted	Adjusted[1]
EBV-EA	2.35	1.70
	(1.62-3.42)	(1.22-2.38)
	p<0.01	p<0.01
EBV-VCA	2.04	1.33
	(1.38-3.03)	(0.93-1.90)
	p<0.01	NS
HHV-6	2.47	2.25
	(1.97-3.11)	(1.78-2.83)
	p<0.01	p<0.01

[1] For EBV, analyses adjusted for influences of age, sex and antibody titres to the other EBV antigen.

For HHV-6, analyses adjusted for influences of age, sex and antibody titres to both EBV antigens.

NS = not significant.

In further analysis of GMT case:control ratios, controlling for degrees of association amongst antibody titres to the 3 antigens was included, as well as adjustments for age and sex variation. The correlation analysis provided a measure of the strength of association amongst the antibody titres to each antigen. By incorporating the correlation analysis results into the GMT ratio calculations, the relative independence of the case:control difference for each antigen was obtained. For example, with control for EBV-VCA antibody levels, does a case control difference still remain for EBV-EA antibody or is their elevation simply correlated with elevated EBV-VCA antibody? The results are shown in Table 3.3.4, adjusted column. There was still a significant, although reduced, difference between cases and controls for antibody to EBV-EA, but not EBV-VCA. The GMT ratio for EBV-EA is now 1.70 (95%CI 1.22-2.38, $p<0.01$), but for EBV-VCA 1.33 (95%CI 0.93-1.90, not significant). These findings suggested that antibody titres to EBV-EA were more closely associated with HD status than EBV-VCA and therefore future analyses were restricted to EBV-EA only. The statistically significant difference between cases and controls remained for HHV-6 antibody when associations between HHV-6 and EBV-EA/EBV-VCA were removed from the analysis. This would suggest that HHV-6 and EBV antibody titres found in HD are in part, at least, independent.

3.3.3 Analysis Of Serological Results Based On Age

MacMahon (1966) proposed that multiple aetiologies existed in HD depending on age. In order to test this hypothesis, the cases and controls were divided into three age groups, a younger (aged 15-34), a middle age group (aged 35-54), and an older group (aged 55 and over). The younger group corresponds to the first incidence curve peak seen in developed countries (MacMahon, 1966; McKinney *et al.*, 1989). Analysis of the GMT ratios were restricted to EBV-EA and HHV-6 and the results are shown in Table 3.3.5. The differences between the cases and controls for EBV-EA in the 3 age groups were similar, although a slightly increased ratio value was found in the middle age group. For HHV-6, the younger age group had a higher GMT ratio compared to the middle and older groups. This result may represent a stronger association between HHV-6 and HD in that particular age group. However, the GMT ratios in the 3 age groups were not statistically different for either EBV-EA or HHV-6.

Table 3.3.5

EBV-EA AND HHV-6 GMT HD CASE : CONTROL RATIOS BY 3 AGE GROUPS (95% CI)

Age Group	EBV-EA[1]	HHV-6[2]
Younger	1.79	2.89
(aged 15-34 years)	(1.01-3.17)	(2.02-4.12)
	p<0.05	p<0.01
Middle	2.51	1.70
(aged 35-54)	(1.13-5.55)	(1.08-2.68)
	p<0.05	p<0.05
Older	1.35	2.01
(aged 55-84)	(0.59-3.11)	(1.30-3.12)
	NS	p<0.01

1 Analyses adjusted for age, sex and antibody titres to HHV-6.

2 Analyses adjusted for age, sex and antibody titres to EBV-EA and EBV-VCA.

NS=not significant.

3.3.4 Analysis Of Serological Results Based On Sibship Size

Certain lifestyle risk factors such as being an only child or child of low birth order have been associated with HD (Gutensohn and Cole, 1980; Bernard *et al.*, 1987). The effect of sibship size on HD using HHV-6 serology data was described in Chapter 2. These analyses were extended to include the EBV-EA serology results. The findings are shown in Table 3.3.6. The ratio values indicate a stronger association of HHV-6 with HD in younger HD cases lacking siblings. This finding was not repeated for EBV-EA. However, the GMT ratio values for HHV-6 in the 'no siblings' and 'some siblings' groups in both age categories were not statistically different.

3.3.5 Comparison Of HHV-6 And EBV Serology With EBV-Associated And Non-Associated HD Cases

An additional group of 21 sera from HD cases were tested for the presence of EBV-VCA, EBV-EA and HHV-6 antibodies to allow a comparison of viral serology with molecularly-determined EBV-associated or non-associated HD cases. This classification was based on the detection of EBER transcripts in RS cells from paraffin-embedded biopsy material (Fig. 3.3.1). Seven of the 21 cases were EBV-EBER RS cell positive (RS+). There was no significant difference between the EBV-RS(+) and EBV-RS(-) groups for antibody levels to the two EBV antigens or HHV-6. There was however an indication that HHV-6 antibody titres tended to be higher in the non-EBV associated cases. Care has to be taken in the interpretation of these results as the study population is modest. The statistical analyses did not include adjustment for age and sex, two possible confounders in the analyses of serological data. Thus, the ability to classify HD cases into two groups depending on EBV-RS cell status was not mirrored by analyses of EBV or HHV-6 serology.

Table 3.3.6

EBV-EA AND HHV-6 GMT HD CASE : CONTROL RATIOS BY AGE GROUP AND SIBLING STATUS (95% CI)

Age Group	EBV-EA[1]	HHV-6[2]
Younger:		
(aged 15-34 years)		
No siblings	2.92	8.33
	(0.34-24.65)	(2.90-23.96)
	NS	p<0.01
Some siblings	1.73	2.53
	(0.98-3.07)	(1.74-3.69)
	NS	p<0.01
Older		
(aged 35-84 years)		
No siblings	2.51	1.48
	(0.34-18.60)	(0.31-7.05)
	NS	NS
Some siblings	1.82	1.82
	(0.99-3.36)	(1.30-2.55)
	NS	p<0.01

1 Analyses adjusted for age, sex and antibody titres to HHV-6.

2 Analyses adjusted for age, sex and antibody titres to EBV-EA and EBV-VCA.

NS=not significant

Figure 3.3.1

DETECTION OF EBER TRANSCRIPTS IN REED-STERNBERG CELLS BY *IN SITU* HYBRIDISATION

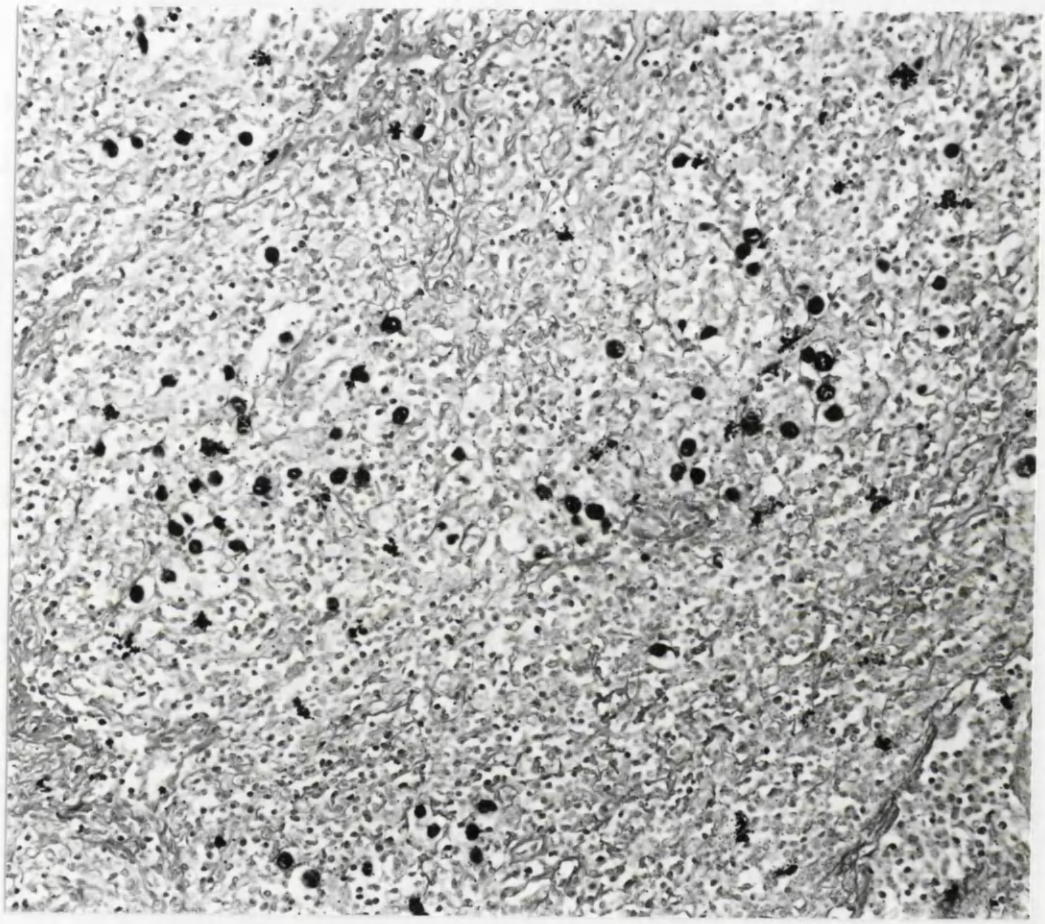


Figure 3.3.1 Detection of EBER transcripts in RS cells by an *in situ* hybridisation assay using formalin-fixed paraffin-embedded HD lymph node tissue. The EBER positive cells are stained black. Kindly provided by Alison Armstrong.

3.4 DISCUSSION

The serology of HHV-6 and EBV was examined in HD using sera derived from an epidemiological case-control study of leukaemias and lymphomas.

In this study, statistically significant differences between the cases and controls for antibody titres to EBV-VCA, EBV-EA and HHV-6 were found. Elevated antibody titres to both viruses have been reported previously (Henle and Henle, 1973; Evans and Gutensohn, 1984; Ablashi *et al.*, 1988; Clark *et al.*, 1990; Chapter 2). In HD, the elevated antibody titres are unlikely to have resulted from polyclonal B cell proliferation as increased humoral responses are restricted to these two viruses. The elevated antibody levels to HHV-6 and EBV could reflect the direct involvement of either virus in the malignancy.

The replication of one herpesvirus may affect the reactivation and replication of another. The results of our correlation analysis suggest that such an interaction between EBV and HHV-6 in HD is not strong and that the serological associations of HHV-6 or EBV with HD are, in part, independent.

However, interactions between HHV-6 and other herpesviruses have been proposed. Elevated HHV-6 and CMV antibody titres have been reported in persons with EBV-induced IM (Linde *et al.*, 1990). Reactivation of herpesviruses can not fully explain the latter phenomenon as antibody levels to measles virus were also raised. Polyclonal B cell proliferation found in IM could explain the rise in antibody titres to a number of unrelated antigens. The same study reported that in IM patients who subsequently seroconverted to CMV, only HHV-6 antibody levels were raised. In the case of CMV infection, antigenic cross-reactivity or selective reactivation of HHV-6 could account for the increased antibody levels to HHV-6.

Chou and Scott (1990) also reported increased IgG antibody titres to HHV-6 in transplant patients with secondary and particularly primary CMV infection. Likewise, Ward *et al.* (1993) have demonstrated HHV-6 reactivation in organ transplant recipients with proven primary EBV or CMV infections. The possibility exists that an interaction between CMV and HHV-6 in HD results in elevated HHV-6 antibody titres. This would seem unlikely however, as higher CMV antibody levels in HD have not been reported consistently (Evans *et al.*,

1978; Langenhuysen *et al.*, 1974; Henderson *et al.*, 1973; Evans and Gutensohn, 1984).

EBV was initially linked to HD based on the findings of serological studies. Our results confirm the presence of elevated antibody titres to EBV antigens in HD. Our analysis also suggests that antibody titres to EBV-EA are more closely associated with disease status than EBV-VCA. A closer association of EBV-EA antibodies with disease status can in part explain the statistically significant correlation with HHV-6 antibody titres which are themselves independently associated with HD. In a previous study, Evans and Gutensohn (1984) proposed that antibody to EBV-VCA was more closely associated with HD compared to EBV-EA. A possible reason for this inconsistency is that the design of statistical testing was different between the two studies. That study used relative risk values to examine the difference between cases and controls, whereas GMT case:control ratios were used here.

There is now strong evidence to indicate a direct role for EBV in the pathogenesis of a subset of HD cases based on molecular studies. Clonal EBV has been detected in HD biopsy tissue (Weiss *et al.*, 1987). More recently EBV-specific reagents have made possible the identification of the virus in RS cells of biopsy material (Pallesen *et al.*, 1991a; Weiss *et al.*, 1991; Armstrong *et al.*, 1992a,b). To-date EBV type A has predominantly been detected in HD tissue (Gledhill *et al.*, 1991; Boyle *et al.*, 1993; Lin *et al.*, 1993).

This ability has facilitated studies examining the epidemiological association of EBV with HD. A stronger association has been suggested for paediatric and older adult cases of HD and also with the MC subtype (Gledhill *et al.*, 1991; Jarrett *et al.*, 1991; Armstrong *et al.*, 1992b, Armstrong *et al.*, 1993). Young adult cases of HD, which make up the first age incidence peak in developed countries and are predominantly of the NS subtype, tend not to be EBV-associated. In this study, EBV-EA GMT case control ratios were not significantly different among the younger, middle and older age groups. Thus, serological and molecular patterns of EBV association with HD did not overlap.

In support of this, there was no significant difference in antibody titres to EBV-VCA and EBV-EA in EBV-associated and non-associated cases, based on the detection of EBER transcripts in RS cells. The statistical analyses is open to criticism as no adjustments for age and sex were included. However, the

findings question the significance and relevance of elevated antibody titres to these particular EBV antigens in HD.

EBV-VCA and EBV-EA are expressed during the lytic cycle of viral replication and presumably are targetted by the immune system, resulting in elevated antibody titres. The pattern of EBV gene expression in RS cells is suggestive of a latent rather than lytic cycle and is similar to that observed in NPC (Young *et al.*, 1988). EBV gene expression in NPC is restricted to EBNA-1 and LMP-1 with an absence of EBNA-2. Expression of LMP-1 has been recognised in RS cells (Herbst *et al.*, 1991b; Pallesen *et al.*, 1991a; Armstrong *et al.*, 1992a) whereas the expression of EBNA-2 appears to be absent (Herbst *et al.*, 1991b; Pallesen *et al.*, 1991a). EBNA-1 is thought to be expressed in RS cells since analysis suggests that the EBV genomes in HD are maintained in an episomal state (Jarrett *et al.*, 1991). At the transcriptional level, LMP-2A and -2B have also been demonstrated in RS cells (Deacon *et al.*, 1993). The role of LMP in RS cells is not known. Molecular analysis of the LMP region has found polymorphism in both the coding and untranslated regions in a series of HD cases (Knecht *et al.*, 1993). However, the regions that encode the protein domains associated with transforming capabilities and immune recognition were conserved. Thus, HD and NPC tumour cells exhibit EBV latent gene expression characteristic of a Lat II pattern (Rowe *et al.*, 1992). Pallesen *et al.* (1991b) reported the expression of the EBV-protein encoded by the BZLF1 gene in a minority of RS cells in 3 of 47 HD cases examined. BZLF1 protein is an immediate early gene product that plays an important role in initiating the EBV lytic cycle (Grogan *et al.*, 1987). These 3 cases were also LMP positive by immunostaining although no EA or VCA were detected in RS cells. Similarly, Joske *et al.* (1992) detected transcripts from the LMP-coding gene in 9 of 20 cases of HD, with only one case also positive for RNA transcripts of the active replication gene BLLF1. Therefore, the stimulating antigens for the immune system, EBV-VCA and EBV-EA, are unlikely to be expressed highly in the tumour cells of HD.

In NPC patients, antibodies to EBV-EA and EBV-VCA of the IgA subclass are detectable and their presence is assumed to represent reactivation of EBV from latency and a pre-NPC condition (De-The *et al.*, 1989). In NPC, viral replication including production of antigens associated with the lytic cycle may be an early event in the path towards malignancy and replicative forms of the virus have been demonstrated in tumour cells (Raab-Traub and Flynn, 1986). Whether

increased EBV lytic cycle replication, perhaps in RS cells, is an early event in HD remains to be clarified. In BL, where elevated antibody titres to EBV lytic antigens have also been recognised, the pattern of latent gene expression in tumour cells is limited to the expression of EBNA-1 only (Rowe *et al.*, 1986). However, Gutierrez *et al.* (1993) were able to detect replicative forms of EBV in BL tumour tissue and proposed that this reactivation may account for the serological changes present in BL.

In the absence of lytic antigen expression within the tumour cells of HD, the host/viral balance could be affected by tumour development or subsequent therapy. Increased EBV replication in a non-neoplastic cell population could account for increased antibody titres to EBV-EA and EBV-VCA.

The significance of an independent serological association of HHV-6 with HD is unclear. It is perhaps relevant that there is a stronger association of HHV-6 with young adult cases of HD. In both the multiple aetiology (MacMahon, 1966) and delayed exposure (Gutensohn and Cole, 1980) hypotheses for the aetiology of HD, viral infection is suspected in this particular age category. To date, molecular evidence linking HHV-6 to HD is incomplete. Torelli *et al.* (1991) identified HHV-6 DNA in tumour tissue from 3 of 25 persons with HD examined by Southern blot or PCR. Interestingly the 3 patients were young adult women with HDNS subtype. Josephs *et al.* (1988) and Gledhill *et al.* (1991), who extended the series previously examined by Jarrett *et al.* (1988), failed to detect HHV-6 in HD cases by Southern blot hybridisation.

Raised HHV-6 antibody titres in HD may reflect late exposure to the virus. Although HHV-6 infection occurs early in life (Briggs *et al.*, 1988), delayed primary infection until adulthood has been associated with clinical illness similar to EBV-IM (Niederman *et al.*, 1988; Steeper *et al.*, 1990; Chapter 4). Delayed exposure to HHV-6 may mirror delayed infection with other agents, including the virus hypothesised to be involved in young adult HD cases and unlikely to be EBV. Alternatively, HHV-6 reactivation may be more common in young adult cases of HD. Reactivation could be induced by either another virus or the disease process itself.

Factors such as therapy after diagnosis may affect HHV-6 antibody titres. As detailed in Chapter 2, the majority of sera were taken from persons receiving anti-tumour therapy. Levine *et al.* (1992b) recently investigated HHV-6 and

EBV serology in patients before and after the initiation of chemotherapy. They found that HHV-6 antibody titres in pre-treatment sera were not significantly different from controls. Significant changes in titres were related to clinical course in HD patients, as HHV-6 IFA titres increased significantly in follow-up of patients who relapsed and decreased significantly over time in patients who did not.

The development of assays that allow the detection of particular HHV-6 antigens may be useful in dissecting the antibody response to the virus in HD. At present the prototype assay (Salahuddin *et al.*, 1986; Briggs *et al.*, 1988) and assay used in this study determine antibody reactivity to the whole range of lytic HHV-6 antigens. More recently, a HHV-6-EA antibody assay (Iyengar *et al.*, 1991) has been described. Interestingly, a higher prevalence of HHV-6-EA specific antibodies, suggestive of viral replication, was found in HD (Iyengar *et al.*, 1991). If HHV-6 were to be present in tumour cells it is possible that no lytic cycle antigens would be expressed. Antigen expression may be absent or restricted to putative latent gene products, in which case measurement of the immune response to lytic cycle associated antigens may be irrelevant. Luppi *et al.* (1993) have reported the detection of integrated HHV-6 *in vivo* in 3 persons, one with HD. Two of the 3 cases were HHV-6 seronegative using an HHV-6 IFA with lytically infected cells as antigen. Likewise, Jarrett *et al.* (1990) were able to detect HHV-6 in PBMCs from seronegative individuals. Thus, future studies should examine the serological response to particular HHV-6 antigens. Similarly, a number of the HD case and control sera in this study are being examined for the serological response to particular EBV latent gene products which might prove useful in distinguishing EBV-associated from non-associated cases (in collaboration with Dr. L.S. Young).

In summary, our analyses identified independent associations of EBV and HHV-6 antibody titres with HD. In the absence of direct evidence linking HHV-6 to HD, antibody levels to HHV-6 may be acting as a marker for exposure to common viruses, particularly in young adult cases. The involvement though of EBV in certain subsets of HD is now well established. Increased antibody titres to EBV lytic cycle antigens however, did not correlate with the presence of EBV in RS cells which questions the relevance of this particular EBV serology in HD.

CHAPTER 4

THE ASSOCIATION OF HHV-6 WITH IM-LIKE ILLNESS. THE PREVALENCE OF ANTIBODY TO HHV-7 BY AGE AND ITS ISOLATION FROM SALIVA

4.1 INTRODUCTION

There are a number of parallels that can be drawn between EBV and HHV-6. Infection with both viruses occurs sub-clinically in the majority of persons during childhood. Primary EBV infection early in life may present with symptoms that resemble an upper respiratory infection and are non-specific. Primary infection with HHV-6 can result in exanthem subitum (Yamanishi *et al.*, 1988). If infection with EBV is delayed until the second decade of life or beyond, IM may result. HHV-6 infection beyond childhood may occasionally result in a similar outcome: Niederman *et al.* (1988) detected HHV-6 specific IgM and high titre IgG antibodies in 3 adult patients with self-limiting, mild illness similar to IM.

Large-scale epidemiological studies demonstrated that IM occurred in patients without pre-existing EBV antibodies and was accompanied by an acquisition of EBV antibodies in the majority of cases (Niederman *et al.*, 1968; Sawyer *et al.*, 1971). Classically, IM refers to illness in young adults associated with characteristic reactive blood smears, fever, sore throat, cervical lymphadenopathy, and frequently serologically detectable heterophile antibodies (Lee *et al.*, 1968; Evans and Niederman, 1989).

The majority of persons who develop EBV-induced IM produce heterophile antibodies. These are antibodies specific for antigens from another species and can be detected by specific laboratory tests. In IM, heterophile antibodies were originally described by Paul and Bunnell (1932) as sheep erythrocyte agglutinins and are present in about 90% of IM cases at some point during the illness. Beef erythrocyte haemolysins and agglutinating antibodies to horse, goat and camel erythrocytes are also demonstrable in IM. The classic heterophile antibody titre is reported as the highest serum dilution at which sheep or horse erythrocytes are agglutinated after adsorption of serum by guinea pig kidney. Heterophile antibodies appear at the onset of illness or shortly after and their presence may be detected for over a year depending on the assay employed (Evans *et al.*, 1975).

Cases of IM-like illness that are heterophile antibody negative can occur. In paediatric populations, EBV-induced IM tends to take a milder course and is often heterophile antibody negative compared to young adults. The most frequently known cause of heterophile negative IM is CMV (Horwitz *et al.*,

1977). Less common causes are drugs, adenoviruses, rubella, toxoplasmosis and HIV (Evans, 1978; Steeper *et al.*, 1987).

