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Human Herpesvirus Virus -6 (HHV-6) infection in Immunocompromised children

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The University of Edinburgh
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Human Herpesvirus Virus -6 (HHV-6) infection in Immunocompromised children

Abstract

At the time of commencing this study HHV-6 was a recently discovered lymphotropic herpesvirus, cultured from the lymphocytes of adult patients with malignancy and HIV infection and subsequently shown to be the cause of the childhood febrile illness exanthem subitum. Whether this virus had any pathogenic role in the symptomatology of immunosuppressed children had not been widely investigated.

The aim of this study was to examine certain aspects of the activity of HHV-6 in a cohort of immunosuppressed paediatric oncology patients. To ascertain whether children presenting with malignant disease had mounted a serological response to this virus, and if so to examine whether HHV-6 played a role in infectious problems occurring in these children when on treatment. A review of the literature was undertaken considering a number of areas relevant to the activity of HHV-6 in immunosuppressed children. Virological data on HHV-6 was examined, as well as the clinical manifestations of HHV-6 infection in the immunocompetent and immunocompromised. The methods used for clinical detection of HHV-6 were also examined and considered for development for the study. Immune deficits and the problems caused by other herpesviruses in paediatric oncology patients were also reviewed.

The current study was designed to include a retrospective serological assessment of HHV-6 antibody responses in samples taken from children at presentation with malignancy and sequentially during treatment. An indirect fluorescent antibody test (IFA) and an enzyme linked immunosorbent assay (ELISA) were used to detect HHV-6 IgG, and selected samples were tested for HHV-6 IgM by IFA. The paediatric patients, whether solid tumour or leukaemic patients, had a similar response to HHV-6 as age matched controls and almost all (97%) had IgG to the virus at the time of presentation. Acquisition of a response to more than one herpesvirus increased with age in the patients and where there was a response to only one virus it was usually HHV-6. Antibody responses to CMV and VZV were similar in patients and controls. Sequential samples from patients during immunosuppressive treatment showed a gradual decline over time in ELISA index for IgG to HHV-6 and no reactivations of HHV-6 were seen. IgM to HHV-6 was produced by one child with a primary CMV seroconversion but HHV-6 reactivation could not be confirmed as the possibility of cross reactive antibody was not excluded by a cross absorption experiment.

A prospective analysis of HHV-6 DNA in saliva and serum was undertaken on new patients presenting between August 1992- August 1993. HHV-6 is secreted in the saliva of those previously infected, and presence of HHV-6 in the serum implies an active viraemia. Saliva was collected prospectively from new patients, from siblings and from volunteer healthy controls. Serum was collected from patients during treatment. A nested PCR for HHV-6 was developed. The sequence of DNA chosen for amplification contained a cleavage site for the restriction endonuclease Hind III which enabled type "A " and "B" HHV-6 to be identified in

any PCR positive samples. HHV-6 DNA (type "B") was commonly found in the saliva of healthy controls (74%) and patients (58%), but patients who were febrile, unwell and neutropaenic less frequently secreted HHV-6 in the saliva supernatant (18%). Whether this was a local effect of chemotherapy on the salivary glands was considered. HHV-6 DNA was not detected in any serum samples, suggesting no evidence of active viraemia.
The potential role of HHV-6 as a serious pathogen in immunosuppressed patients remains to be fully elucidated, but in this limited study of a small paediatric cohort of oncology patients no evidence of deleterious virus activity was found.

I declare that the experimental work and composition of this thesis was undertaken entirely by myself.

Human Herpesvirus Virus -6 (HHV-6) infection in Immunocompromised children

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I developed an interest in viral infections in children as a result of caring for a little boy with HIV. The courage of this child and his loving family will be with me always. The inability to effectively treat his infection was a source of great personal sadness, but spurred me on to learn more. I would like to thank him for the chance to have been part of his life, for his humour and his encouragement. I would like to dedicate this work to him.

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Human Herpesvirus Virus -6 (HHV-6) infection in Immunocompromised children

Aims of this study

The aim of this study was to examine certain aspects of the activity of human herpesvirus virus -6 (HHV-6) in a cohort of immunosuppressed paediatric patients. To ascertain whether children presenting with malignant disease had mounted a serological response to this virus, and if so to examine whether HHV-6 plays a role in infectious problems suffered by children immunosuppressed on chemotherapy for treatment of malignant disease.

Questions:

1) Have children presenting with malignancy an immune response to HHV-6, and if so does it reflect the same pattern as that of normal children?

2) Is there evidence of HHV-6 primary infection / reinfection / or reactivation of infection in this group of children during the course of treatment and if so does this relate to any clinical problems?

Plan of investigation

A review of the literature concerning HHV-6 was undertaken to examine a number of areas relevant to the question of the activity of this virus in immunosuppressed children. The basic virological data on HHV-6 was examined, as well as the clinical manifestations of infection in the immunocompetent and immunocompromised. The methods developed for clinical detection of HHV-6 were also examined and considered for use / adaptation for this study. The immune deficits of paediatric oncology patients and the problems caused by herpesviruses in these patients were also considered.

With this background the current study was designed, to include a retrospective and prospective arm. A retrospective collection of sera obtained from children at presentation with malignant disease and sequential sera collected during treatment for malignancy were tested for an antibody response to HHV-6.

A prospective analysis of salivary HHV-