In the absence of detectable heterophile antibody, the detection of EBV specific IgM or EBV-EA-diffuse(D)-component specific IgG antibodies are crucial for the diagnosis of EBV-induced IM. IgM antibodies specific for EBV-VCA are usually present in sera at the time of clinical presentation. These IgM antibodies may persist for 3 to 6 months. Antibodies to EBV-EA-D appear later than EBV IgM antibody and are only present in 75% of typical EBV-induced IM cases (Henle *et al.*, 1971).

The extent to which primary HHV-6 infection is responsible for non-EBV associated and perhaps heterophile antibody negative IM is unclear. Often patients with glandular fever or IM-like illness do not have a diagnosis of EBV-induced IM substantiated following virus serological investigations. The ability to attribute another viral cause to IM after tests for EBV are negative would be advantageous. Heterophile antibody negative IM-like syndromes must be separated from potentially more serious illnesses such as acute leukaemia and malignant lymphoma.

The majority of HHV-6 serological studies in health and disease have utilised assays that detected HHV-6 specific IgG antibodies. I have developed an IFA to detect HHV-6 specific IgM antibodies since their presence is likely to reflect active viral replication perhaps as a result of primary infection. Sera from patients who presented with symptoms suggesting an IM-like illness were chosen to be screened for HHV-6 specific IgM and IgG antibodies. The sera had previously been tested for EBV specific IgM and heterophile antibodies. Over two thirds of the sera were from persons who were EBV IgM antibody negative.

The serological response to a virus is likely to consist of antibody specificities directed against a number of viral antigens. For example, antibodies to EBV-VCA arise following primary infection and persist for life, thereby acting as a marker for past infection. Antibodies to EBV-EA appear transiently following initial infection. In IM, EBV-EA-D specific antibodies may be present for up to 6 months (Lamy *et al.*, 1982). During EBV reactivation, often found in immunocompromised patients (Lange *et al.*, 1980), pregnant women (Fleisher and Bolognese, 1983) and elderly people (Sumaya, 1977), the persistence or

recurrence of elevated EBV-EA antibodies is found. In the case of EBV, the detection of EA specific antibodies has been used successfully to identify active infections and establish disease associations (Ooka *et al.*, 1991).

The availability of a screening test to detect HHV-6-EA antibodies may prove useful in distinguishing active viral replication and be used to examine the relationship of the virus with disease. Serological assays for HHV-6 use multiple antigens as substrates. Some HHV-6 proteins synthesised during the HHV-6 replication cycle have been identified (Balachandran *et al.*, 1989; Littler *et al.*, 1990; Balachandran *et al.*, 1991; Okuno *et al.*, 1992; Neipel *et al.*, 1992) including early antigens expressed in the presence of DNA synthesis inhibitors. The HHV-6 prototype IFA, described in 2.2.4, was adapted to restrict viral antigen expression to early antigens only. The sera from patients with IM-like illness were tested for HHV-6-EA specific IgG antibodies by this adapted IFA.

The mode of transmission of HHV-6 is at present unknown. Although the majority of HHV-6 laboratory isolates have been derived from peripheral blood samples, the ubiquitous nature of the virus and its prevalence in young children suggests that the virus is relatively easy to acquire. In parallel with EBV, a likely vehicle for transmission is saliva. EBV can be cultured from throat washings from 10-20% of normal healthy adults, from 50% of renal transplant recipients, and from the majority of seriously ill leukaemia and lymphoma patients (Strauch *et al.*, 1974; Chang *et al.*, 1973). IM has also been spread by blood transfusion and open heart surgery as the "postpump perfusion" syndrome (Gerber *et al.*, 1969). There have been few reports describing the successful isolation of HHV-6 from saliva (Pietroboni *et al.*, 1988a; Levy *et al.*, 1990a; Harnett *et al.*, 1990), suggesting the virus is difficult to isolate from this source. In this study, attempts to isolate virus from saliva were made using three different protocols.

In 1990, Frenkel *et al.* isolated a novel herpesvirus from the mitogen-stimulated purified CD4+ve T cells of a healthy person. This new virus had the morphology of a herpesvirus and limited DNA homology with HHV-6. The virus was named human herpesvirus-7 (HHV-7). Since that time further isolates of HHV-7 have been made from saliva (Wyatt and Frenkel, 1992) and the peripheral blood of a person with chronic fatigue syndrome (Berneman *et al.*, 1992b).

The seroprevalence of HHV-7 in the general population has been studied to a limited extent only (Wyatt *et al.*, 1991). In order to investigate the age at which infection with HHV-7 occurs, sera were collected from hospitalised children. The sera were screened for antibodies to not only HHV-7, but HHV-6 and EBV, to compare the ages at which infection by these three viruses occurred. The ability of HHV-7 to infect different cell lines *in vitro* was also investigated. To date, HHV-7 has been propagated in mitogen-stimulated CBMCs. The preparation and supply of cord blood limits the amount of virus that can be obtained and a permissive continuous cell line would aid further studies on this new virus.

4.2 MATERIALS AND METHODS

4.2.1 Source Of Sera

Two hundred and thirty two serum samples were kindly provided by Dr. G.B. Clements and Mr. J Stewart at the Regional Virus Laboratories, Ruchill Hospital, Glasgow. The sera were taken from persons with a wide range of symptoms associated with IM and had been tested for the presence of EBV-IgM antibodies and heterophile antibodies using an Monospot test (Ortho Diagnostics). Fifty two of the sera were found to be EBV-IgM seropositive and 79 were heterophile antibody positive.

Eleven serum samples from children aged 1 to 3 years were kindly provided by Dr. J. Fox, University College and Middlesex School of Medicine, London. Sequential serum samples from 6 patients taken at 0, 2 and 6 months which, at the initial or second sample, were HHV-6 IgM positive were kindly provided by Dr. H. Kangro, St. Bartholomews Hospital, London.

Two hundred and thirteen serum samples taken from hospitalised children for diagnostic investigations were kindly provided by Dr. P. Mackie, Royal Hospital for Sick Children, Glasgow.

4.2.2 Indirect Immunofluorescence Assays

(i) HHV-6, EBV-VCA, and CMV IFAs

The IFAs used to detect antibodies to HHV-6 and EBV-VCA have been described in 2.2.4 and 3.2.3. A change from the protocol outlined in 2.2.4 was that the FITC-labelled rabbit anti-human IgG conjugate only was used. The assay to detect CMV specific IgM antibodies (Gullsorb) was used according to the manufacturers instructions.

(ii) HHV-6 IgM IFA

The original HHV-6 IFA was modified to detect HHV-6 specific IgM antibodies. Prior to screening, sera were pretreated with Gullsorb IgG Inactivation Reagent to remove competitive HHV-6 IgG class antibodies and the target for rheumatoid factor antibodies. The product of this treatment is a 1:10 dilution of

sera devoid of IgG antibodies. Ten microlitres of sera at a 1:10 dilution were spotted onto each well of an HHV-6 antigen slide and incubated for 3 hours in a moisture chamber at 37°C. The slide was washed four times with PBS over 30 minutes. Ten microlitres of a 1:25 dilution of FITC-labelled rabbit anti-human IgM heavy chain specific antisera (Dako) were spotted onto each well and incubated as above for a further 40 minutes. The slides were then washed as above and viewed under UV light. Sera from 232 patients with symptoms associated with IM were screened by this HHV-6 IgM antibody assay. The same sera were also screened for HHV-6 IgG specific antibodies as detailed above (see 4.2.2.i).

(iii) HHV-6-EA IFA

The assay was based on a method designed to restrict CMV antigen expression to EA only in infected fibroblasts (The *et al.*, 1974). In the CMV protocol the DNA inhibitor cytosine arabinoside (Ara-C) was used at a concentration of 20µg/ml to block DNA synthesis completely as judged by thymidine incorporation followed by autoradiography.

In order to determine the optimal concentration of Ara-C which inhibited DNA synthesis of J JHAN cells, the ability of serial dilutions of Ara-C to inhibit the uptake of thymidine was measured. Briefly, 10^5 J JHAN cells in a volume of 90µl complete medium plus 10%FCS were placed in wells of a flat-bottomed 96-well tissue culture plate (Flow Laboratories). A stock solution of Ara-C (Sigma) in PBS at a concentration of 1mg/ml was sterilised by autoclaving. A series of ten-fold dilutions were set up to give a final concentration ranging from 0.1ng/ml to 100µg/ml Ara-C when 10µl of the dilution were added to each well. As controls, J JHAN cells without Ara-C or complete medium plus 10%FCS without cells were included. Each Ara-C dilution and controls were tested in triplicate. The plate was incubated at 37°C in 5% $C0_2$ overnight. Wells were pulsed with 1µCi tritiated methyl thymidine (Amersham) for 8 hours at 37°C. The contents of wells were harvested onto glass fibre filters (Flow Laboratories) using a Dynatech Titretek Cell Harvester. Incorporation of thymidine was measured on a Beckman scintillation counter. The lowest concentration of Ara-C at which complete inhibition of thymidine incorporation was last seen was 1µg/ml. In the design of the HHV-6-EA IFA, this concentration of Ara-C was used throughout.

Infection of cells with murine sarcoma virus is enhanced by pretreatment with the polycation diethylaminoethyl-Dextran (DEAE-D) (Duc-Nguyen, 1968). A similar pretreatment step with DEAE-D has been reported to enhance HHV-6 infection *in vitro* (Levy *et al.*, 1990b). DEAE-D (Sigma) at a final concentration of 25µg/ml was added to each of two flasks containing 10^6 J JHAN cells/ml (total volume 25ml). Flasks were incubated at 37°C for 30 minutes. Cells were washed twice in complete medium. Cell pellets were resuspended in 8ml cell-free HHV-6 infected tissue culture medium previously harvested from HHV-6 infected J JHAN cultures when maximum cytopathic effect was apparent and passed through a 0.45µm pore filter (Gelman Sciences). The cells were incubated at 37°C for 4 hours and 17ml of complete medium was added. To one flask 1µg/ml Ara-C was added. The flasks were incubated at 37°C for 3 days.

To determine the enhancing effect of DEAE-D treatment on HHV-6 infection, two flasks were set up as above but without addition of Ara-C. The cells in one flask were mock-treated with medium without DEAE-D. After 3 days the level of infectivity of cells was determined by preparation of HHV-6 antigen slides and IFA using HHV-6 positive and negative sera. The level of HHV-6 antigen positive cells in the DEAE-D treated culture was higher (43.5% compared to 34.9%) than in the untreated J JHAN cells. However, the increase in the number of HHV-6 infected cells following DEAE-D treatment was not sufficient to merit its continued use.

The preparation of HHV-6-EA IFA slides and the IFA itself were identical to that described in 2.2.4 except that the FITC-labelled anti-human IgG conjugate only was used. Three groups of sera were screened by both the HHV-6-EA IFA and the prototype HHV-6 IFA. One group was sera from persons with IM-like illness, the second group was 11 sera from children aged 1 to 3 years and the third set was sequential serum samples from 6 persons containing at least one HHV-6 IgM positive sample. All sera were screened at a 1:10 dilution.

4.2.3 Isolation Of Virus From Saliva

(i) Preparation of blood mononuclear cells

Umbilical cord bloods were kindly provided by Royal Maternity and Southern General Hospitals, Glasgow and Royal Alexandra Hospital, Paisley. Adult

peripheral blood was obtained from healthy persons within the Department of Veterinary Pathology. Mononuclear cells were separated from whole blood by gradient centrifugation. Collected blood, already diluted 1:2, was further diluted two-fold in HBSS and 25ml layered onto 15ml Ficoll Hypaque (Pharmacia). The gradient was centrifuged at 800*g* for 30 minutes and the interface collected and washed twice in HBSS. Mononuclear cells were resuspended at 10^6 cells/ml in complete medium containing 20% FCS and stimulated with 5µg/ml phytohaemagglutinin-M (PHA) (BCL) for 3 days. After PHA-stimulation, cultures were refed with medium supplemented with 5U/ml human recombinant IL-2 (Cetus or Amersham).

(ii) Saliva co-cultures

Saliva samples were collected from volunteers within the Department of Veterinary Pathology. The volunteers were asked to provide a saliva specimen into a sterile universal container (Sterilin) and saliva was stored at 4°C until use. Saliva was never stored for longer than 4 hours before being processed. Initially 4 saliva samples were collected. The samples were spun in a microcentrifuge for 5 minutes. The supernatant was removed and diluted 1:1 in RPMI 1640 and passed through a 0.45µm filter (Gelman Sciences). The cell pellet was washed five times with RPMI 1640 containing 800U/ml penicillin, 800µg/ml streptomycin, 300µg/ml gentamicin and 5µg/ml fungizone, following pelleting by 2 minutes centrifugation. The cell pellet was then freeze thawed 3 times and resuspended in 100µl complete medium. Either 100µl of cell-free saliva or saliva cells were added to $2.5x10^6$ PBMCs previously stimulated with PHA for 48 hours in a 1ml volume and incubated for 1.5 hours at 37°C. The volume was then made up to 5ml with complete medium plus 20%FCS. Cultures were fed every 3 days and tested at time-points by IFA for the expression of viral antigens using HHV-6 positive and negative sera. Cultures were kept until the majority of cells were dead as determined by their failure to take up the dye crystal violet.

The protocol was subsequently altered to that described by Levy *et al.* (1990a). Briefly, whole saliva samples were diluted 1:3 in RPMI 1640, passed through a 0.45µm filter and 0.4ml inoculated onto $3x10^6$ PBMCs stimulated previously with PHA. As above, cells were examined for viral antigens by IFA.

Following the study by Wyatt and Frenkel (1992), the protocol was further modified to the procedure used to isolate HHV-7. Saliva samples were diluted 1:3 in complete medium containing 20%FCS and centrifuged at 2000*g* for 10 minutes. The supernatant was passed through a 0.45µm filter and the samples mixed 1:1 with 10^6 PHA-stimulated CBMCs/ml. As above, cells were sampled at time-points for the expression of viral antigens with HHV-6 positive and negative sera using IFA. HHV-7 positive and negative serum controls were also included.

4.2.4 Electron Microscopy

Cells from 2 saliva/CBMCs co-cultures were examined for the presence of viral particles by electron microscopy (EM). Cells in culture medium were mixed (1:1v/v) with a fixative of 1.3% paraformaldehyde/1.6% gluteraldehyde and centrifuged at 400*g* for 7 minutes. One millilitre of fresh fixative was then added. Post-fixation of the cells was carried out using Osmium fix in Millong's buffer. The samples were dehydrated through a series of acetone washes. The samples were then impregnated with araldite resin and embedded and cured in EM capsules. Sections were cut and examined by EM using a Zeiss transmission microscope. The processing of the cell pellets following the initial fixation step and their subsequent examination was carried out by staff in the EM unit of the department under the supervision of Dr. Helen Laird.

4.2.5 Propagation Of HHV-7 *In Vitro*

The original isolate of HHV-7 (RK-strain) was kindly provided by Dr. N. Frenkel, National Institute of Allergy and Infectious Diseases, USA. Virus was propagated in PHA-stimulated CBMCs. Virus was passaged onto uninfected cells when greater than 50% of cells appeared infected by altered morphology in culture or by expression of viral antigens by IFA. HHV-7 infected CBMCs were cocultivated with a number of continuous cell lines. Infected CBMCs were mixed with the target cell line at a ratio of 1:5. Viral cytopathic effect characterised by large cells and viral antigen expression using IFA with HHV-7 positive and negative sera were used to examine the co-cultures for evidence of HHV-7 infection.

4.2.6 HHV-7 IFA

The HHV-7 IFA was identical to the HHV-6 IFA described in 2.2.4. HHV-7 (RK-strain)-infected CBMCs were used as a source of antigen. Uninfected CBMCs were used as controls. Sera from 213 children from the Royal Hospital for Sick Children and 29 adults from within the Department of Veterinary Pathology were tested by IFA for antibodies to HHV-7 at a serum dilution of 1:10 in HBSS. The children's sera were also tested for the presence of antibodies to HHV-6 and EBV-VCA by IFA. The sera of children aged 1 to 4 years (12-59 months) seropositive at a 1:10 dilution for either HHV-7 of HHV-6 were re-screened at a dilution of 1:80. The serology results in the 1+2 years and 3+4 years age groups were statistically analysed in three groups based on antibody titres (10, 10-40, ≥80), for correlation between HHV-6 and HHV-7 antibody titres using a Spearman's p rank correlation analysis, on the statistical package Minitab.

4.2.7 Identification Of Two Herpesvirus Isolates

Two viral isolates were derived from saliva samples following co-cultivation with CBMCs. Viral antigen expression was determined using characterised human sera which consisted of HHV-6 positive and negative and HHV-7 positive and negative controls. The two isolates were tested by IFA for reactivity with mouse monoclonal antibodies (MoAbs) to known human herpesviruses. The IFA was similar to that described in 2.2.4 except that an FITC-labelled goat anti-mouse IgG conjugate (Sigma) was used. The antibody reagents were: a pool of HHV-6 specific MoAbs used at a 1:10 dilution, provided by Dr. J. Fox, University College and Middlesex School of Medicine, London; a MoAb that reacts with EBV-VCA (DuPont) used at a 1:10 dilution; a MoAb that reacts with the late nuclear protein of CMV (DuPont) used at a 1:10 dilution.

4.3 RESULTS

The serology of HHV-6 has mainly relied on IFAs and ELISAs that have been designed to detect IgG class antibodies specific to a range of viral antigens. In this study the HHV-6 IFA was adapted to detect either antibodies of the IgM subclass, which are present following primary infection, or antibodies to HHV-6-EA which are likely to be detectable following active viral replication.

4.3.1 HHV-6 Serology In IM-Like Illness

A total of 232 serum samples from persons with a variety of symptoms associated with IM-like illness were screened by both HHV-6 IgG and IgM IFAs. One hundred and seventy seven of the sera were HHV-6 IgG antibody positive. Ten serum samples were HHV-6 IgM seropositive (all 10 were also HHV-6 IgG seropositive) with titres ranging from 10 to 160 (Table 4.3.1). The same group of sera had previously been tested for the presence of both heterophile and EBV IgM antibodies. Eight of the 10 HHV-6 IgM positive sera were both heterophile and EBV IgM seropositive, suggestive of primary EBV infection. A further patient, although EBV IgM seronegative, was heterophile antibody positive and had a raised EBV IgG titre, consistent with a diagnosis of EBV-induced IM. Two of the HHV-6 IgM positive sera were also CMV IgM seropositive (Table 4.3.1).

Only one person in this study population was HHV-6 IgM seropositive, heterophile antibody and EBV IgM seronegative. This person was also seropositive for EBV-VCA and EBNAs (data not shown) indicative of prior exposure to EBV. The serological findings suggested either primary or reactivated HHV-6 infection in this individual and an absence of CMV or EBV infection or reactivation.

4.3.2 Development Of HHV-6-EA IFA

The 232 sera from persons with IM-like illness were also tested for antibodies to HHV-6-EA by the modified IFA. Only 3 of the sera were found to be positive by this assay (Fig. 4.3.1). A further two groups of sera were screened in which the detection of EA antibodies would be predicted. Sequential serum samples from 6 individuals who had detectable HHV-6 IgM antibodies in either the initial or second serum samples were tested. None of these sera were HHV-6-EA

Table 4.3.1

RESULTS OF HERPESVIRUS SEROLOGY IN 10 HHV-6 IgM SEROPOSITIVE SERA FROM PERSONS WITH IM-LIKE SYMPTOMS

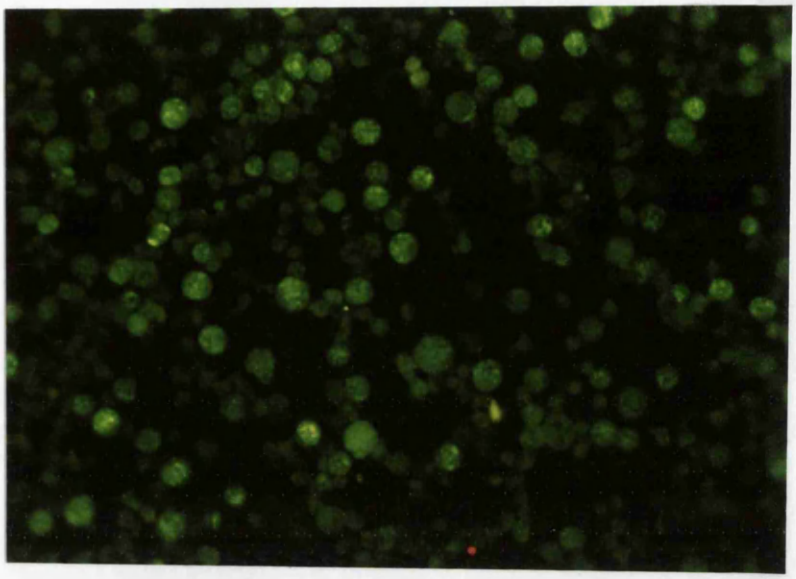
		HHV-6 Serology		EBV Serology			
Patient No.	Age(yr)/Sex	IgM	IgG	IgM	IgG	CMV-IgM	Heterophile Antibody
1	?/M	80	1280	+	320	-	+
2	18/M	10	80	+	160	-	+
3	?/F	40	5120	+	ND	-	+
4	18/F	40	1280	+	1280	-	+
5	15/M	40	5120	+	1280	-	+
6	Child/F	20	1280	+	320	-	+
7	21/F	160	320	+	160	+	+
8	15/F	160	2560	+	320	+	+
9	17/F	80	2560	-	5120	-	+
10	21/F	160	1280	-	80	-	-

ND = not done, yr = years.

Figure 4.3.1

DETECTION OF ANTIBODY TO HHV-6-EARLY ANTIGEN BY IFA

A.



B.

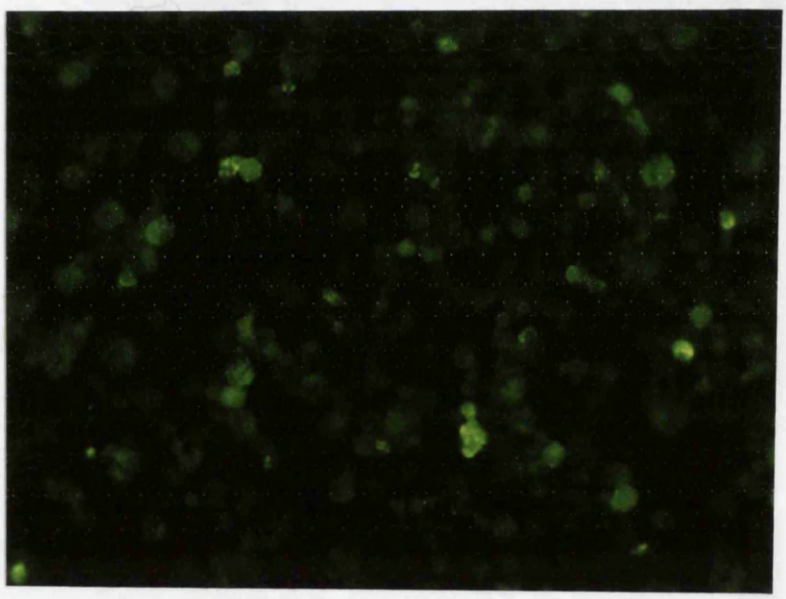


Figure 4.3.1 Immunofluorescent microscopy of an IFA designed to detect HHV-6-EA antibody. Infected J JHAN were treated with an inhibitor of DNA synthesis to prevent expression of HHV-6 late proteins and used as a source of antigen. Three of 232 sera from persons with IM-like illness were positive by this assay at a 1:10 serum dilution. Staining patterns obtained with two sera from patients with IM-like illness are shown. Both sera were HHV-6 antibody positive using the IFA described in 2.2.4. A. Positive staining which suggests the serum sample contained HHV-6-EA antibody B. Negative staining which suggests the serum was HHV-6-EA antibody negative (x250).

antibody positive. In a group of 11 sera from young children, where recent HHV-6 infection was presumed, two serum samples were scored positive by HHV-6-EA IFA (Table 4.3.2). These 2 serum samples were derived from the same child and were taken 5 weeks apart. A stronger reactivity was observed with the first serum sample.

The modified IFA appeared unsuitable for the detection of HHV-6-EA specific antibodies. The level of EA present in Ara-C treated infected J JHAN may be limited and thus, affect the sensitivity of the assay. Possibly the method of fixation did not preserve the reactive epitopes of HHV-6-EA. Alternatively, the positive staining pattern visualised with a limited number of serum samples was non-specific. However, the same sera tested on uninfected Ara-C treated J JHAN were negative.

4.3.3 Isolation Of Virus From Saliva

The first protocol adopted to isolate virus from both cell-free and cellular saliva was unsuccessful. This procedure involved washes of saliva cells in high concentrations of antibiotics and freeze-thawing in order to avoid bacterial and fungal contamination in tissue culture. Such pretreatment of saliva may affect the viability of infectious virus. However, no virus was isolated from cell-free saliva cultures where such pretreatment was not necessary.

The isolation protocol of Levy *et al.* (1990a) was adopted for a further 8 saliva samples. At day 9 of co-culture with PBMCs, no viral antigen expression was detected by IFA with HHV-6 positive and negative control human sera. After 13 days, 2 co-cultures were positively stained with both the HHV-6 positive and negative sera. No viable material from these cultures was available to passage the infection to fresh PBMCs. Attempts to passage the infection with stored tissue culture supernatants were unsuccessful. However, the reactivity with characterised human sera suggested that the infectious agents were not HHV-6.

Two co-cultures of cell-free saliva and CBMCs were set-up according to the procedure of Wyatt and Frenkel (1992) used to isolate HHV-7. In both cases viral antigen expression was detected at 7 and 10 days of co-culture respectively. Up to 50% of cells were stained with both the HHV-6 positive and negative control sera (both HHV-7 seropositive). Reactivity was detected with

Table 4.3.2

ANTIBODY STATUS OF CHILDREN'S SERA TO HHV-6 ANTIGENS

Sample Number	HHV-6-prototype IFA	HHV-6-EA IFA
1	+	-
2	+	-
3	+	-
4	+	+
5	+	+
6	+	-
7	+	-
8	+	-
9	+	-
10	+	-
11	+	-
HHV-6 positive control	+	-
HHV-6 negative control	-	-

an HHV-7 seropositive control, but not with an HHV-7 negative control (weakly HHV-6 seropositive). The infection was passaged in both cases to fresh PHA-stimulated CMBCs maintained on IL-2. Examination of infected CBMCs by electron microscopy detected the presence of herpesvirus particles in both co-cultures (Fig. 4.3.2).

4.3.4 Prevalence Of Antibody To HHV-7, HHV-6, And EBV By Age

The ages of acquisition of antibodies to HHV-7, HHV-6 and EBV were examined using sera from 213 children ranging in age from <2 months to >10 years. There was a decline in the prevalence of antibodies to all 3 viruses over the first 6 months of age (Table 4.3.3) consistent with the decline of maternally-derived antibodies. A rise in the seroprevalence of the 3 viruses in 10+11 month old children suggested primary infection in a number of children. As previously reported the majority of children (31/41, 82.9%) had seroconverted to HHV-6 by the age of 2 years. Similarly, the majority of 1+2 year olds were HHV-7 seropositive (27/41, 65.9%). Although fewer children were HHV-7 seropositive in this age group compared to HHV-6 the difference was not statistically significant. The rate of HHV-7 seropositivity rose to 28/30 (93.3%) in 3+4 year olds compared to 21/30 (70.0%) seropositive for HHV-6, a statistically significant difference. In the 1+2 year olds, the lowest rate of seropositivity to the 3 viruses was found for EBV (21/41, 51.2%). Table 4.3.4 reports the number of sera in each age group that were either dual or triple seropositive for HHV-7, HHV-6, and EBV.

Our results differed from the initial study of HHV-7 seroprevalence in children which reported that infection was uncommon in 1+2 year olds (Wyatt *et al.*, 1991). In 1+2 year olds where HHV-6 infection is common, it was possible that antibodies cross-reactive with HHV-7 could have been detected at our screening dilution of 1:10. The problem of cross-reactive antibodies was addressed by Wyatt *et al.* (1991) who investigated HHV-7 antibody titres in patients with exanthem subitum. There was some evidence that low titres to HHV-7 (<80) were present in sera during the convalescent phase although concurrent HHV-7 infection could not be ruled out.

We therefore screened the sera in the 1+2 and 3+4 year old age groups for HHV-6 and HHV-7 antibodies at a dilution of 1:80. At this higher dilution 17/41 (41.5%) of the 1+2 and 13/25 (52.0%) of the 3+4 year olds were HHV-7

Figure 4.3.2

DETECTION OF HERPESVIRUS PARTICLES IN SALIVA CO-CULTURE CELLS BY ELECTRON MICROSCOPY

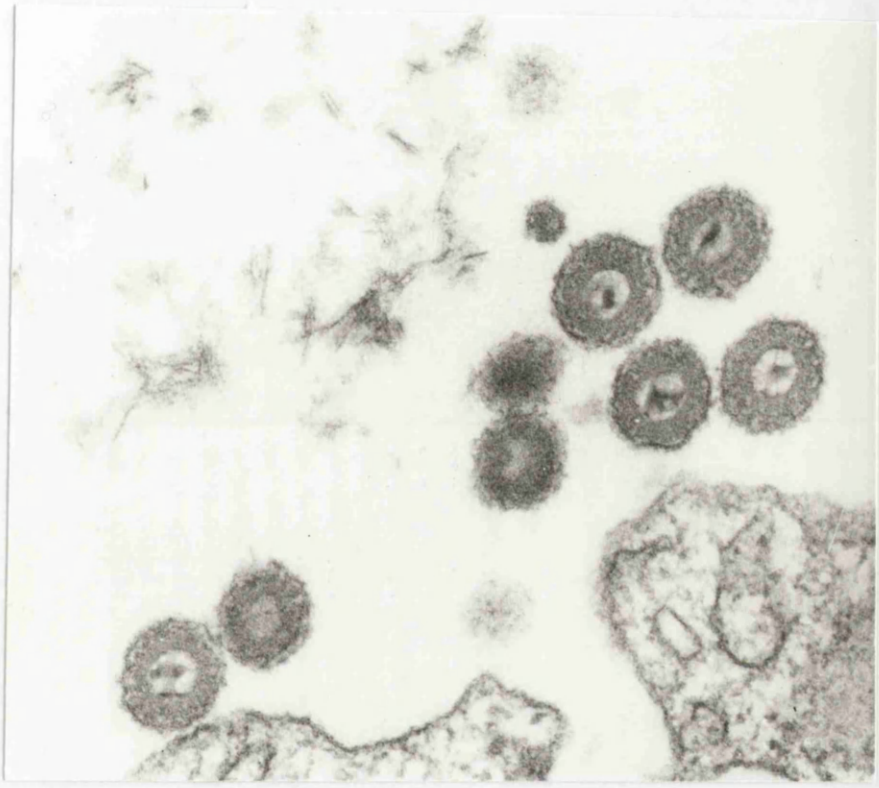


Figure 4.3.2 Detection of herpesvirus particles in a co-culture of CBMCs and cell-free saliva (from volunteer RJ) by electron microscopy. The virus particles shown are extracellular (x100,000).

Table 4.3.3

SEROPOSITIVITY TO HHV-7, HHV-6, AND EBV BY AGE

AGE	No. of Sera	No. HHV-7 Seropositive	No. HHV-6 Seropositive	No. EBV Seropositive
Months				
<2	18	17 (94.4%)	15 (83.3%)	17 (94.4%)
2+3	11	8 (72.7%)	4 (36.4%)	11 (100%)
4+5	13	2 (15.4%)	4 (30.8%)	8 (61.5%)
6+7	10	2 (20.0%)	3 (30.0%)	5 (50.0%)
8+9	10	6 (60.0%)	6 (60.0%)	5 (50.0%)
10+11	12	7 (58.3%)	7 (58.3%)	7 (58.3%)
Years				
1+2	41	27 (65.9%)	34 (82.9%)	21 (51.2%)
	41	17 (41.5%)*	20 (48.8%)*	
3+4	30	28 (93.3%)	21 (70.0%)	21 (70.0%)
	25	13 (52.0%)*	13 (52.0%)*	
5+6	18	15 (83.3%)	14 (77.8%)	13 (72.2%)
7+8	16	12 (75.0%)	14 (87.5%)	11 (68.8%)
9+10	16	15 (93.8%)	11 (68.8%)	12 (75.0%)
>10	18	14 (77.8%)	18 (100%)	13 (72.2%)
Adults	29	28 (96.6%)	ND	ND

* Sera screened at dilution of 1:80

ND = Not Done

Table 4.3.4

DUAL AND TRIPLE SEROPOSITIVITY TO HHV-7, HHV-6, AND EBV BY AGE

AGE	No. of Sera	No. HHV-7/ HHV-6 Seropositive	No. HHV-7/ EBV Seropositive	No. HHV-6/ EBV Seropositive	No. HHV-7/ EBV/HHV-6 Seropositive
Months					
<2	18	0 (0%)	6 (33.3%)	0 (0%)	11 (61.1%)
2+3	11	0 (0%)	4 (36.4%)	1 (9.1%)	3 (27.3%)
4+5	13	0 (0%)	1 (7.7%)	1 (7.7%)	1 (7.7%)
6+7	10	0 (0%)	1 (10.0%)	1 (10.0%)	1 (10.0%)
8+9	10	2 (20.0%)	3 (30.0%)	1 (10.0%)	1 (10.0%)
10+11	12	1 (8.3%)	1 (8.3%)	0 (0%)	4 (33.3%)
Years					
1+2	41	12 (29.3%)	3 (7.3%)	6 (14.6%)	11 (26.8%)
3+4	30	5 (16.7%)	5 (16.7%)	0 (0%)	15 (50.0%)
5+6	18	3 (16.7%)	2 (11.1%)	1 (5.6%)	9 (50.0%)
7+8	16	3 (18.8%)	1 (6.3%)	3 (18.8%)	7 (43.8%)
9+10	16	2 (12.5%)	4 (25.0%)	0 (0%)	8 (50.0%)
>10	18	3 (16.7%)	0 (0%)	2 (11.1%)	11 (61.1%)

seropositive, compared to 20/41 (48.8%) and 13/25 (52.0%) for HHV-6 in these age groups respectively. Analysing the serology results in the 1+2 and 3+4 year old age groups following division into 3 groups on the basis of antibody titre (<10, 10-40, ≥80), little correlation was found between HHV-6 and HHV-7 antibody titres. For the 1+2 year olds the Spearman's p rank correlation analysis = 0.021 and for the 3+4 year olds, the value was 0.193. Although not totally ruling out the presence of cross-reactive antibodies it would appear that in our study population HHV-7 infection occurred at a younger age than previously reported.

4.3.5 Propagation Of HHV-7 In Continuous Cell Lines

A disadvantage of the present system used to propagate HHV-7 is the requirement for CBMCs. A number of continuous cell lines were tested for their ability to support HHV-7 infection. Table 4.3.5 describes the results. None of the cell lines initially tested were permissive for HHV-7 infection. Following the success of Berneman *et al.*, (1992a) to infect the immature T cell line SUP-T1 with the JI strain of HHV-7, we successfully infected SUP-T1 cells with HHV-7 (RK-strain) and the two putative isolates of HHV-7 (DC and RJ) derived from saliva (see 4.3.3). In all cases infection was suspected by the appearance of large cells as well as balloon-like cells (Fig. 4.3.3) and confirmed by identification of viral antigens using an IFA with HHV-7 positive and negative sera (Fig. 4.3.4). Adherent cells were present in infected but not uninfected cultures. All 3 viruses were passaged onto uninfected cells at a ratio of 1:5 infected to uninfected cells. In the case of HHV-7 (RK-strain), infection of SUPT-1 cells was maintained following inoculation with 0.45μm-filtered tissue culture supernatant derived from infected SUP-T1 cells.

SUP-T1 cells infected with the RK-strain of HHV-7 or putative HHV-7 isolates, DC or RJ, were used as a source of antigen in an IFA using monoclonal antibodies against known human herpesviruses (Table 4.3.6). No reactivity was found with a pool of monoclonal antibodies raised against HHV-6 and a monoclonal antibody specific for EBV-VCA. Very weak reactivity was found with all 3 isolates using a monoclonal antibody specific for the late nuclear protein of CMV. Our preliminary analyses would suggest that the saliva herpesvirus isolates are further isolates of HHV-7.

Table 4.3.5

ABILITY TO INFECT CELL LINES WITH HHV-7 (RK-STRAIN)

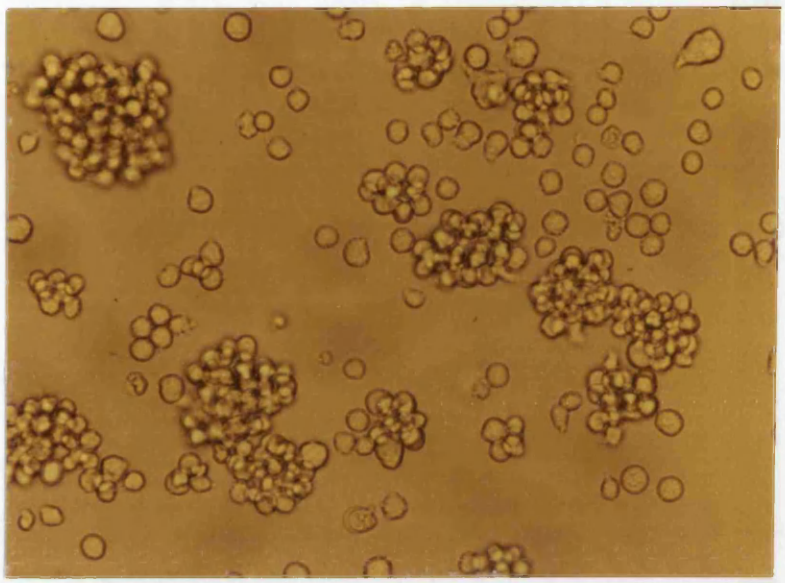
Cell line	Cell Type	Origin	HHV-7 Infection
GHI	T	ALL	-
HPB	T	ALL	-
Peer 78	T	ALL	-
JM	T	ALL	-
CEM	T	ALL	-
C8166	HTLV-1 Trans.	Cord Blood	-
U937	Promonocytic	HL	-
L540	-	HD	-
HO	-	HD	-
HSB-2	T	ALL	-
SUP-T1	T	NHL	+
CBMCs	T and B	-	+

ALL=acute lymphoblastic leukaemia, HL=histiocytic lymphoma, HD=Hodgkin's disease, NHL=Non-Hodgkin's lymphoma, CBMCs=PHA-stimulated cord blood mononuclear cells, Trans=transformed.

Figure 4.3.3

MORPHOLOGY OF HHV-7 (RK-STRAIN) INFECTED SUP-T1 CELLS IN CELL CULTURE

A.



B.

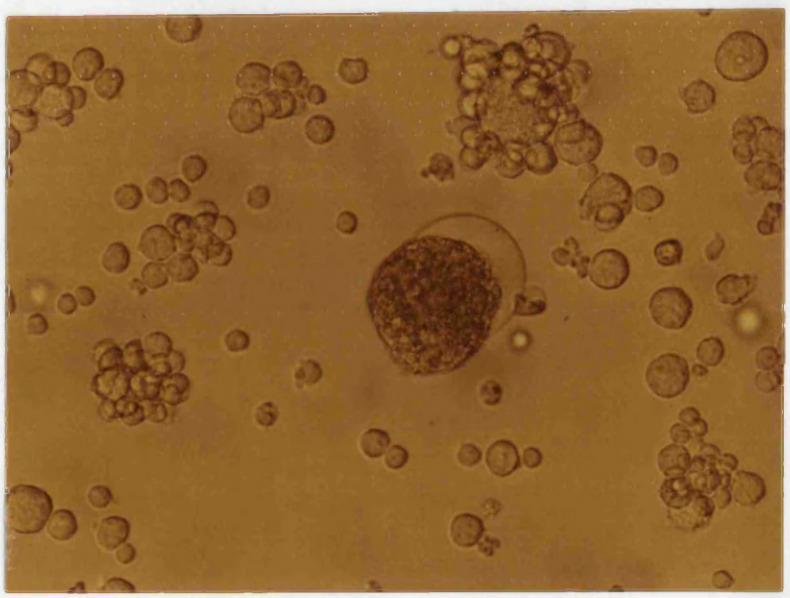
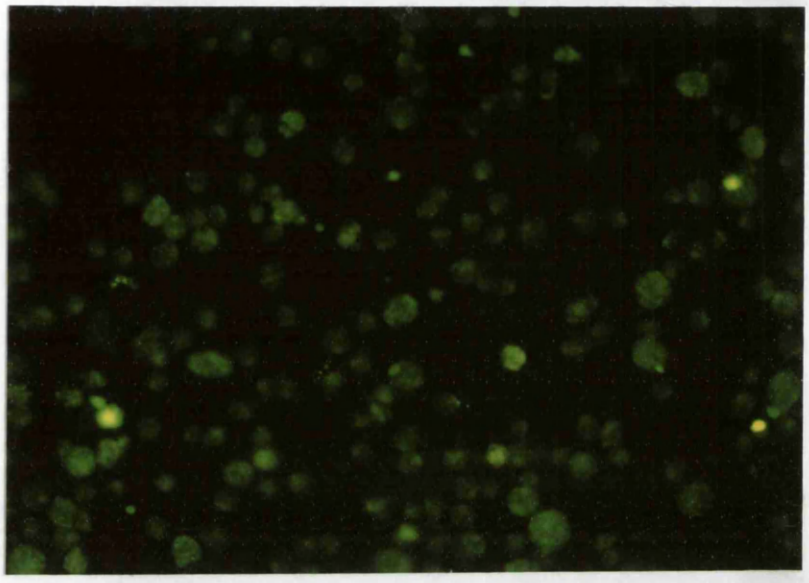


Figure 4.3.3 Appearance of HHV-7 infected and uninfected SUP-T1 cell cultures as viewed by light microscopy. A. Uninfected culture. B. HHV-7(RK-strain) infected culture which characteristically contains large, balloon-like cells (x200).

Figure 4.3.4

DETECTION OF VIRAL ANTIGENS IN HHV-7 (RK-STRAIN) INFECTED SUP-T1 CELLS BY IFA

A.



B.

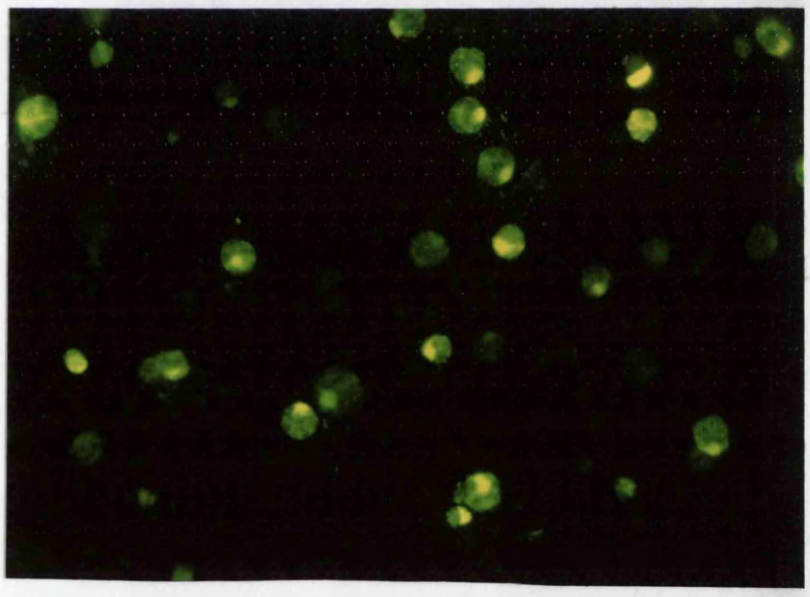


Figure 4.3.4 Immunofluorescent microscopy of HHV-7 (RK-strain) infected SUP-T1 using human sera. A. HHV-7 antibody negative, HHV-6 antibody positive serum. B. HHV-7 antibody positive, HHV-6 antibody negative serum. Both sera were used at a 1:10 dilution (x250).

Table 4.3.6

REACTIVITY OF HHV-7 (RK-STRAIN) AND PUTATIVE HHV-7 ISOLATES RJ AND DC WITH SPECIFIC ANTIBODY REAGENTS

Antibody to:	B95.8 (TPA-treated)	CMV infected Fibroblasts	HHV-6 infected J JHAN	HHV-7(RK) infected SUP-T1	DC isolate infected SUP-T1	RJ isolate infected SUP-T1
EBV-VCA(Mab)	+	-	-	-	-	-
CMV-LNP(Mab)	-	+	-	+/-	+/-	+/-
HHV-6(Mab)	-	-	+	-	-	-
HHV-6+/HHV-7+ /EBV+/CMV+ hu serum	+	+	+	+	+	+
HHV-6-/HHV-7+/EBV+/CMV+ hu serum	+	+	-	+	+	+
HHV-7-/HHV-6+/EBV-/CMV+ hu serum	-	+	+	-	-	-

VCA=viral capsid antigen, LNP=late nuclear protein, hu=human, Mab=monoclonal antibody.

4.4 DISCUSSION

The strongest association between HHV-6 and disease at present is that primary infection can result in the childhood illness exanthem subitum (Yamanishi *et al.*, 1988). Beyond childhood the clinical outcome of HHV-6 infection is less clear.

A number of early case reports suggested HHV-6 infection was associated with IM-like illness in adults (Kirchesch *et al.*, 1988; Niederman *et al.*, 1988; Irving and Cunningham, 1990) allowing parallels in the epidemiology of EBV and HHV-6 to be drawn. The majority of children are infected sub-clinically with EBV and HHV-6 early in life. However, if primary infection is delayed until the second decade of life or beyond then IM can result. In our group of patients with a wide variety of symptoms associated with IM, only one case of probable HHV-6-induced IM-like illness was identified. This patient was diagnosed by her General Practitioner as having a post-viral syndrome involving fatigue and depression over a period of 5 months. It is not possible to determine if HHV-6 infection or reactivation occurred either as a primary event or subsequently during the course of illness. Interestingly, the clinical presentation of this case is similar to a group of patients with chronic fatigue who have a pattern of illness suggestive of glandular fever and are associated serologically with HHV-6 (Read *et al.*, 1990). HHV-6 active replication as a result of viral reactivation may occur with increased frequency in adults with chronic fatigue syndrome (CFS) (Josephs *et al.*, 1991; Buchwald *et al.*, 1992). However, the clinical relevance of HHV-6 reactivation in CFS is unclear.

The majority of IM cases are attributable to primary EBV infection. The remainder of cases, which usually lack heterophile antibodies, can be attributable to a number of other infections particularly CMV and perhaps less frequently, HHV-6. Horwitz *et al.* (1992) have suggested that in a study group of 253 patients with heterophile negative IM-like illness, HHV-6 infection was responsible for 5% of the cases. Other EBV-negative IM cases have been associated with IgM antibody to HHV-6 (Irving and Cunningham, 1990; Steeper *et al.*, 1990).

We detected HHV-6 IgM antibodies in the sera of 9 persons with apparent EBV-induced IM. These findings may represent a dual infection with EBV and HHV-6. Dual infection with EBV and HHV-6 has been described previously (Bertram

et al., 1991) although reactivation of HHV-6 following primary EBV infection was suggested to occur more frequently. The detection of HHV-6 IgM antibodies can occur not only following primary infection, but also virus reactivation (Fox *et al.*, 1990b). An as yet undefined interaction between EBV during primary infection and latent HHV-6 may result in reactivation and increased HHV-6 IgG antibody titres. Likewise, a similar interaction between CMV and HHV-6 resulting in the frequent reactivation of the latter has been reported in immunosuppressed organ transplant recipients (Ward *et al.*, 1991; Ward *et al.*, 1993) and immunocompetent patients (Irving *et al.*, 1990) with proven primary CMV infection. HHV-6 reactivation has also been reported in children with acute measles infection (Suga *et al.*, 1992).

The effect on clinical outcome of either dual infection or reactivation of HHV-6 in EBV-induced IM is unknown. Hepatic dysfunction has been noted to be more significant in patients with dual EBV/HHV-6 infections than in patients with either infection alone (Bertram *et al.*, 1991). Similarly the role of HHV-6 in interstitial pneumonitis in bone marrow transplant patients may be complicated by dual infection with CMV (Carrigan, 1992). It is clear that HHV-6 serological results in diseases such as IM must be interpreted in the context of findings of serological assays for other infectious agents such as EBV and CMV.

Antibodies to specific viral antigens can be more appropriate in detecting recent or active viral infection. Antibodies to EBV-EA and CMV-EA are detectable in the sera of persons following primary infection and episodes of active replication. Our HHV-6-EA IFA failed to detect HHV-6-EA specific antibodies in sera where their presence might be expected. It is probable that the assay was not of an adequate sensitivity although a similar experimental approach had been used successfully to detect antibodies to CMV-EA.

Using a different approach Iyengar *et al.* (1991) developed an HHV-6-EA antibody assay by purifying HHV-6-EA from infected cell lysates with an HHV-6-EA MoAb. Antibodies to HHV-6-EA were found in 30% of healthy individuals and 29% of persons with heterophile negative IM suggesting recent viral replication. Interestingly 62.5% of HD sera were HHV-6-EA antibody positive indicating that HHV-6 replication occurs at a greater frequency in that malignancy.

There are a number of possible modes of transmission of HHV-6. Congenital transmission of HHV-6 has been reported (Dunne and Demmler, 1992; Aubin *et al.*, 1992). The detection of HHV-6 in oropharynx and salivary glands of healthy adults (Fox *et al.*, 1990a; Gopal *et al.*, 1990) and the isolation of virus from saliva (Harnett *et al.*, 1990; Levy *et al.*, 1990a) suggests the virus may be transmitted through contact with infected oral secretions.

We were unable to isolate infectious HHV-6 from saliva by 3 different isolation protocols. Despite this, a number of the same saliva specimens were used in the study of Jarrett *et al.* (1990) and found to be positive for HHV-6 by PCR. The HHV-6 PCR strategy used in that study does not amplify sequences from other human herpesviruses including HHV-7, providing strong evidence that HHV-6 was present in the saliva. Our results suggest that the virus was not present in an infectious form. Others have also been unsuccessful in the isolation of HHV-6 from this source (Yanagi *et al.*, 1990) although Pietroboni *et al.* (1988a), Levy *et al.* (1990a) and Harnett *et al.* (1990) have reported the isolation of HHV-6 from saliva. Successful isolation may be dependent on the methodology used such as centrifugal enhancement (Harnett *et al.*, 1990). Based on the procedure of Levy *et al.* (1990a), infectious agents from two saliva samples were isolated that, after limited analyses, were unlikely to be HHV-6. Levy *et al.* (1990a) propagated herpesvirus isolates in PBMCs in the course of virus isolation. It is possible that HHV-6 isolates originated from reactivated virus from the PBMCs used in co-culture. Wyatt and Frenkel (1992) suggested that HHV-7 isolation from saliva may have been outreplicated by HHV-6 rescued from PBMCs.

The HHV-6 saliva isolates of Harnett *et al.* (1990) were propagated in CBMCs which should be free from HHV-6 infection. However, in their study, the authors identified isolates by reactivity with characterised human sera. From our own and others' results (Clark *et al.*, 1993; Wyatt *et al.*, 1991; Berneman *et al.*, 1992a), the majority of adult human sera are HHV-7 seropositive. The clarification of these isolates as HHV-6 would provide conclusive proof of the ability to isolate the virus from saliva.

Preliminary analyses suggests that in our study 2 further isolates of HHV-7 have been made from the saliva of healthy adults. Molecular studies to identify these viruses have confirmed that they are HHV-7 (Z. Berneman, personal communication). HHV-7 has been isolated from both peripheral blood and

saliva. Two further groups have recently reported the frequent isolation of HHV-7 from saliva (Black *et al.*, 1993; Hidaka *et al.*, 1993) and one group has isolated HHV-7 from the peripheral blood of a child with an illness similar to chronic EBV infection (Kawa-Ha *et al.*, 1993). As yet the cell tropism of HHV-7, both *in vivo* and *in vitro*, has not been fully recognised. *In vitro* the virus can be propagated in activated CBMCs and PBMCs (Frenkel *et al.*, 1990; Berneman *et al.*, 1992a,b). Double immunofluorescence studies showed that HHV-7 JI strain predominantly infected T lymphocytes (Berneman *et al.*, 1992a). The authors failed to infect all continuous cell lines tested but one, SUP-T1 cells. Likewise, we were unable to infect a number of lymphoid cell lines with HHV-7 RK-strain. Recently we have been able to infect SUP-T1 with not only HHV-7 RK, but also the two putative HHV-7 isolates. To date infection with these 3 viral isolates has been maintained by passaging infected cells onto uninfected SUP-T1 cells. An HHV-7 permissive continuous cell line will facilitate future studies such as serological investigations where the supply and preparation of CBMCs proved to be a limiting factor.

At present it is not known whether HHV-7 infection is associated with human disease. Epidemiological information is scarce. We found that infection with HHV-7 and HHV-6 was common in children by the age of 2 years. By 4 years of age 93.3% of children were HHV-7 seropositive. In contrast, Wyatt *et al.* (1991) found that HHV-7 infection occurred at an older age in children than HHV-6. Sera in our sample population were derived from hospitalised children from a different geographical locale which may have influenced the findings. Our sample numbers were considerably larger than the 25 sera from children aged from newborn to 58 months screened by Wyatt *et al.* (1991). This would be the most likely explanation for the different results.

Wyatt *et al.* (1991) suggested that antigenic cross-reactivity between HHV-6 and HHV-7 may interfere with IFAs. A similar situation had previously been proposed to occur between HHV-6 and CMV (Larcher *et al.*, 1988) although this proved to be unfounded for various HHV-6 serological assays (Morris *et al.*, 1988; Buchbinder *et al.*, 1989; Coyle *et al.*, 1992). Our analyses did not support the idea of cross-reactive antibodies between HHV-6 and HHV-7 affecting the IFAs.

The HHV-7 serological results confirm the widespread nature of HHV-7 infection in the population and indicate that primary infection may occur even

earlier in life than previously proposed. The relevance of HHV-7 infection to human disease remains to be answered by future research.

CHAPTER 5

AN *IN VITRO* MODEL TO STUDY VIRAL INVOLVEMENT AND MECHANISMS OF AUTOIMMUNITY IN SJÖGREN'S SYNDROME

5.1 INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by lymphocytic infiltration and inflammation of exocrine glands, principally salivary and lacrimal, leading to diminished or absent glandular secretion. The majority of infiltrating cells in SS salivary glands are T cells with a predominantly T helper/inducer phenotype (Lindahl *et al.*, 1985). The infiltrate contains a smaller population of B cells. In lacrimal glands the infiltrate similarly contains a mixture of T and B cells although B cells predominate (Pepose *et al.*, 1990). The term SS is generally used but sicca syndrome and less frequently Mikulicz disease (Morgan and Castleman, 1953) have been used for various expressions of the same condition. Multi-organ involvement, notably of the lungs (Constantopoulos *et al.*, 1984), kidneys (Bardana and Montanaro, 1990), and skin (Bloch *et al.*, 1965), emphasises the systemic nature of SS. SS may be present as a primary disease process (primary SS) or in association with another connective tissue disease (secondary SS) such as rheumatoid arthritis, systemic lupus erythematosus and occasionally scleroderma (reviewed by Aziz *et al.*, 1992).

The aetiology of SS is unknown but is thought to be an interaction of genetic, hormonal and environmental factors. There is an increase in the frequency of HLA-B8, DR3 and Dw52 antigens in patients with primary SS (Bardana and Montanaro, 1990; Arnett *et al.*, 1988; Fei *et al.*, 1991) and secondary SS has been linked to HLA-DR4 (Moutsopoulos *et al.*, 1979). Association with HLA may vary with ethnic group. In Japan, a strong association between primary and secondary SS with rheumatoid arthritis and HLA-DRw53 has been reported (Moriuchi *et al.*, 1986). SS is more common in middle aged to elderly females than in males, suggesting hormonal influences in the development of disease.

Viruses are amongst environmental agents that have been considered as aetiological factors in SS (Flescher and Talal, 1991). Research has mainly focused on two families of viruses, the retroviruses and herpesviruses. More recently, contradictory evidence for an association of Hepatitis C virus with SS has been reported (Haddad *et al.*, 1992; Aceti *et al.*, 1992; Mariette *et al.*, 1993).

The role of retroviruses in autoimmunity in general has recently been reviewed (Krieg and Steinberg, 1990; Venables and Brookes, 1992a). Clinical features of

SS including parotid gland enlargement were reported in intravenous drug users (Smith *et al.*, 1988) who were at a high risk from HTLV-1, -11, and HIV infection (Robert-Guroff *et al.*, 1986). Similarly, a sicca syndrome or SS-like illness has been described in persons infected with HIV-1 (Itescu *et al.*, 1989). Talal *et al.*, (1990) found antibodies that cross-reacted with the p24 (gag) protein of HIV-1 in 30% of SS patients. The authors suggested retroviral expression in at least a subset of SS patients. Type A intracisternal retroviral particles have been reported in co-cultures of SS salivary gland homogenates and the H-9 cell line (Garry *et al.*, 1990). In Japan, where HTLV-1 infection is endemic, the virus has been suggested to play a part in the pathogenesis of a subset of cases of primary SS (Eguchi *et al.*, 1992). Green *et al.* (1989) developed a transgenic mouse line that expresses the tax gene of HTLV-1. These mice developed similar pathology in the salivary gland to that found in SS. Thus, separate lines of evidence suggest that retroviral infection may play a role in SS.

The herpesviruses CMV and EBV have been proposed as possible causal agents of SS (Burns, 1983; Fox *et al.*, 1986b). More recently HHV-6 has also been linked with SS. Higher antibody titres to HHV-6 were reported in SS (Ablashi *et al.*, 1988) although not consistently (Baboonian *et al.*, 1990). HHV-6 DNA and protein have been described in major (Fox *et al.*, 1990a) and minor (Krueger *et al.*, 1990) salivary glands. HHV-6 has been detected rarely in biopsy tissue from persons with NHL however, there has been a significant overrepresentation of HHV-6 positive NHLs occurring in the context of SS (Josephs *et al.*, 1988; Jarrett *et al.*, 1988). The development of malignancy is a recognised feature of autoimmune disease (Kinlen, 1992) including SS (Kassan *et al.*, 1978).

The risk of lymphoma in SS has been estimated to be 44 times greater than expected (Kassan *et al.*, 1978). The risk of lymphoma may be higher in patients with parotid gland swelling (Relative Risk=66.7) compared to those without (Relative Risk=12.5). The autoimmune disorder may precede the development of lymphoma by intervals as long as 20 years. The natural history of this progression has been separated into 3 categories. The first stage comprises the characteristic features of SS. The term "pseudolymphoma" or "pre-lymphoma" has been used to describe the second stage (Talal *et al.*, 1967; Anderson and Talal, 1972) where the clinical picture is suggestive of malignancy but which cannot be classified as such after tissue biopsy. The

third stage is the development of a malignant lymphoma. The lymphomas may involve salivary glands or major parenchymal organs such as lungs, kidney, and gastrointestinal tract (Talal and Bunim, 1964).
The identification of viruses in salivary gland tissue has made use of fixed tissues, relying on either frozen or paraffin-embedded sections for immunostaining, DNA hybridisation and PCR assays. Labial salivary glands have been cultured *in vitro* for immunological and virological studies (Cremer *et al.*, 1974) and Lamey *et al.* (1984) developed a protocol for the tissue culture of epithelial cells from such glands. I have adapted this procedure to obtain salivary gland cell populations derived from minor salivary glands taken from persons undergoing investigation for xerostomia and therefore, potential SS cases. The primary salivary gland cultures provided an opportunity to investigate mechanisms of autoimmunity associated with SS, and to examine these cultures for the presence of viruses associated with the disease.

Although there are no universally accepted diagnostic criteria for SS, a number of classification schemes have been proposed. These can be based on objective tests such as measurement of salivary or tear flow rates, however, all schemes require the presence of lymphocytic infiltrates around salivary glands in biopsy specimens (Fox *et al.*, 1986c; Skopouli *et al.*, 1986; Homma *et al.*, 1986; Manthorpe *et al.*, 1986). Parotid gland biopsy has been advocated in preference to labial glands in these investigations on the grounds that the gland is most affected in the disease process (Ferguson *et al.*, 1990). However to reduce the risk of damage to important anatomical structures, biopsy of the minor salivary glands of the lower lip has been used for many years as the histological changes there appear to correlate with those in the parotid and submandibular glands (Wise *et al.*, 1988; Bertram and Hjorting-Hansen, 1970; Chisholm *et al.*, 1970). Thus, minor salivary glands can be obtained from persons, usually presenting with xerostomia, who are undergoing investigation for the presence of lymphocytic infiltration.

A common feature of SS is the presence of autoantibodies directed against nuclear autoantigens particularly La (SS-B) (Akizuki *et al.*, 1977; Harley *et al.*, 1986; Harley, 1987). SS patients with anti-La antibodies have more active and severe disease (Harley *et al.*, 1986). La is a transcription termination factor for RNA polymerase III and selected RNA polymerase II transcripts (Rinke and Steitz, 1982; Stefano, 1984; Madore *et al.*, 1984; Gottlieb and Steitz, 1989a,b) including the EBV EBER transcripts (Lerner *et al.*, 1981; Howe and Shu, 1988).

The mechanism by which anti-La antibodies are generated is unknown. Molecular mimicry with a possible viral protein, feline sarcoma virus gag leader protein, has been suggested (Kohsaka *et al.*, 1990). McNeilage *et al.* (1990) detected a primary reaction against an immunodominant epitope of the La protein in the sera of SS patients which in later stage disease was followed by the appearance of anti-La antibodies directed against other regions of the La molecule. The initial immunodominant region of La contains the epitope with homology to the viral gag protein. Alternatively, the antigen itself may be the initial target that is presented to the immune system. Baboonian *et al.* (1989) reported a translocation of La from the cell nucleus to the cytoplasm and cell surface following viral infection of a continuous cell line. Cell surface expression could promote the presentation of La epitopes to the immune system. The effect of viral infection of salivary gland epithelial cells on the cellular location of La was investigated.

An immunohistological feature of salivary glands in SS is the aberrant expression by epithelial cells of MHC class II molecules (Lindahl *et al.*, 1985; Fox *et al.*, 1986a) possibly induced by the cytokine interferon-γ (IFN-γ) (Fox *et al.*, 1986a). The effect of IFN-γ on MHC class II expression and the cellular location of La in salivary gland cultures was also examined.

The ability to culture salivary gland epithelilal cells *in vitro* allowed a novel approach to investigate the association of EBV and HHV-6 with SS. The presence of these two viruses was examined by PCR in epithelial cell cultures derived from patients diagnosed with SS and from persons lacking histological evidence of lymphocytic infiltrates in salivary glands. Such persons were given a diagnosis of non-specific sialadenitis.

A number of salivary gland epithelial cell populations were also examined by immunostaining for the expression of putative retroviral gene products in collaboration with workers at the Kennedy Institute of Rheumatology, London. Amongst the antibody reagents used was an antisera raised against a peptide predicted from an open reading frame of an endogenous retrovirus, HTLV-1 related endogenous sequence-1 (HRES-1) (Perl *et al.*, 1989) and a MoAb directed against HTLV-1 p19 antigen.

Finally, the numbers of salivary gland cells that result from the tissue culture of minor salivary glands are limited. Previously, Fox *et al.* (1986a) reported a

salivary gland cell line derived from a parotid gland tumour. We attempted to immortalise primary salivary gland epithelial cells using an adenovirus/SV40 hybrid transforming virus that contains the large T antigen of SV40. The virus can infect epithelial cells which are permissive for adenovirus and the SV40 large T is thought to interact with Rb and p53 to promote immortalisation and transformation (Levine *et al.*, 1991). SV40 has previously been used to immortalize human cell types including keratinocytes and bronchial epithelial cells (Steinberg and Defendi, 1983; Reddel *et al.*, 1991).

5.2 MATERIALS AND METHODS

5.2.1 Buffers And Solutions

Denaturing (Alkali) Buffer	Sodium Hydroxide	0.5M
	Sodium Chloride	1.5M
	adjusted to >pH12.0	
Denhardt's Solution 100x	Ficoll	2%
	Bovine Serum Albumin(Fraction V)	2%
	Polyvinylpyrollidone	2%
Hybridisation Buffer	Formamide	35%
	Denhardt' solution	5x
	Sodium laurylsulphate	0.1%
	Tris pH7.4	50mM
	EDTA pH8.0	10mM
	SSC	3x
	Dextran sulphate	10%
	Geneblock (ILS)	250ug/ml
10x T4 Kinase Buffer	Tris Cl (pH7.6)	0.5M
	Magnesium Chloride	0.1M
	Dithiothreitol	50mM
	Spermidine HCl	1mM
	EDTA (pH8.0)	1mM
10x Gel Loading Buffer for Non-Denaturing PAGE	Bromophenol Blue	0.42%
	Glycerol	50%
	made up in TE	
Neutralising Buffer	Tris base	0.5M
	Sodium Chloride	3M
	Conc. Hydrochloric Acid	3.3%
	adjusted to pH8.0	

NIB Buffer	Potassium Chloride	50mM
	Tris pH8.3	10mM
	Magnesium Chloride	1.5mM
	Gelatin	100ug/ml
	NP 40/Tween 20	0.45%
SSC	Sodium Chloride	0.15M
	Sodium Citrate	0.015M
TAE Buffer	Tris	40mM
	Sodium Acetate	20mM
	Sodium Chloride	20mM
	EDTA	2mM
	adjusted to pH8.0	
TBE Buffer	Tris	90mM
	Boric Acid	90mM
	EDTA	2.25mM
	adjusted to pH8.0	
TE Buffer	Tris (pH8.0)	10mM
	EDTA (pH8.0)	1mM

All chemicals were supplied by Sigma.

5.2.2 Source Of Salivary Glands

Salivary glands were kindly provided by the Department of Oral Medicine and Pathology, Glasgow Dental School and Hospital, under the supervision of Dr. P-J. Lamey. The minor labial salivary glands were obtained by excision biopsy from patients undergoing investigation for the symptom of xerostomia. Occasionally biopsies were obtained from individuals without evidence of xerostomia, but where unrelated surgery required the removal of minor salivary gland tissue. The lobules of minor salivary gland were placed immediately in transport medium consisting of RPMI 1640, 20%FCS, 800U/ml penicillin, 800µg/ml streptomycin, 2mM L-glutamine, 25mM hepes, 150µg/ml gentamicin, 2.5µg/ml fungizone (all Life Technologies). The biopsies were transported to the LRF Virus Centre and processed immediately. Serum samples were also obtained at the same time and stored in aliquots at -80°C.

5.2.3 Tissue Culture Of Salivary Gland Biopsies

The salivary gland biopsy was placed in a 60mm tissue culture dish (Corning) containing 2ml of salivary gland (SG) medium (Dulbecco's MEM Nutrient mix F12 (HAM) medium supplemented with 15mM hepes buffer, 10% FCS, 400U penicillin, 400µg/ml streptomycin, 2mM glutamine) (all Life Technologies). The tissue was cut into 5-10 smaller pieces using sterile fine forceps and scissors. The pieces of salivary gland were transferred to a $25cm^2$ tissue culture flask (Costar) and 2ml SG medium added. The flask was gassed with CO_2 and incubated at 37°C. If enough pieces of tissue were available 2 such flasks were set up. Initially an enzymatic digestion step was included after mechanically dissecting the biopsy. The tissue was incubated in 2ml SG medium containing 1U Dispase (BCL) for 1hr in a shaking water bath at 37°C and then placed in tissue culture flasks as above.

Tissue culture flasks from different manufacturers or treated Costar flasks were used in initial experiments to optimise conditions for the successful outgrowth of cells from tissue explants. The types of flask employed were $25cm^2$ tissue culture flasks obtained from Bibby, Nunc and Costar and Costar flasks pre-coated with extracellular matrix (ECM) (Biological Industries) or coated with laminin, a constituent of ECM. Laminin (Sigma) was dissolved in PBS to a final concentration of 50µg/ml and stored in 1ml aliquots at -80°C. One millilitre of the stock laminin was added to a $25cm^2$ Costar flask to provide the

manufacturers recommended concentration of 2µg/cm^2 and incubated for 45 minutes at room temperature. Excess fluid was removed and the flask briefly washed with PBS. The laminin-coated flasks were then used as above for the culture of salivary gland tissues.

For experiments that involved live cell fluorescence (see section 5.2.8), pieces of salivary gland tissue were placed directly onto plastic coverslips in a tissue culture flask (Leighton tubes, Costar). These cultures were fed with 1ml of SG medium.

The SG medium in salivary gland tissue cultures was changed twice every week with total medium replacement. Outgrowth of cells from pieces of tissue was usually observed 7-10 days after initiation of the culture. Cultures were maintained until at least 3 of the pieces of tissue in any one flask produced an outgrowth of cells.

5.2.4 Adenovirus Type-2 Infection Of Salivary Gland Cell Cultures

Adenovirus type-2 was kindly provided by Quality Biotech Ltd. at a titre of 10^8 plaque-forming units (PFU) per ml and stored at -80^{o}C. The epithelial cell line Hep-2 was maintained in complete medium plus 10%FCS. Hep-2 or salivary gland cells were inoculated with either 1 or 10 PFU per cell of adenovirus type-2. A rough estimate of salivary gland cell numbers was obtained by viewing under the light microscope. Cells of non-epithelial morphology were removed from salivary gland cultures by brief exposure to trypsin-EDTA (1x) (Life Technologies) twice in the preceeding 7 days before inoculation with virus as detailed in 5.2.10. Hep-2 cell numbers were calculated using a counting chamber, placed in a flask overnight to allow adherence and the appropriate amount of virus added. Cells were harvested and cytocentrifuged 24 or 48 hours later onto microscope slides (see 5.2.6).

5.2.5 Treatment With Interferon-γ

Salivary gland cultures were treated with SG medium containing human recombinant IFN-γ (BCL) at 500U/ml for 3 days. The medium was changed daily and fresh IFN-γ added. The cells were cytocentrifuged onto slides and fixed as described below (5.2.6).

5.2.6 Preparation Of Cytocentrifuge Slides

Cytocentrifuge slides were prepared from salivary gland or Hep-2 cell cultures. The supernatant was removed from the flask and the cell monolayers washed once with PBS. Two millilitres of Trypsin-EDTA solution were added and the flask incubated at 37°C until all of the adherent cells had lifted from the surface, aided by shaking the flask. The cells were washed once in SG medium and once with HBSS. The cell number was counted and adjusted to 10^5 cells per 100µl. Cytocentrifuge slides were prepared by centrifugation at 400rpm for 10 minutes on a Cytospin 2 (Shandon). Each slide (Fisons) was prepared with 10^5 cells. The slides were air-dried and fixed in the appropriate fixative for 10 minutes. Cells derived from adenovirus infected or IFN-γ treated cultures and subsequently stained with La-specific monoclonal antibodies were fixed in ice-cold acetone. Cells subsequently stained for cytokeratins were fixed with ice-cold methanol (BDH). Fixed slides were stored at -20°C until use.

5.2.7 Indirect Immunofluorescence Assay

Salivary gland epithelial cells were probed with MoAbs diluted in PBS comprising: (i) A combination of 2 anti-La MoAbs, SW1 and SW5, both IgG2b isotype (Smith *et al.*, 1985) used at a 1:50 dilution (kindly provided by Dr. P. Venables, Kennedy Institute). (ii) Anti- cytokeratin-18 and -19 MoAbs (Sigma) used at dilutions of 1:100 and 1:20 respectively. (iii) Anti-adenovirus group antigen MoAb, IgG1 isotype (Chemicon), used at a dilution of 1:200. (iv) Anti-SV40 large T antigen specific MoAb (Chemicon) used at a 1:10 dilution. (v) MoAb AGF2.3 (kindly provided by Dr. G. Brown, Birmingham University) which reacts with a nuclear membrane antigen in selected transformed cell lines (Lord *et al.*, 1988), used neat. (vi) Anti-HLA-DR MoAb (the Binding Site) used at a dilution of 1:20. (vii) Anti-human fibronectin MoAb (Sigma) used at a 1:200 dilution. (viii) Anti-CD25 MoAb (Scottish Antibody Production Unit) was used at a 1:10 dilution as a negative primary antibody control in experiments where enough cytocentrifuge slides were available.

Thirty microlitres of the antibody dilution were spotted onto the slide and incubated at room temperature for 1hr in a moisture chamber. The slides were washed over 20 minutes with 2 changes of PBS and incubated for a further 30 minutes at room temperature with a 1:20 dilution of FITC-conjugated goat anti-mouse IgG (Sigma). The slides were then washed as above and mounted

using 2.5% 1,4 diazobicyclo-2.2.2.octane in PBS/glycerol(1:1v/v) and viewed under a Leitz Laborlux fluorescent microscope with either a x25 or x50 water immersion lens (both Leitz).

For double staining of La and adenovirus antigen, a mixture of two conjugates was used - a sheep anti-mouse IgG2b antibody (FITC-labelled) used at 1:20 and a tetramethyl rhodamine isothiocyanate (TRITC)-labelled sheep anti-mouse IgG1 antibody used at 1:10 (both The Binding Site).

5.2.8 Live Cell Fluorescence

For cells grown on Leighton tube coverslips and probed with MoAbs, the procedure was identical to that detailed in 5.2.7 except that the primary and secondary antibody incubations were carried out on viable cells prior to fixation. Particular care was taken to ensure that cells on coverslips did not dry out during the assay. Viability of the cells after completion of the IFA was checked by incubating the coverslips in 0.2% trypan blue (Sigma) for 1 minute followed by a wash in PBS. The cells were then fixed in ice cold acetone/methanol (1:1v/v) before mounting and viewing under the fluorescent microscope.

Following treatment with IFN-γ, salivary gland cells were examined for the cell surface expression of HLA-DR molecules and La antigen. Cells were trypsinised into suspension and washed once in SG medium and once in PBS. The cells were resuspended in 30µl of PBS/0.2% bovine serum albumin/0.1% sodium azide (diluent) (all Sigma), containing either the anti-HLA-DR or anti-La MoAbs. The cells were incubated on ice for 30 minutes and washed twice in diluent. The cells were resuspended in 30µl of FITC-labelled goat anti-mouse IgG and incubated for a further 30 minutes on ice. The cells were washed as above, fixed in 1% paraformaldehyde (Sigma), spotted onto a Teflon-coated Multitest slide and viewed under the fluorescent microscope.

5.2.9 Photography

Photographs of cell cultures and IFA slides were taken using an Olympus PM-10AK system attached to the appropriate microscope. Fuji 50asa 35mm slide film was used for light field photographs and Fuji 400asa 35mm slide film for fluorescent field photographs.

5.2.10 Harvesting Primary Culture Cells For Polymerase Chain Reaction

For the first PCR experiments, salivary gland cultures were harvested when a sizeable outgrowth of cells was evident by viewing under the light microscope. Tissue explants were removed from the cultures by dislodgement with a 1ml pipette, taking care not to score the surrounding cell monolayer. The tissue was then discarded. The flask was washed twice with 5ml PBS to remove any remaining suspension cells and debris. The adherent cells were removed by scraping the surface of the flask with a cell scraper (Costar) in a 1ml volume of PBS. The cell suspension was transferred to two microcentrifuge 1.5ml flip-top tubes and pelleted in a microcentrifuge at maximum speed for 30 seconds. The supernatant was discarded and cell pellets stored at -80^{o}C until used for PCR. An equal number of control tubes containing PBS were set up at the same time for use as negative controls. PBS (0.5ml) was added to flip-top tubes, centrifuged, PBS removed and control tubes stored at -80^{o}C. Air controls were also set up in order to identify airborne contamination of samples during handling. Empty flip-top tubes that had been open for the duration of the cell harvesting procedure in the safety cabinet were utilised and stored at -80^{o}C until use.

For the second set of PCR experiments, care was taken to remove adherent cells lacking epithelial cell morphology prior to harvesting the cell pellets. When sufficient outgrowth of cells from tissues had occurred, the pieces of tissue were removed as above. A trypsinisation step was utilised to remove non-epithelial cells from cultures. The flask was washed with 5ml PBS and the supernatant containing cells in suspension and debris removed. Two millilitres of Trypsin-EDTA solution were added and the flask incubated at 37^{o}C for 3min. The flask was shaken gently until cells of non-epithelial morphology were seen to lift from the flask surface into suspension. The trypsin-EDTA was removed and the flask washed twice with 3ml SG medium. The culture was then fed with fresh SG medium. This trypsinisation step was repeated 3 days later and cultures fed with fresh SG medium.

The remaining adherent cells were then harvested a further 3 days later. The flasks were washed with PBS and 2ml trypsin-EDTA added and incubated at 37^{o}C until all of the adherent cells had lifted from the surface, aided by gentle shaking of the flask. The cell suspension was made up to a volume of 10ml with SG medium in a 10ml conical-tube (Costar) and centrifuged at 250*g* for 5

minutes. The supernatant was removed and the cells washed twice in 5ml PBS. The cells were counted and aliquoted at 10^5 cells per 1.5ml flip-top tube, pelleted in a microcentrifuge and cell pellets stored at -80°C until use. Negative controls consisting of PBS were set up as above. No air controls were included for the second set of PCR samples.

5.2.11 Polymerase Chain Reaction

(i) HHV-6 PCR

The primers used in the PCR experiments were derived from the pZVH14 clone of HHV-6 (Josephs *et al.*, 1986) and amplify a DNA fragment of 161 base pairs (Jarrett *et al.*, 1990). This clone does not have any known homology to other human herpesviruses (Josephs *et al.*, 1986) and the primers do not amplify sequences from cells infected by EBV, CMV, or HHV-7 (data not shown). The nucleotide sequences of the 5' and 3' primers are:

5'> TCTCACAGCCCAGGACAATGGATTATATAT < 3'

5'> TGAGATCATTCTCCCGTTCTTGAGGG < 3'

Tubes containing cell pellets and control tubes (PBS/air) were digested with Proteinase K (Sigma) in NIB buffer. Twenty-five microlitres of NIB buffer containing 1.5µg Proteinase K were added to each tube and overlayed with mineral oil (Sigma). The tubes were incubated at 56°C for 30 minutes in a waterbath and then steamed for 10 minutes in a boiling waterbath to inactivate the Proteinase K. Twenty-five microlitres of the master mix were added through the mineral oil to give a final reaction volume of 50µl. The master mix contained reagents that gave a final concentration per reaction of 1U Amplitaq (Cetus), 50picomoles (pmol) of each primer and 200µmol/L of each deoxynucleoside triphosphate in 10mM Tris (pH8.4), 50mM KCl, and 1.5mM $MgCl_2$. The positive control consisted of a cell pellet containing 10^5 J JHAN plus 100 HHV-6-infected J JHAN cells.

Thermal cycling was performed using an Intelligent Heating Block (Cambio). Reactions were initially denatured by heating to 94°C for 5 minutes followed by 40 cycles of 94°C for 0.1 minute, 50°C for 0.1 minute, and 72°C for 1.5 minutes. The products from the HHV-6 PCR reactions (cell pellets and

controls) were assayed subsequently using nested primers which amplify an internal fragment of 90 base pairs. The nucleotide sequences of the 5' and 3' nested primers are:

5' > TCCACTACTTAAAACCGA < 3'

5' > TGATGAAGTATGTGATAG < 3'

Five microlitres of a 50-fold dilution of the reaction products in H_2O were used per assay. Reaction conditions and thermal cycling were as above except that the 72°C step was omitted.

(ii) EBV PCR

The primers used amplify a 140bp DNA sequence within the BamH1-W region of the EBV genome and have been described previously (Deacon *et al*, 1991). The 5' and 3' primers were respectively:

5' > TGACTTCACCAAAGGTCAGG < 3'

5' > AGGACCACTTTATACCAGGG < 3'

The cell pellets were treated with a Proteinase K digestion step in NIB buffer as detailed for HHV-6 in section 5.2.11.(i) above. Apart from the different primer set the reaction mixture and conditions of thermal cycling were the same as for HHV-6. No nested primers were used in the EBV assay. The positive control was a pellet of 10^4 B95.8 cells.

(iii) β-globin PCR

As a control to determine the presence of amplifiable DNA in the sample tubes, a region of the human β-globin gene was also amplified using identical reaction conditions to those for HHV-6 and EBV PCR assays. The primer sequences have previously been described (Saiki *et al.*, 1988). The 5' and 3' primers were respectively:

5' > ACACAACTGTGTGTTCACTAGC < 3'

5' > CTCCTGAGGAGAAGTCTGC < 3'

This set of primers amplifies a 110bp region of the β-globin gene.

(iv) Analysis of samples

Reaction products were analysed by polyacrylamide gel electrophoresis (PAGE). Twenty microlitres of the PCR reaction product were added to 5µl of gel loading buffer. Ten microlitres of this were analysed on an 8% polyacrylamide gel on a Bio-Rad Mini Protean II gel system. Acrylamide (8%) was made up from stock solutions of PAGE[1] Liquidacrylamide (30%) and PAGE[1] Bisacrylamide (2%) (both BCL) in TBE and polymerised by the addition of 0.06% (w/v) ammonium persulphate and 0.12% (v/v) N,N,N',N'-tetramethylethyldiamine. DNA molecular weight markers, PhiX174 Hae III markers (Life Technologies) were run on each gel. Electrophoresis was carried out at 100 Volts (V) for approximately 1 hour in a TBE buffer until the dye front had reached the bottom of the gel. The gel was subsequently stained using ethidium bromide (Sigma) at a concentration of 0.5mg/L. The gel was immersed in this solution for 15 minutes, the buffer removed and replaced with distilled H_2O. The stained gel was viewed under UV-light using a Foto/Prep I transilluminator (Fotodyne) and the gel photographed using a Polaroid MP-4 Land Camera with Polaroid High Speed 4x5 Instant Sheet film. In PCR experiments with HHV-6 nested primers and β-globin primers, the results were scored following visualisation of ethidium bromide stained gels.

For identification of reaction products by hybridisation with specific probes, the gels were washed first in denaturing buffer for 8 minutes followed by 8 minutes in neutralising buffer. Electrophoresed products were then transferred to a nylon membrane, Hybond-N (Amersham), using a Bio-Rad Trans-Blot Cell. The gels were electroblotted overnight at 15V in TAE buffer. Filters were hybridised to an oligonucleotide probe specific for each amplified sequence. The DNA sequence of the oligonucleotide probes were as follows:

HHV-6 5' > CTTCCATCGAGGCCTCATCTATCACATACT < 3'

EBV 5' > AGCGCGTTTACGTAAGCCAGACAGCAGCCAATTGTCAGTT < 3'

The probes were synthesised "in-house" employing an automated oligonucleotide synthesiser (Model 381A, Applied Biosystems). Probes were end-labelled with γ ^{32}P-Adenosine Triphosphate (ATP) (specific activity

3000Ci/mmol) (ICN). Seventy picomoles of probe were mixed with 10µl of 10xkinase buffer, 2µl T4 kinase (Life Technologies), 70pmol γ ^{32}P-ATP, the volume made up to 100µl with ultrapure H_2O and incubated at 37°C in a waterbath for 1 hour. The probe was added to the top of a pre-packed Sephadex G-50 column (Pharmacia) previously equilibrated with TE, and 400µl TE added immediately. The solution was allowed to run through. A fresh 400µl TE was added and the eluted solution collected, providing 400µl of ^{32}P-labelled probe.

Filters were prehybridised in 2.5ml of hybridisation buffer in a 50ml Falcon conical tube on a roller in a 37°C hotroom for 1 hour. Fifteen microlitres of the probe were added to each filter and incubated on the roller at 37°C overnight. Filters were washed twice in 6xSSC, 0.1% SDS (Sigma) at room temperature and a further 2 washes of 30 minutes at 60°C. Filters were then exposed to Hyperfilm-MP (Amersham) at -70°C.

(v) Avoidance of contamination

Great care was taken to avoid contamination of reactions. The culturing of salivary gland cells and their subsequent harvesting as cell pellets was carried out in a safety hood where routine virus propagation was not done. The master mixes for all of the PCR experiments were prepared outside the department. Positive displacement pipettes were used throughout the setting up of reactions. Positive controls were set up in a different safety cabinet after the sample reactions had been completed. In the course of these experiments no contamination of PBS or air controls occurred.

5.2.12 Ultrastructural Examination Of Salivary Gland Cells By Electron Microscopy

The ultrastructure of cells cultured from a salivary gland biopsy was examined by EM. The medium was removed from the flask and 2ml of paraformaldehyde/gluteraldehyde fixative added. The cell monolayer was scraped off immediately using a cell scraper. The supernatant was removed and centrifuged at 400*g* for 7 minutes. Fresh fixative was added to the cell pellet. The pellet was processed and examined by EM as outlined in 4.2.4.

5.2.13 Inoculation With A Transforming Virus

Salivary gland cultures were inoculated at a multiplicity of infection of 100PFU/cell with an adenovirus type-5/SV40 recombinant virus (Van Doren and Gluzman, 1984). This virus was the kind gift of Professor P. Gallimore, CRC Laboratories, Birmingham, UK. Virus stock was stored at -80^{o}C at a titre of $1x10^{9}$ PFU/0.2ml. Infected cultures were fed twice weekly. Cultures that reached confluence were split either 1:2 or 1:5. Cytocentrifuge slides of a culture that had been propagated in such a manner for over 20 passages were prepared, fixed in acetone, and stained with a MoAb specific for SV40 large T antigen.

Experimental work with the adenovirus type-5/SV40 recombinant virus was carried out in a Category 2 containment facility within a Class II microbiological safety cabinet. Waste infectious material was treated first with Virkon Virucidal Disinfectant followed by autoclaving. As is the practice in this laboratory, no glassware or sharps were used apart from microscope slides and the cytocentriguge slide holders and rotor bucket were treated with Virkon and 70% ethanol following each use. The cytocentrifuge rotor bucket was sealed and opened only within the safety cabinet.

5.3 RESULTS

5.3.1 Primary Salivary Gland Cultures

The tissue culture conditions were successful in promoting the outgrowth of cells with an epithelial morphology from salivary gland tissue explants. The protocol was based on the the studies of Lamey *et al.* (1984), although certain modifications were made. In this study, Dulbecco's MEM/F12 medium supplemented with 10%FCS was used as opposed to Medium 199 (Gibco-BRL) supplemented with 20%FCS. Also, salivary gland cultures were grown in tissue culture flasks rather than on sterile glass coverslips housed in petri-dishes.

Initially flasks from three manufacturers were used. The most satisfactory growth of salivary gland cells was obtained using Costar 25cm^2 flasks and therefore, the majority of biopsies were cultured in such flasks. The use of Costar flasks either pre-coated with ECM or laminin, a constituent of ECM, did not promote greater cellular outgrowth from tissue explants. However, cells adhered at a faster rate on laminin or ECM when passaging salivary gland epithelioid cell lines. Greater cell numbers in these flasks were not apparent compared to those untreated.

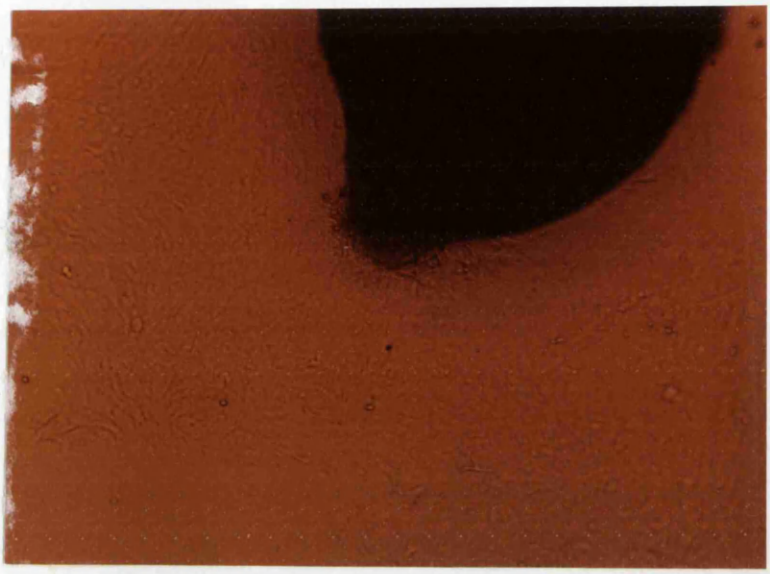
The majority of salivary gland biopsies yielded outgrowth of cells to varying degrees. Figure 5.3.1 shows the cellular outgrowth from a piece of labial salivary gland biopsy. Frequently the cell numbers obtained were insufficient for further experimentation. In a number of cultures, fibroblast cell growth masked the presence of epithelial-like cells. In this study salivary gland cells were not routinely passaged. The culture conditions were designed to produce *in vitro* epithelial cells with differentiation states as close to their *in vivo* equivalent and not to derive epithelioid cell lines as has been done previously (Lamey *et al.*, 1984). Salivary gland cells were able to grow on plastic coverslips housed in Leighton tubes although not as successfully as in Costar flasks and fibroblast contamination was more common.

The majority of cells in culture had an epithelial morphology. The main visible contaminant was fibroblast-like cells. These cells were less adherent to the flask surface and were selectively removed from cultures following brief exposure to trypsin-EDTA. Two approaches were used to confirm the epithelial nature of cellular outgrowths. First, the ultrastructure of a salivary gland cell

Figure 5.3.1

LABIAL SALIVARY GLAND CELL CULTURE

A.



B.

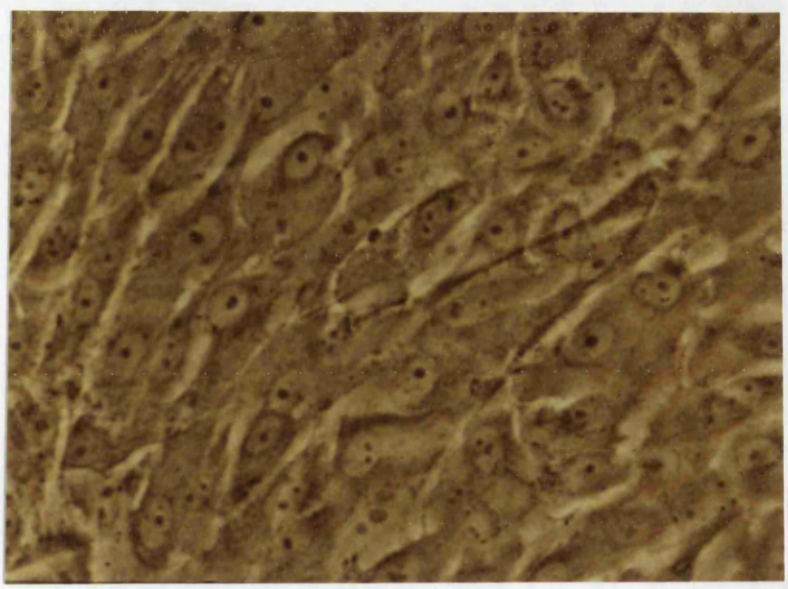


Figure 5.3.1 Salivary gland cell cultures. A. Cells growing out from a piece of labial salivary gland biopsy (x40). B. Monolayer of cells with epithelial morphology from a salivary gland culture (x400).

population was examined by electron microscopy. The presence of epithelial cell-junctions, namely desmosomes with tonofilament bundles, was identified (Fig. 5.3.2). Secondly, the cells were examined for cytokeratin expression. One hundred percent of a salivary gland cell culture examined stained positive with either an anti-cytokeratin no.18 or 19 antibody by IFA (Fig. 5.2.3).

5.3.2 Viral-Infection Of Salivary Gland Cells

Following inoculation with 10PFU adenovirus type-2 a proportion of cells stained positively with antibodies against adenovirus group antigen (Fig. 5.3.4). Control salivary gland epithelial cells probed with the two anti-La MoAbs displayed a uniform staining pattern confined to the cell nucleus (Fig. 5.3.5). Following inoculation with either 1 or 10 PFU of adenovirus per cell, the staining pattern altered in a proportion of cells either 24 or 48hr later. Some of the cells had a similar distribution of La within the nucleus as untreated cells although the staining appeared brighter and more granular. A number of the cells revealed a more punctate nuclear staining pattern particularly obvious in the region of the nuclear membrane (Fig. 5.3.5). Double-staining for both La and adenovirus suggested that this altered nuclear staining pattern was confined to adenovirus infected cells (Fig. 5.3.6). No cytoplasmic La was detected 48hr following viral infection.

5.3.3 Effect Of IFN-γ On Salivary Gland Cells

Following treatment with high-doses of IFN-γ (500U/ml), viable salivary gland cells in suspension were examined by IFA for the cell surface expression of HLA-DR and La antigens. Cell membrane expression of HLA-DR molecules was observed to varying degrees on all cells (Fig. 5.3.7). No cell surface expression of La was detected. However, following treatment with IFN-γ, salivary gland epithelial cells displayed clear cytoplasmic as well as nuclear staining using anti-La MoAbs (Fig. 5.3.7).

5.3.4 Live Cell Fluorescence Of Coverslip Cultures

Viable salivary gland or Hep-2 cells cultured on coverslips and either untreated or inoculated with 10PFU of adenovirus per cell, were examined by IFA for the cell surface expression of La. No surface expression of La was detected 48hr after infection on Hep-2 cells. In a number of coverslip cultures which were

Figure 5.3.2

ULTRASTRUCTURAL EXAMINATION OF SALIVARY GLAND CELLS BY ELECTRON MICROSCOPY

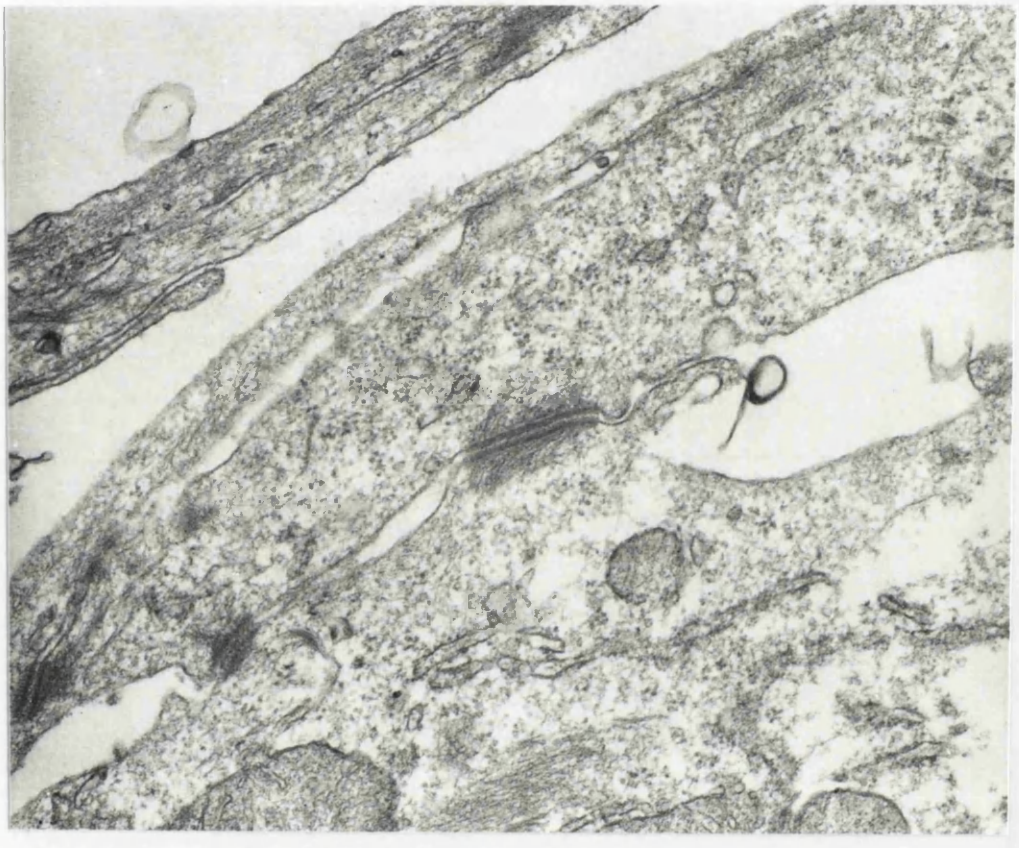
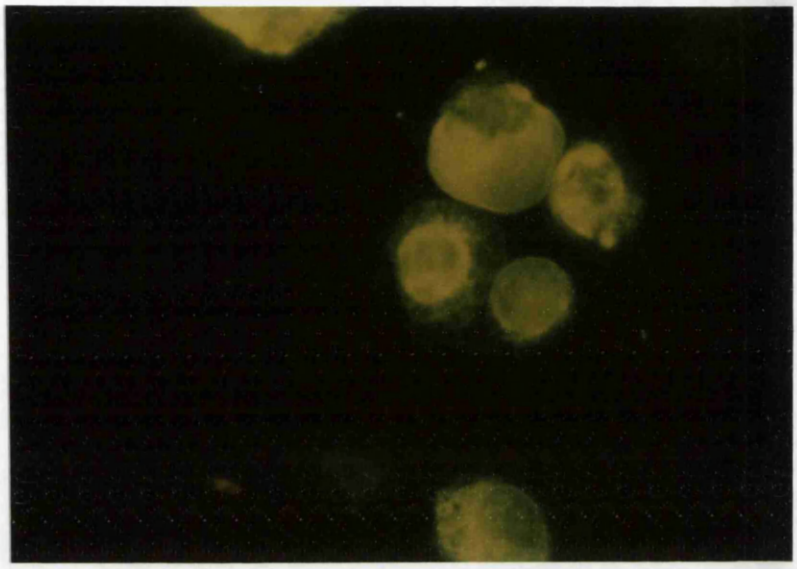


Figure 5.3.2 Ultrastructural examination of salivary gland cells. Examination revealed the presence of epithelial cell-junctions. The tonofilament bundles and desmosomes of the cell-junction in the centre of the photograph are clearly visible (x40,000).

Figure 5.3.3

EXPRESSION OF CYTOKERATIN POLYPEPTIDES BY SALIVARY GLAND CELLS

A.



B.

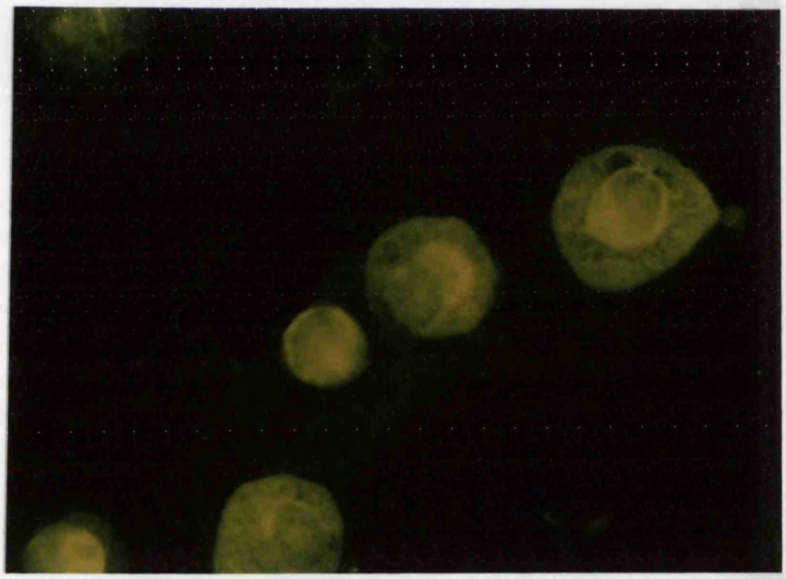


Figure 5.3.3 Expression of cytokeratin polypeptides in salivary gland cells. Immunofluorescent microscopy of salivary gland epithelial cells using MoAb to 2 different cytokeratins A. cytokeratin no.18. B. cytokeratin no.19. 100% of the cells exhibited positive staining with both cytokeratin MoAbs (x500).

Figure 5.3.4

ADENOVIRUS INFECTION OF SALIVARY GLAND EPITHELIAL CELLS

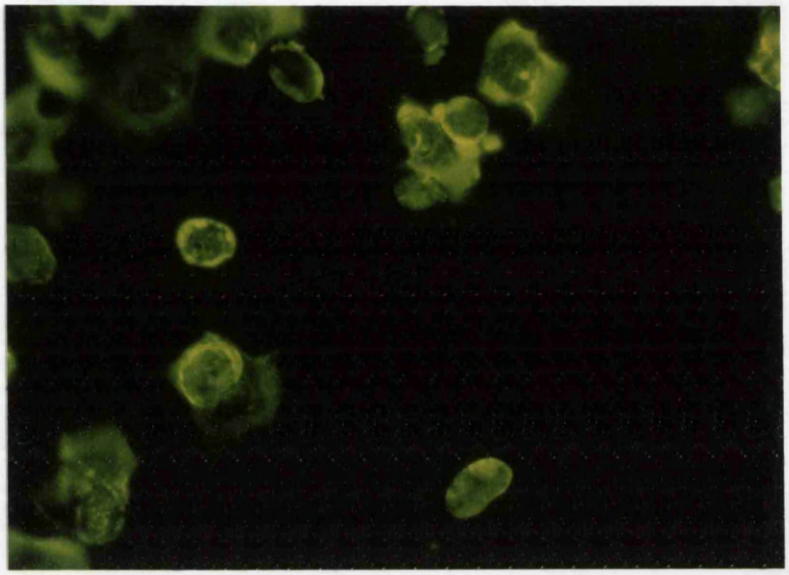
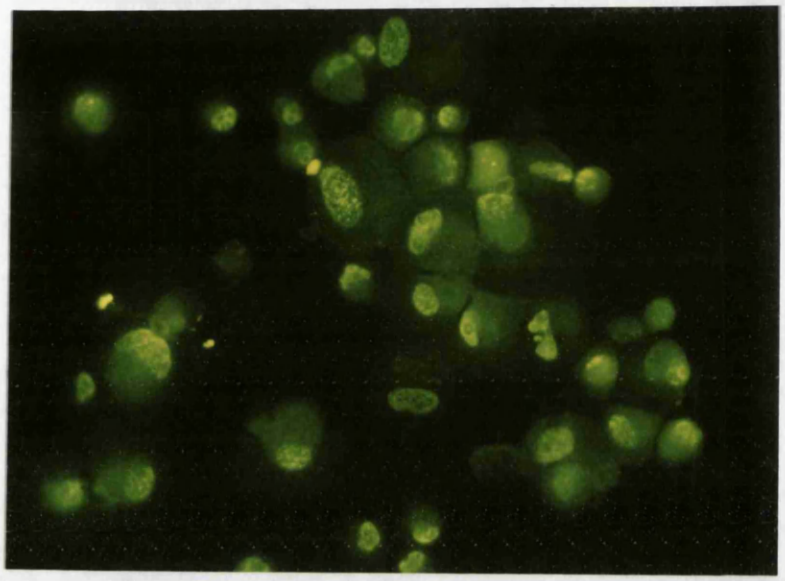


Figure 5.3.4 Immunofluorescent microscopy of infected salivary gland cells using anti-adenovirus group antigen MoAb. The cells were infected for 48hr with adenovirus type 2 (10PFU/cell) (x250).

Figure 5.3.5

DETECTION OF LA ANTIGEN IN ADENOVIRUS-INFECTED AND UNINFECTED SALIVARY GLAND CELLS

A.



B.

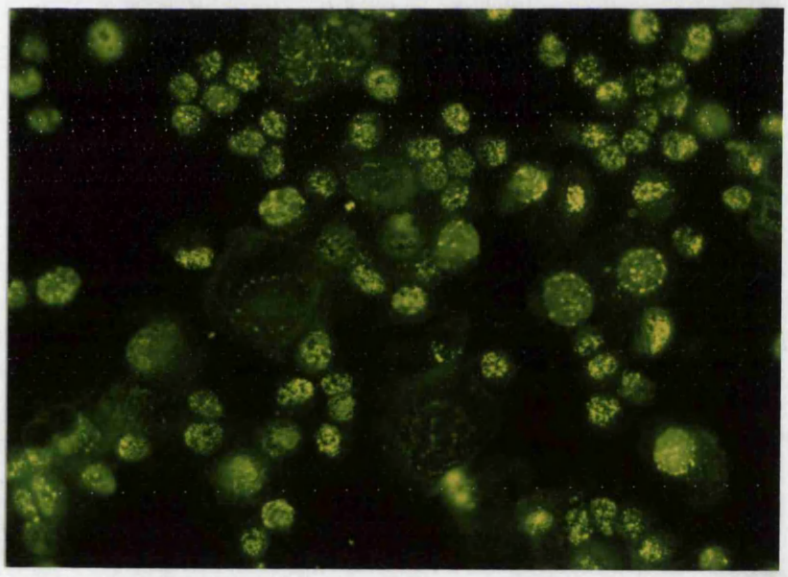


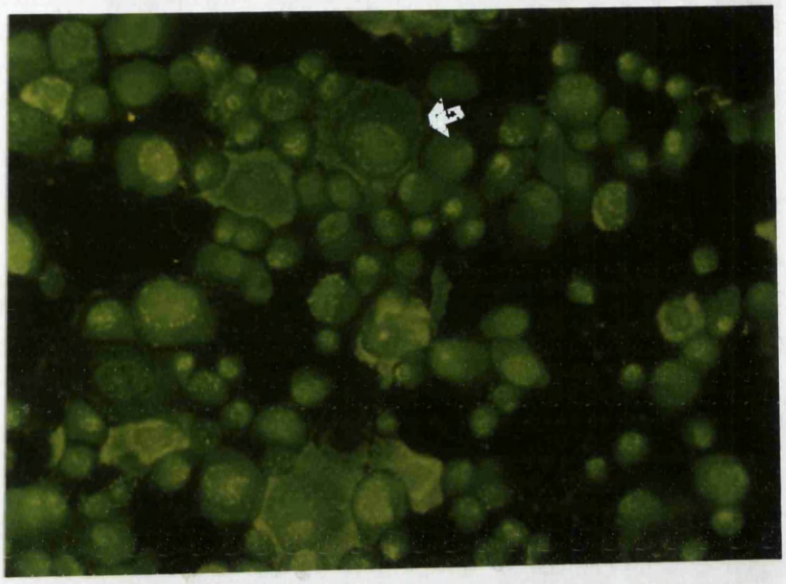
Figure 5.3.5 Immunofluorescent microscopy of salivary gland cells using anti-La MoAbs.

A. Uninfected cells. The anti-La MoAbs produce a uniform staining pattern confined to the cell nucleus. B. 48hr after infection with adenovirus type-2 (10PFU/cell). A number of cells display a more punctate staining pattern in the region of the nuclear membrane (x250)

Figure 5.3.6

DOUBLE-STAINING OF LA AND ADENOVIRUS ANTIGENS IN INFECTED SALIVARY GLAND CELLS

A.



B.

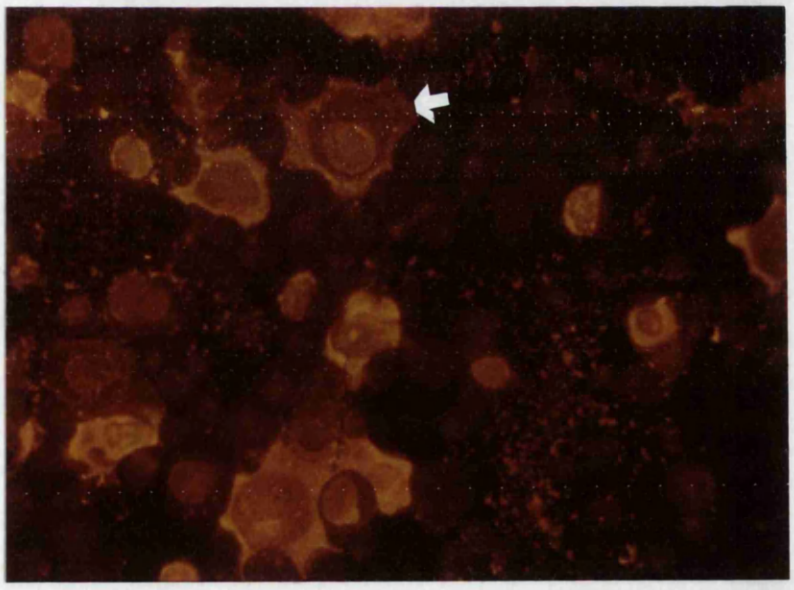


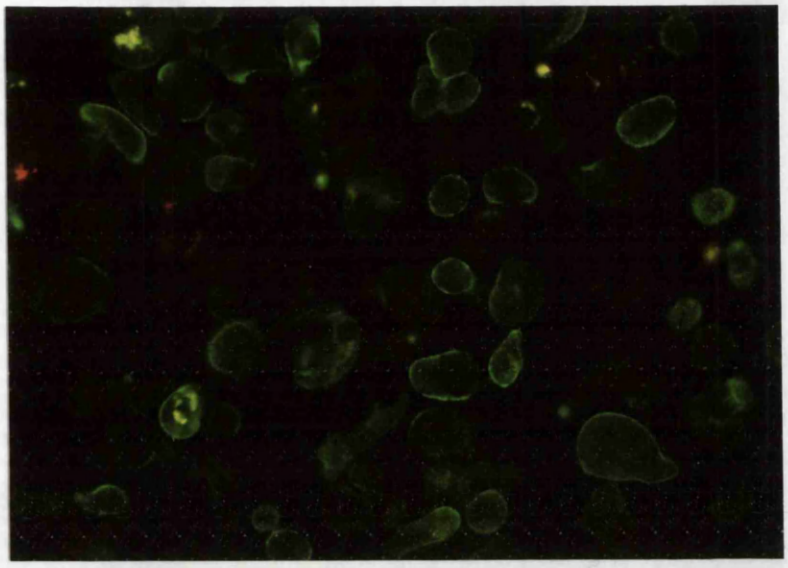
Figure 5.3.6 Immunofluorescent microscopy of salivary gland cells double-stained using anti-La and anti-adenovirus MoAbs, 48hr after infection with adenovirus.

A. Anti-La MoAbs. Many of the smaller cells display a uniform staining pattern of La similar to uninfected cells. A proportion of the cells exhibit a more speckled staining pattern in the region of the nuclear membrane. B. Anti-adenovirus MoAb. Detection of adenovirus antigen appears to be restricted to cells with an altered distribution of La within the nucleus (x250).

Figure 5.3.7

EXPRESSION OF HLA-DR AND LA BY SALIVARY GLAND CELLS TREATED WITH INTERFERON-γ

A.



B.

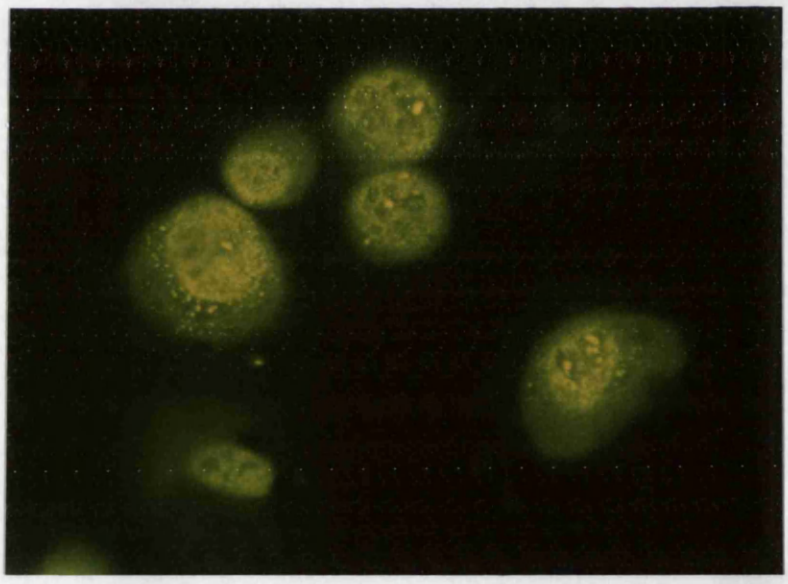


Figure 5.3.7 Immunofluorescent microscopy of salivary gland cells treated with 500U/ml IFN-γ for 72hr.

A. Viable cells probed with anti-HLA-DR MoAb showing cell surface fluorescence (x250). B. Acetone-fixed cells reacted with anti-La MoAbs revealing cytoplasmic as well as nuclear expression of La (x500).

either infected or uninfected with adenovirus, treated or untreated with IFN-γ, areas of the cell monolayer stained positively with the anti-La MoAbs. The staining pattern was found over individual cells or areas of cells (Fig. 5.3.8). Bachmann *et al.* (1991) reported an extracellular location for La under certain laboratory conditions. The authors suggested that extracellularly the La antigen was associated with fibronectin in the ECM. Cultures derived from the same salivary gland biopsy were stained by live cell IFA for either La or fibronectin. The distribution of fibronectin as expected was found in regions surrounding cells (Fig. 5.3.9) and was different from the areas stained positive with the anti-La antibodies.

To investigate with which feature of the cell monolayer the anti-La antibody stained areas was associated, viable salivary gland cells stained with the La antibodies were examined using confocal microscopy by Dr. A. Entwistle (Ludwig Institute, London). The positively stained areas were considered to be non-specific and were not associated with any specific feature of the cell monolayer (data not shown). Interestingly Hep-2 cells, either untreated or treated with IFN-γ, showed no positive staining with the same antibodies (data not shown). Thus, the non-specific reaction was a feature of salivary gland cells cultured on coverslips.

5.3.5 Detection Of HHV-6 And EBV By PCR

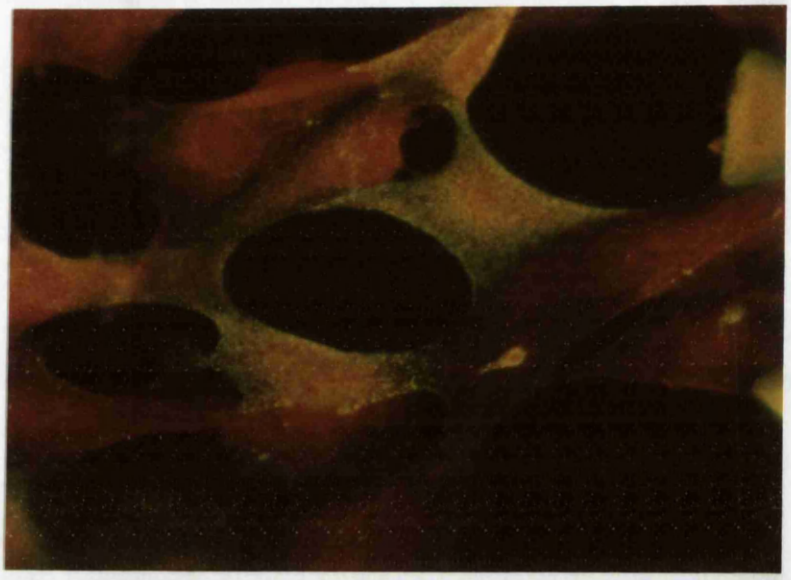
Initially salivary gland cell populations cultured from 11 different biopsies were assayed by PCR for HHV-6. The results are shown in Table 5.3.1. Following the first round of amplification one strongly positive and 2 weakly positive bands were detected by autoradiography after a 7-day exposure. Using the HHV-6 nested primers only these 3 positive samples gave rise to fragments of the correct size, which were visualised on ethidium bromide stained gels. The 3 positive samples were derived from 2 persons diagnosed with SS (1 primary and 1 secondary) according to the criteria proposed by Daniels, (1984) and one person diagnosed with non-specific sialadenitis. The 8 HHV-6 negative samples were derived from 1 person with SS and 7 persons with non-specific sialadenitis.

An unsatisfactory element of the first group of PCR samples was that cell pellets were not uniform in cell number. In addition, Kondo *et al.* (1991) described monocytes as a possible site of HHV-6 latency *in vivo*. Under tissue

Figure 5.3.8

REACTIVITY OF VIABLE SALIVARY GLAND CELL MONOLAYERS WITH ANTI-LA ANTIBODIES

A.



B.

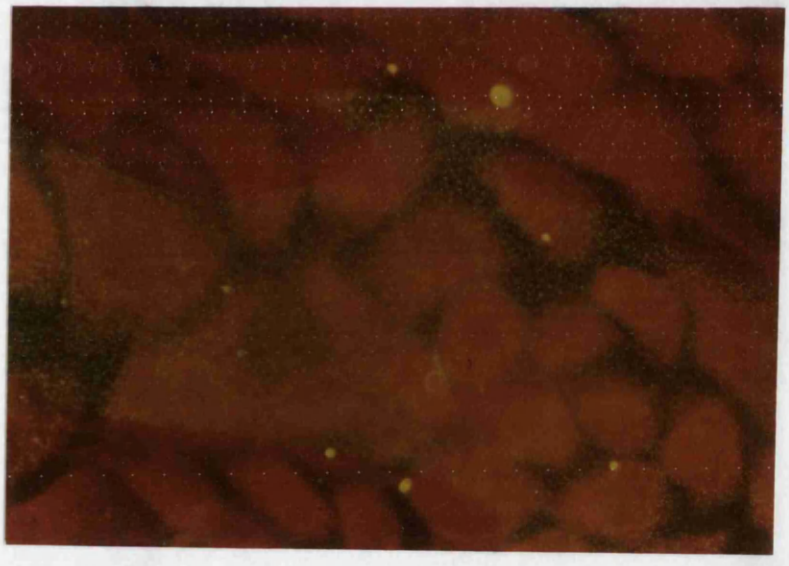


Figure 5.3.8 Immunofluorescent microscopy of salivary gland viable cell monolayers probed with anti-La MoAbs.

A. Positive fluorescence corresponding to the surface area of individual cells.

B. An area of positive fluorescence spanning a number of cells (x500). Confocal microscopy did not identify these positively-stained areas with any feature of the cell monolayer.

Figure 5.3.9

DISTRIBUTION OF FIBRONECTIN IN SALIVARY GLAND CELL MONOLAYERS

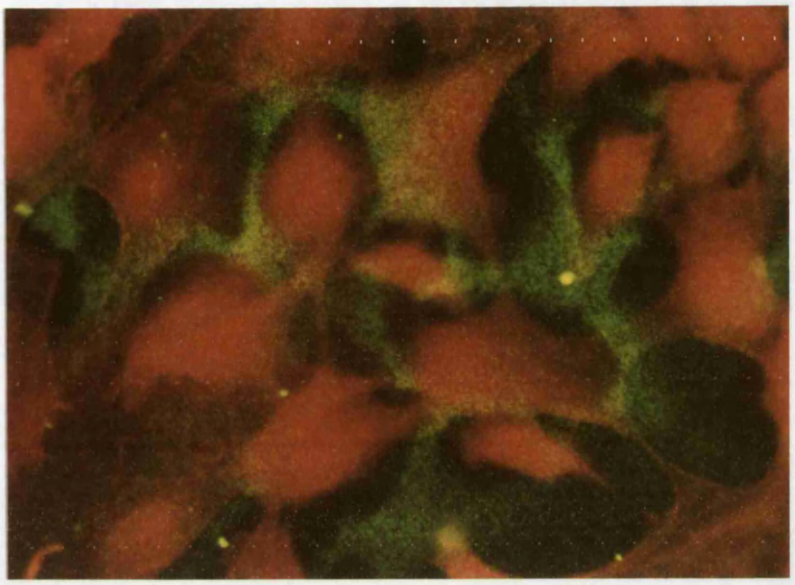


Figure 5.3.9 Immunofluorescent microscopy of a viable salivary gland cell monolayer using an anti-fibronectin MoAb. Fibronectin is detected in regions surrounding cells. The distribution of fibronectin does not appear to correspond to the staining pattern obtained with anti-La MoAbs on similar cell monolayers (Fig. 5.3.8) (x500).

Table 5.3.1

DETECTION OF HHV-6 BY PCR IN *IN VITRO* CULTURED SALIVARY GLAND CELLS - 1

Patient Ref.	Age/Sex	HHV-6 PCR Result	Diagnosis
A	18/M	-	NSS
B	60/F	-	NSS
C	57/F	-	NSS
D	71/F	+	2^{y} SS
E	66/M	+	NSS
F	69/F	-	1^{y} SS
G	46/F	+	1^{y} SS
H	55/F	-	NSS
I	80/F	-	NSS
J	34/M	-	NSS
K	?/M	-	NSS

NSS=non-specific sialadenitis; 2^{y}=secondary; 1^{y}=primary.

culture conditions monocytes are adherent cells. In order to minimise the risk of monocyte contamination of salivary gland cultures, trypsinisation steps were introduced prior to the harvesting of cells in a second group of samples. The trypsinisation step was successful in removing fibroblasts and in the one culture examined, 100% of cells were cytokeratin positive by IFA.

A further 10 cell populations were tested for HHV-6 by PCR. EBV PCR investigations were performed on 9 of the 10 samples. β-globin PCR was included to confirm there was amplifiable DNA in sample tubes. A uniform cell pellet number of 10^5 was used except for EBV PCR where only 10^4 cells were available for 3 of the samples. The results of the PCRs are shown in Table 5.3.2. Two of the 10 were HHV-6 positive following amplification with the first set of primers and hybridisation with the HHV-6-specific probe (Fig. 5.3.10). No further positive samples were detected following amplification with the HHV-6 nested primers and visualisation of amplified product on ethidium-bromide stained gels (Fig. 5.3.10). The 9 samples tested for EBV by PCR were negative following hybridisation with the EBV-specific probe (Fig. 5.3.11). All of the 10 samples were β-globin gene positive demonstating the presence of amplifiable DNA (Fig. 5.3.11).

The two HHV-6 positive epithelial cell populations were grown from biopsies from persons with non-specific sialadenitis. Epithelial cell cultures from biopsies from 3 persons diagnosed with SS (1 primary and 2 secondary) (Daniels, 1984) and a further 5 cultures from persons with non-specific sialadenitis were negative for HHV-6 and EBV by PCR.

5.3.6 Immortalisation Of Salivary Gland Epithelial Cells

Using a recombinant adenovirus type-5 containing the large T antigen of SV40, attempts were made to infect and immortalise primary salivary gland cells. The majority of cultures infected with this virus developed massive outgrowths of fibroblast-like cells. However, in one infected culture, epithelial-like cells grew to confluence and were then passaged at least once per week for 4 months. The morphology of these cells remained unchanged over this time period although the cells ceased to grow after 4 months. Expression of SV40 large T antigen was detected in the nucleus of 100% of these cells (Fig. 5.3.12). It is probable that the transforming virus altered the growth properties of the cells in this culture, but was insufficient to immortalise them.

Table 5.3.2

DETECTION OF HHV-6 AND EBV BY PCR IN *IN VITRO* CULTURED SALIVARY GLAND CELLS - 2

Patient Ref.	Age/Sex	HHV-6 PCR Result	EBV PCR Result	β-globin PCR Result	Diagnosis
1	49/F	-	-	+	2^{y} SS
2	70/F	-	-	+	NSS
3	?/F	-	-	+	NSS
4	47/F	-	-	+	NSS
5	76/F	-	-*	+	NSS
6	65/F	-	-*	+	NSS
7	41/F	+	-	+	NSS
8	60/F	-	-*	+	1^{y} SS
9	52/F	+	-	+	NSS
10	73/F	-	-	+	2^{y} SS

* = 10^4 cells used per reaction; NSS=non-specific sialadenitis; 1^{y}=primary; 2^{y}=secondary

Figure 5.3.10

DETECTION OF HHV-6 IN CULTURED SALIVARY GLAND EPITHELIAL CELLS BY PCR

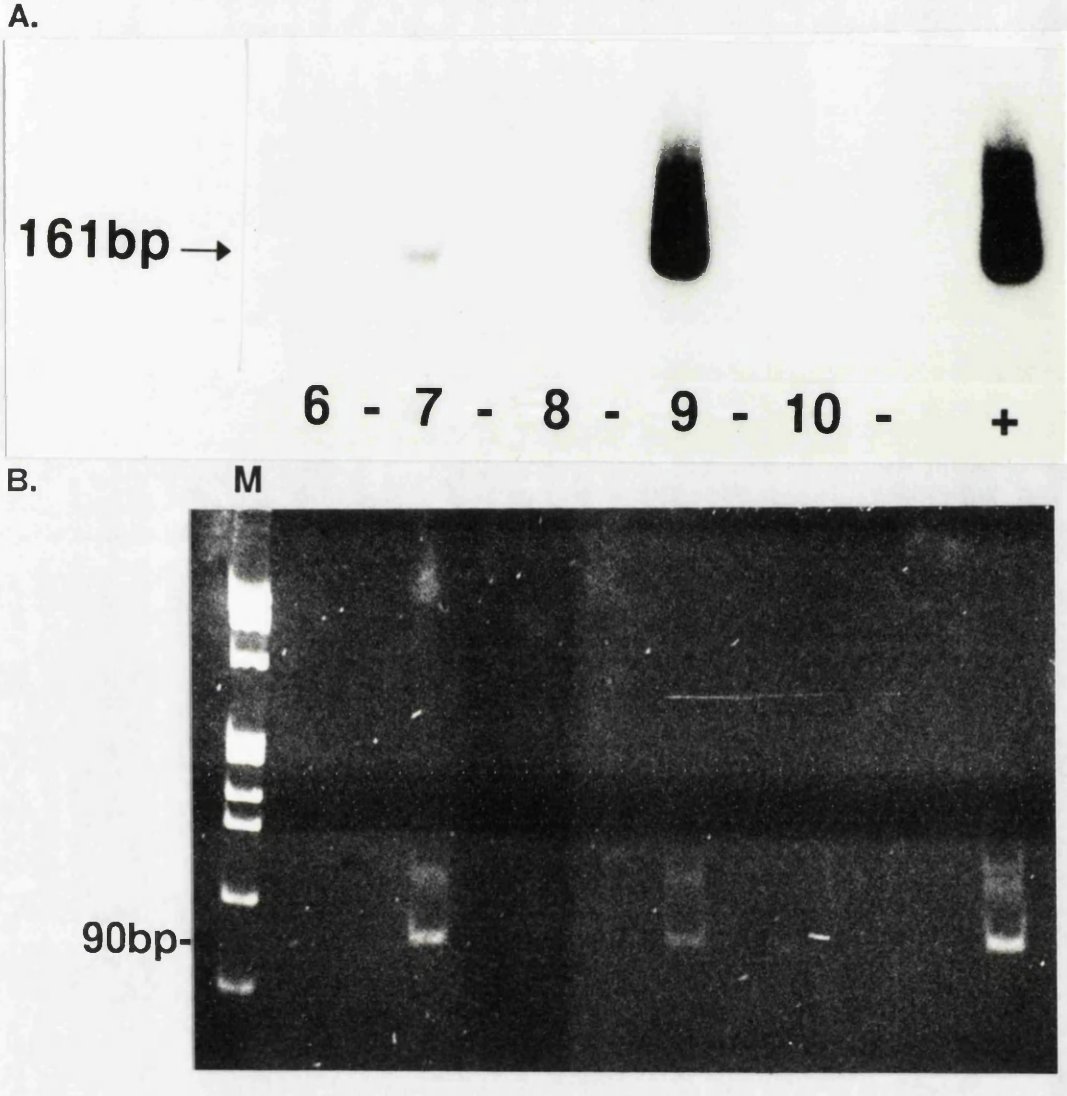


Figure 5.3.10 Detection of HHV-6 in the second group of salivary gland epithelial cell cultures by PCR.

A. Autoradiograph of samples 6-10 following one round of HHV-6 PCR. Samples 7 and 9 are positive.

B. Ethidium bromide stained polyacrylamide gel of nested HHV-6 PCR on samples 6-10 and their controls. Positive bands of the correct size are found for samples 7 and 9 only.

Numbers correspond to sample numbers; - = accompanying negative control; + = positive control; M = DNA molecular weight markers.

Figure 5.3.11

RESULTS OF EBV AND β-GLOBIN PCR USING CULTURED SALIVARY GLAND EPITHELIAL CELLS

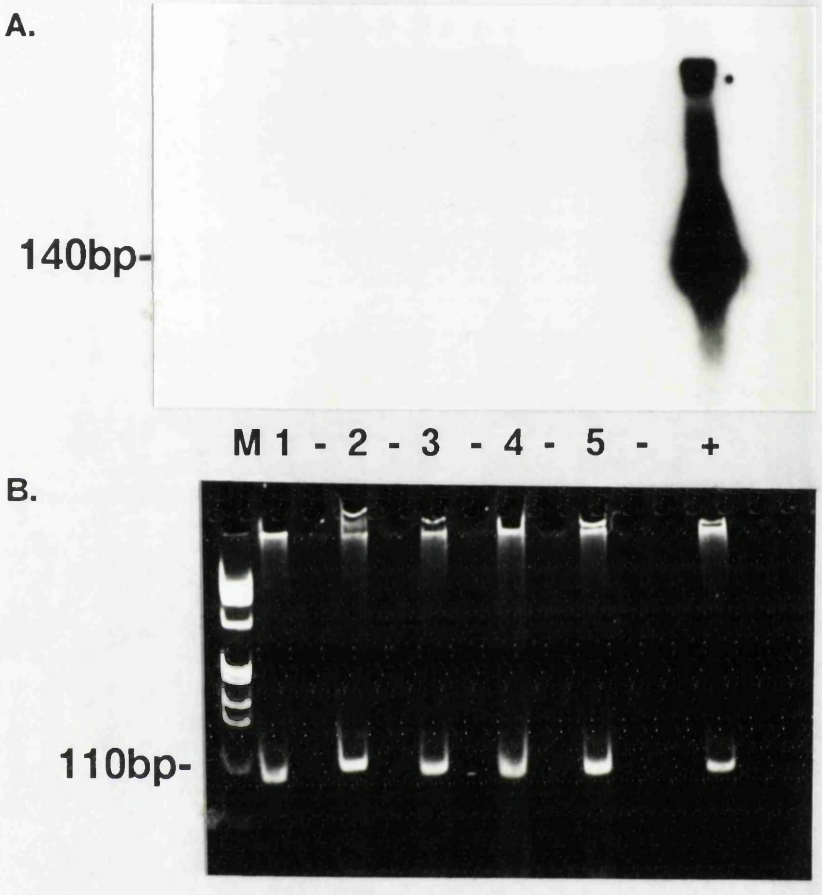


Figure 5.3.11 Results of EBV and β-globin PCR on salivary gland epithelial cells.

A. Autoradiograph of samples 1-5 following EBV PCR. All the samples are negative.

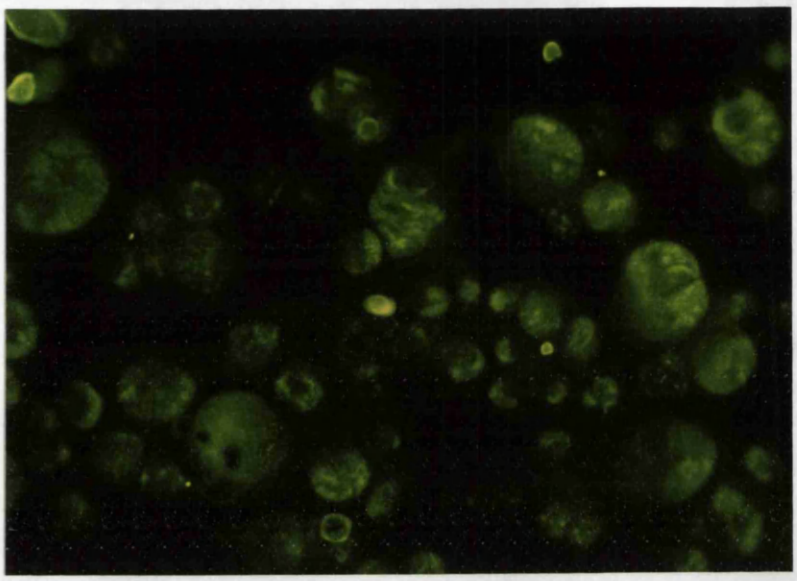
B. Ethidium bromide stained polyacrylamide gel of β-globin PCR on samples 1-5 and their controls. Bands of the correct size (110bp) are found for each of the salivary gland epithelial cell samples, demonstrating the presence of amplifiable DNA.

Numbers correspond to sample numbers; - = accompanying negative control; + = positive control; M = DNA molecular weight markers.

Figure 5.3.12

CHARACTERISATION OF ANTIGEN EXPRESSION IN SALIVARY GLAND CELLS INFECTED WITH A TRANSFORMING VIRUS

A.



B.

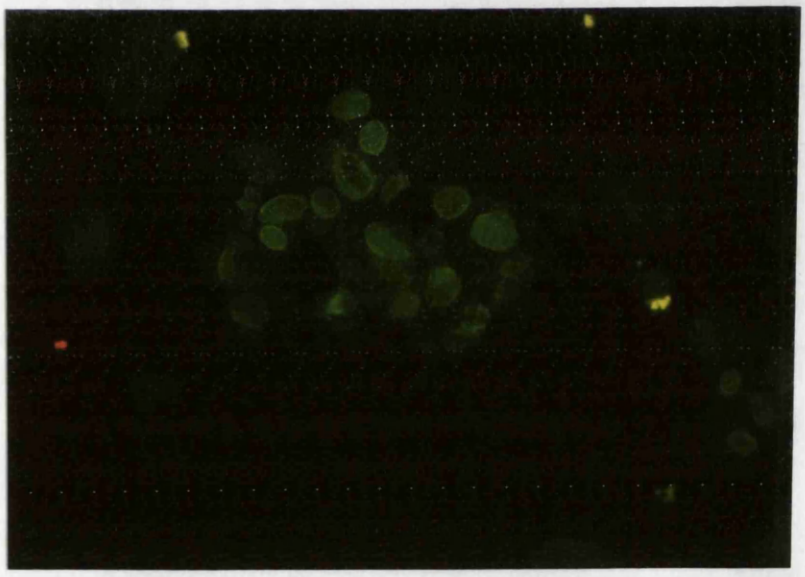


Figure 5.3.12 Immunofluorescent microscopy of salivary gland cells infected with an adenovirus/SV40 hybrid transforming virus. A. Cells probed with anti-SV40 large T antigen MoAb showing nuclear staining in 100% of cells. The cells were from a salivary gland culture that had been infected with the virus and passaged once a week for 4 months (x500).

B. Cells probed with AGF2.3 MoAb showing nuclear membrane staining in the majority of cells. This antibody detects a nuclear membrane antigen expressed by certain transformed epithelial cell lines (x250).

Following inoculation with the transforming virus, primary salivary gland cells showed a brighter staining with the AGF2.3 antibody which detects a nuclear membrane protein present in certain transformed epithelial cells (Fig. 5.3.12). The presence of this antigen was either absent or weakly-stained in untreated cells. These results provided further evidence for the epithelial nature of primary salivary gland culture cells.

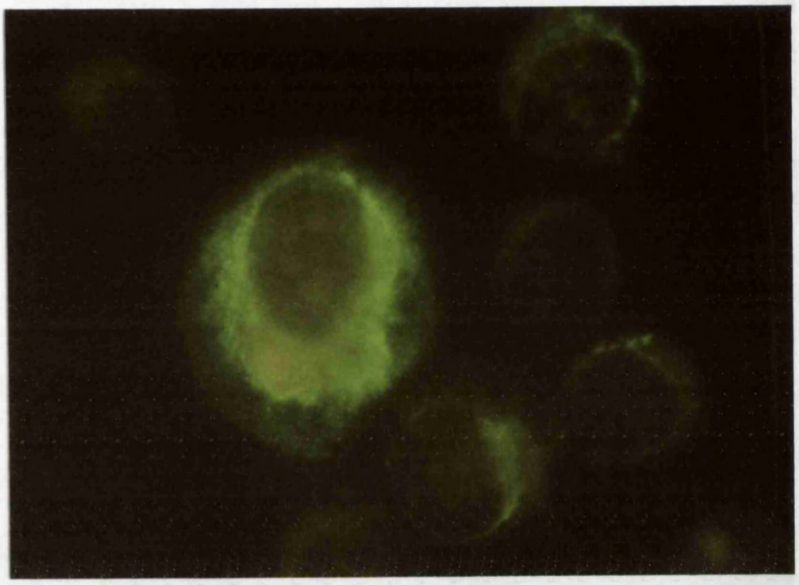
5.3.7 Detection Of Putative Retroviral Antigens In Salivary Gland Cultures

A number of primary salivary gland cultures grown in our laboratory were examined by workers at the Kennedy Institute of Rheumatology for expression of antigens that reacted with antibodies raised against retroviral antigens. The results of these investigations have previously been reported (Shattles *et al.*, 1992; Brookes *et al.*, 1992). A MoAb (197 Ab) that reacts with HTLV-1 p19 detected an antigen, designated 197 Ag, expressed in primary salivary gland cells (Fig. 5.3.13). The antigen detected was unlikely to be HTLV-1 derived as persons from which these cultures were derived were HTLV-1 seronegative. Primary salivary gland cells were also found to express an antigen detected with an antisera raised against a predicted amino acid sequence of a protein of HRES.1 (Fig. 5.3.13). Thus, these particular antibody reagents may be detecting antigens in salivary gland tissue encoded by retroviruses.

Figure 5.3.13

DETECTION OF PUTATIVE RETROVIRAL ANTIGENS IN CULTURED SALIVARY GLAND CELLS

A.



B.

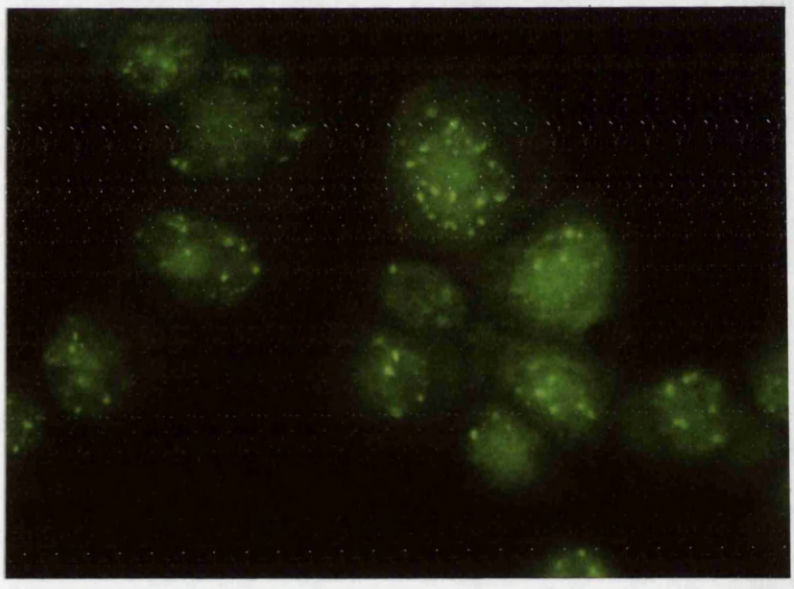


Figure 5.3.13 Immunofluorescent microscopy of salivary gland epithelial cells using antibodies directed against retroviral antigens.

A. Cells probed with 197 MoAb showing cytoplasmic staining. This antibody reacts with HTLV-1 p19 antigen. The putative antigen it reacts with in salivary gland tissue has been designated 197 antigen.

B. Cells probed with purified antisera against a predicted peptide of HRES-1 showing bright, speckled nuclear staining. The antigen detected in salivary gland tissue has been proposed to be an endogenous retroviral product. Photographs kindly supplied by Dr. Sharon Brookes.

5.4 DISCUSSION

The aetiology of SS and the mechanism by which autoimmune responses develop in the disease remain unknown. I have utilised salivary gland epithelial cell cultures as a model to investigate viral involvement and mechanisms of autoimmunity associated with SS.

Examination of the ultrastructure of cultured salivary gland cells by electron microscopy by ourselves and others (Lamey *et al.*, 1984), revealed cell-junctions characteristic of epithelial cells. The epithelial components of salivary gland consist of ductal, acinar and myoepithelial cells. The cells in culture expressed both cytokeratin no. 18 and 19. In an immunohistological study, Geiger *et al.* (1987) identified cytokeratin no. 18 expression in both the ductal and acinar elements of salivary glands. Cytokeratin no.19 was only demonstrated in the ductal component. Thus, expression of cytokeratins suggests that the cultured cells are ductal in origin although rapid acinar to ductal transdifferentiation has been observed in cultured human pancreas (Hall and Lemoine, 1992). Similarly, based on the detection of the enzyme 11β-hydroxysteroid dehydrogenase, it has been suggested previously that the majority of cells in primary explant cultures are ductal (Lamey *et al.*, 1984).

The salivary gland cultures provided an opportunity to examine mechanisms by which the La antigen could be presented to the immune system. The role of membrane expression of nuclear antigens including La in autoimmunity has recently been reviewed (Venables and Brookes, 1992b). I was able to demonstrate an altered nuclear distribution of La in adenovirus-infected cells. This is in contrast to the study of Baboonian *et al.* (1989) where similar conditions of infection of a continuous epithelial cell line, Hep-2, resulted in cytoplasmic and cell membrane expression of La. However, similar experiments carried out in this project were unable to repeat this phenomenon. A translocation of La to the cytoplasm and cell surface has also been observed in HSV-1 infected CV-1 cells (Bachmann *et al.*, 1989; Bachmann *et al.*, 1992). It is possible that methodological differences such as virus titre at inoculation, and time at which cells were examined post-infection may explain the difference in results. Alternatively, the differences may be due to the use of primary salivary gland cultures, which are a more representative cell type to study, rather than continuous cell lines.

A number of other experimental stimuli have been proposed that alter the cellular location of La. UV-irradiation of human keratinocytes or stimulation with estradiol resulted in cell surface La expression (Furukawa *et al.*, 1990; Bachmann *et al.*, 1990; Furukawa *et al.*, 1991) and EBV-transformed cell lines also express cytoplasmic La (Smith *et al.*, 1985). Serum-starvation of cells was reported to result in a translocation of La to the cell surface and an extracellular phase of the antigen observed (Bachmann *et al.*, 1991). IFN-γ treatment can result in cytoplasmic or membrane expression of La depending on the cell type (Venables and Brookes, 1992b), although this has not been examined previously in salivary gland cultures.

IFN-γ treatment of salivary gland epithelial cells resulted in a translocation of La from the nucleus to the cell cytoplasm. Exposure to IFN-γ also resulted in the cell surface expression of HLA-DR molecules. IFN-γ positive lymphocytes are found in normal salivary glands (Brett *et al.*, 1992) probably as a result of constant antigenic challenge from resident microbial flora (Bocci, 1988). In SS, the infiltrating mononuclear cell population, including many activated T cells, is likely to provide a greater source of this cytokine. Bottazzo *et al.* (1983) hypothesised that IFN-γ induced aberrant expression of MHC class II molecules in association with self-antigens could result in an autoimmune response such as thyroid autoimmunity. Our results suggest that a similar mechanism may be operating in SS. As well as HLA-DR, the expression of MHC class II subregion products HLA-DP and -DQ might be an important feature of salivary gland epithelium in SS (Thrane *et al.*, 1993). Our model for the *in vitro* culture of salivary gland cells should prove useful in studying the cytokine-induced expression of different MHC class II products.

Although autoantibodies to La are a common feature, they are not detected in all cases of SS. The number of patients with SS who have detectable autoantibodies to La can vary from 25-87% depending on the assay used (Slobbe *et al.*, 1991). Viral infection of salivary glands may be important as an initial target for the immune system and, in areas of high IFN-γ secretion, a translocation of La to the cytoplasm and cell surface might occur. *In vivo*, cytoplasmic La has been demonstrated in conjuctival epithelial cells imprinted from SS patients (Yannopoulos *et al.*, 1992). A possible consequence of the cycling of La through different cellular compartments, perhaps including an extracellular phase, is the processing and presentation of the antigen in association with MHC molecules, including class II. Such an event could

stimulate a cell-mediated immune response. Alternatively, surface expression of La could act as a target for autoantibody-mediated damage.

Shattles *et al.* (1992) using an antibody, 197 MoAb, directed against the p19 (gag) protein of HTLV-1 detected an antigen, 197 Ag, in 3 of 6 *in vitro* cultured salivary gland cell cultures grown in our laboratory. The 197 Ag expression was induced by IFN-γ at high doses in PBMCs and an epithelial cell line. Immunohistochemical staining detected the antigen in 31% of primary and 24% of secondary SS patients, 21% of patients with sicca symptoms and 12.5% of patients with other connective tissue diseases. The antigen was not expressed in 13 normal salivary glands. None of the patients included in the study of Shattles *et al.* (1992) was HTLV-1 seropositive. The 197 Ag may be a product of an endogenous retrovirus, induced by IFN-γ and thus secondary to inflammation.

Salivary gland cells cultured *in vitro* were shown to express another antigen which is possibly a product of a human endogenous retrovirus, HRES-1 (Brookes *et al*, 1992). Expression of this antigen however was not disease-specific. Elevated antibody titres to a peptide of HRES-1 were also found in SS (Banki *et al.*, 1992; Brookes *et al.*, 1992). The importance of expression of this antigen and 197 Ag in salivary gland epithelial cells is at present unknown. These antigens could be presented to the immune system as antigens that have bypassed tolerance or act as superantigens (Choi *et al.*, 1991) and activate T cells expressing particular T cell receptor (TCR)β genes. Interestingly, Vβ2 and Vβ13 genes were found to be predominantly expressed in T cells in lip specimens from SS patients (Sumida *et al.*, 1992). In sicca syndrome in the context of HIV infection, an overrepresentation of particular TCR rearrangements was found (Dwyer *et al.*, 1993). In this case, the proliferation of T cells in salivary glands may be antigen driven rather than stimulated by a superantigen (Dwyer *et al.*, 1993). At present the presence of TCR restriction in autoimmunity remains inconclusive (Navarrete and Bottazzo, 1993).

The role of hormones in SS and autoimmunity is not clearly defined, but may affect endogenous retroviral and La expression. Estradiol treatment followed by progesterone stimulated a human endogenous retroviral sequence in a human breast cancer cell line (Ono *et al.*, 1987). Estradiol-treatment of human keratinocytes can result in the expression of La at the cell surface (Furukawa *et*

al., 1991). Thus, the occurrence of SS predominantly in elderly women may reflect hormonal influences which could be important in the development of SS. A number of different techniques have been used to identify viral DNA or protein in salivary glands and determine if the presence of a virus occurs with increased frequency in SS. PCR has the advantage of sensitivity whereas *in situ* hybridisation techniques reveal the cellular localisation of the virus. The mononuclear cell infiltrate in SS glands may harbour either HHV-6 or EBV infected cells that provide a source of virus when PCR is applied to fixed tissues. Krueger *et al.* (1990) observed HHV-6 infected mononuclear as well as epithelial cells in salivary gland tissue sections from SS patients. A possible site of HHV-6 latency is monocytes/macrophages (Kondo *et al.*, 1991). In this study, the sensitivity of PCR has been applied to purified salivary gland epithelial cell populations to investigate viral presence.

The initial findings of 3 HHV-6 positive cultures, 2 of which were grown from persons with SS had to be interpreted cautiously as the cultures could possibly have harboured contaminating monocytes. Also, uniform cell numbers amongst samples were not available. Although monocytes/macrophages have adherent properties under tissue culture conditions, we are confident that the refined protocol adopted for the second set of PCR assays removed cells of non-epithelial morphology including residual macrophages.

In this second group of samples, two of ten primary salivary gland epithelial cell cultures were HHV-6 positive although the presence of HHV-6 was not associated with SS. This is the first demonstration of HHV-6 in primary salivary gland epithelial cell cultures of which we are aware. Rhim *et al.* (1989) reported the ability to transform primary human parotid gland epithelial cell cultures with an adenovirus/SV40 hybrid virus, but did not detect HHV-6, EBV, or CMV in these cell populations by PCR. HHV-6 has previously been localised to epithelial cells in salivary glands using avidin-biotin detection systems in fixed tissues (Fox *et al.*, 1990a; Krueger *et al.*, 1990). Endogenous biotin in salivary tissue however, may interfere in assays utilising biotin-labelled reagents (Green *et al.*, 1992). Our results confirm that HHV-6 is present in salivary gland epithelium, which may be a site of replication and persistence for the virus. Whether HHV-6 infection in genetically-predisposed individuals, or HHV-6 infection of a particular cell type, is involved in the pathogenesis of SS remains to be clarified.

There is no clear consensus regarding the increased detection of EBV in salivary gland tissue in SS compared to normal controls using *in situ* hybridisation (Venables *et al.*, 1989; Mariette *et al.*, 1991; Karameris *et al.*, 1992) or PCR (Deacon *et al.*, 1991; Mariette *et al.*, 1991; Deacon *et al.*, 1992) as methods of detection. The EBV genome has been detected by in situ hybridisation and PCR with increased frequency compared to controls and also latent and lytic antigens identified in the lacrimal glands of SS patients (Pflugfelder *et al.*, 1993). The inability to detect EBV in any of the cultures tested is not likely to have resulted from the insensitivity of the PCR assay. The most likely explanation is that EBV infected cells were not present in culture at the time of harvesting. This does not exclude the presence of EBV infected cells at the initiation of cultures as such cells may be unable to persist as a result of lytic infection.

The association between HHV-6 or EBV and lymphomas occurring in the context of SS is stronger than with the autoimmune disease itself (Josephs *et al.*, 1988; Jarrett *et al.*, 1988; Fox *et al.*, 1989). It is possible that the infiltrating lymphocytes found in salivary glands in SS provide a larger than usual susceptible population for infection by either of these herpesviruses. EBV, which can immortalise B cells *in vitro*, or HHV-6 could facilitate B cell hyperreactivity which is a feature of SS. B cell hyperreactivity may give rise to oligoclonal lymphocyte populations which have been detected by receptor gene rearrangement studies in SS salivary and lacrimal glands (Freimark *et al.*, 1989; Pepose *et al.*, 1990). The emergence of a neoplastic cell may require a chromosomal alteration. Detectable t(14;18) translocations have been shown in SS lymphomas but were not found in pre-lymphoma biopsies, even those which exhibited oligoclonal immunoglobulin gene rearrangements (Pisa *et al.*, 1991).

In summary, the ability to grow salivary gland epithelial cells in culture has allowed preliminary investigations into mechanisms of autoimmunity associated with SS and to search for viruses linked with the disease. This model provides a basis for future research aimed at clarifying the role of cytokines, particularly IFN-γ, in the autoimmune process, and to investigate the presence of retroviral gene products in salivary gland epithelia.

CHAPTER 6

GENERAL DISCUSSION

6.1 HHV-6 AND LYMPHOMA/LEUKAEMIA

An important aim of this study was to examine the role of HHV-6 in a range of human lymphomas and leukaemias. The availability of sera from a large epidemiological survey of such malignancies has allowed the combination of epidemiological data with serological investigations. The number of sera in the disease and control groups plus the design of the original study from which sera were derived have made this the most complete examination of HHV-6 serology in malignancy so far. An important consideration in the design of a seroepidemiological survey is to obtain sera from as appropriate controls as possible. Antibody titres to viral antigens can vary with age and sex. For HHV-6, higher antibody levels are found in children (Yanagi *et al.*, 1990) and females (Clark *et al.*, 1990). An initial study described the presence of higher HHV-6 seropositivity in ALL (Ablashi *et al.*, 1988). In a later matched case-control study such a finding was not found (Levine *et al.*, 1992a) and the younger age distribution of ALL cases is likely to have proved misleading in the original study (Ablashi *et al.*, 1988). The results presented here were derived from a case-control study and adjustments for age and sex were included in all of the statistical analyses. Significant differences in HHV-6 antibody titres between cases and controls were identified in AML, LG-NHL, and HD.

The relevance of elevated antibody titres in each of these three malignancies remains unclear. An increase in HHV-6 replication and expression of viral antigens is one possible explanation. Factors such as the disease process itself or subsequent therapy could lead to reactivation of the virus from latency. The sites of HHV-6 replication and latency *in vivo* are not fully characterised. Good evidence exists for replication occurring in salivary glands (Fox *et al.*, 1990a). Latency may be established in a lymphoid cell as the virus was detected by PCR in a rare cell in PBMCs (Gopal *et al.*, 1990; Jarrett *et al.*, 1990). The inability to isolate HHV-6 from blood in healthy individuals suggests the virus is present in a non-infectious latent form, possibly in monocytes/macrophages (Kondo *et al.*, 1991). Molecular studies have failed to identify HHV-6 in tumour tissue from AML cases and the majority of NHLs using southern blot hybridisation or PCR as methods of detection (Josephs *et al.*, 1988; Jarrett *et al.*, 1988; Torelli *et al.*, 1991). It seems unlikely therefore that HHV-6 can be present in the tumour cells of these malignancies.

The detection of virus in the RS cells of HD may be problematic owing to their frequent paucity in the total cell population. One possible way around this would be to obtain single RS cells from biopsy tissue and examine the genome for evidence of viral sequences. Recently Trumper *et al.* (1993) were able to pick single RS cells and by general amplification of cDNA examine gene expression pattern including genes characteristic of haematopoietic lineages.

Features of the biology and epidemiology of HD are suggestive of a viral aetiology (discussed Chapter 3). Only EBV has consistently been associated with the disease and the results presented in this thesis confirmed the presence of elevated antibody titres to EBV antigens in HD. The availability of sensitive assays for the detection of EBV nucleic acid and protein have clearly demonstrated the presence of clonal EBV in HD tumour tissue and localised viral gene expression to within the RS cell (Gledhill *et al.*, 1991; Pallesen *et al.*, 1991a; Armstrong *et al.*, 1992b). EBV-associated HD has been identified more frequently in paediatric and older cases of HD, and in the MC subtype group (Jarrett *et al.*, 1991; Armstrong *et al.*, 1993; Weinreb *et al.*, 1992). A preliminary study presented here did not however, find a correlation between raised EBV antibody titres to lytic cycle antigens and HD cases with detectable virus within RS cells. The detection of replicative EBV within RS cells has recently been reported. Brousset *et al.* (1993) found that biopsy tissue from 3 of 40 EBV-associated HD cases stained positive with antibodies to the ZEBRA protein. One of the cases was also stained with anti-EA-restricted-component antibody, which indicated early gene expression. Thus, EBV replication in RS cells can occur although it would appear to be a rare event. It remains a possibility that such a form of replication, including abortive lytic cycles, could affect antibody titres to antigens such as EBV-EA and EBV-VCA.

The elevated EBV antibody titres may reflect a particularly severe past infection which could account for the altered antibody response that has been detected preceeding diagnosis (Mueller *et al.*, 1989). A consequence of severe infection is the manifestation of IM which has been associated with the development of HD. RS-like cells containing LMP have been detected in acute IM (Isaacson *et al.*, 1992) and may be progenitor RS cells. Likewise RS-like cells which are LMP positive have been found in NHL (Khan *et al.*, 1993). IM has a peak incidence in young adults and it is possible this severe EBV infection is directly associated with the development of EBV-associated HD. However, EBV-associated HD cases are found less frequently in the young adult age group. It

is tempting to speculate that another virus is involved in the pathogenesis of young adult HD. Perhaps of some relevance is the identification of a stronger association of HHV-6 antibody titres to HD in this age category. The development of sensitive *in situ* detection assays are necessary to clarify the role of HHV-6 in HD. In the absence of direct involvement, it is possible that the HHV-6 antibody levels are acting as a marker for delayed infection to common infectious agents. One of these may prove to be a virus associated with HD.

The associations of HHV-6 and EBV antibody with HD were, in part, independent of each other. Therefore, the elevated antibody titres to HHV-6 in HD were unlikely to represent a consequence of an interaction with EBV. This can not be excluded however, and interactions between HHV-6 and other viruses have been proposed. Primary infection with EBV or CMV can affect HHV-6 antibody levels, most likely by stimulating reactivation. Alternatively *in vitro* studies have reported that EBV-negative B cell lines not permissive for HHV-6 infection become permissive following infection with EBV (Lusso *et al.*, 1992). Only a small minority of the cells were productively infected. Latent or non-productive infection was proposed in others. The nature of this interaction is not known although the authors hypothesised that either EBV infection could result in the expression of the membrane receptor for HHV-6 or the presence of EBV in the same cell could somehow bypass putative cellular factors restricting HHV-6 expression. If this phenomenon occurs *in vivo*, then an expanded number of EBV-infected B cells would provide an increased target cell population for HHV-6 infection. A reversal of this situation has been proposed to occur between HHV-6 and HIV-1 (Lusso *et al.*, 1993). HHV-6 infection of natural killer cells is cytopathic and also induces the expression of CD4, thereby predisposing them to infection with HIV-1. Thus, there is evidence that HHV-6 infection can potentially suppress the natural anti-viral immunity of the host.

6.2 HHV-6 AND HHV-7 INFECTION IN CHILDREN AND ADULTS

Serological assays to detect antibodies to particular HHV-6 antigens could prove valuable in future studies. To-date an IFA has been the most commonly used HHV-6 antibody detection assay. At present, tests that detect a whole range of lytic cycle antigens and one that detects HHV-6-EA antibodies have been developed. Levine *et al.* (1992b) found that HHV-6 antibody titres in the same sera varied depending on the assay. In that study an IFA and ELISA were used that utilised chronically-infected cells and purified virus respectively

as HHV-6 antigen. As for EBV, antibody profiles to various antigens may vary in health and disease.

The ubiquitous nature of HHV-6 and the fact that the majority of healthy persons are seropositive makes the interpretation of HHV-6 serology in health and disease difficult. As used here, a case-control study has proved valuable in highlighting the association of HHV-6 with particular malignancies. Another approach to examine the role of HHV-6 in particular conditions is the detection of seroconversion at the time of illness. This would seem to have its drawbacks since IgM antibodies to HHV-6 are detected not only during primary infection, but also reactivation (Fox *et al.*, 1990b). The majority of persons with detectable HHV-6 IgM antibodies with IM-like symptoms in this study showed serological evidence of primary EBV infection. Therefore, apparent HHV-6 seroconversions should be considered in the context of findings to other viruses. As for serology, the detection of HHV-6 DNA in tissue samples from healthy persons and the ability to isolate the virus, mainly from saliva, limits the conclusion that can be taken from similar findings in disease. For a common virus that persists in the host, a method of choice to examine disease associations is the *in situ* identification of virus in tissue where its presence is not normally found. There are already a range of HHV-6 specific MoAbs and DNA probes which hopefully will further help characterise the relationship between HHV-6 and disease.

An important value of serology is that with relatively simple technology the seroprevalence of a virus can be examined in large numbers of people. The sole requirement is that the virus can be propagated in tissue culture to provide a constant source of antigen. Therefore, an IFA was the method chosen to study the prevalence of the recently discovered HHV-7. The results have shown that, like HHV-6, infection with the virus is common with 28/29 adults seropositive. Again, similar to HHV-6, infection with HHV-7 occurs early in life as the majority of children had HHV-7 antibody by the age of 2 years. Saliva is likely to be an important vehicle for HHV-7 transmission as the virus was isolated from this source (Wyatt *et al.*, 1991; Chapter4). The availability of HHV-7 specific DNA probes (Berneman *et al.*, 1992a; Black *et al.*, 1993) will further help determine the role of HHV-7 in human disease which is at present unknown. In a large series of HD and NHL biopsy samples HHV-7 was not detected by Southern blot hybridisation (R. Jarrett, personal communication in collaboration with Z. Berneman).

In addition to the association with certain malignacies, viruses have been associated with particular chronic diseases. In order to study viral involvement in SS an *in vitro* model was developed. This was based on the ability to culture epithelial cells from labial salivary gland biopsies. Preliminary studies did not find a link between the presence of HHV-6 or EBV in such cultures and SS. The results of this study do however indicate the important role IFN-γ may play in precipitating an autoimmune response in SS. The salivary gland cultures proved useful as a model to study mechanisms of autoimmunity including autoantibody production. Similarly, they can be used to further study the possible role of endogenous retroviral antigen expression in SS.

An important consideration for future studies on HHV-6 is the relevance of different viral strains in human disease. A number of studies have reported nucleotide sequence and antigenic variations between HHV-6 isolates which can be categorised into 2 groups (Jarrett *et al.*, 1989; Wyatt *et al.*, 1990; Schirmer *et al.*, 1991; Gompels *et al.*, 1993; Dewhurst *et al.*, 1993). Such findings culminated in the recent assignment of new nomenclature for HHV-6 as two subgroups - variant A and variant B (Ablashi *et al.*, 1993). Initial investigations have reported the almost exclusive involvement of HHV-6 variant B in symptomatic primary infection in children (Dewhurst *et al.*, 1993). At present there is no serological assay available for large scale screening that distinguishes between variant A and variant B. The development of such an assay will be of great value in future studies of the epidemiology of HHV-6.

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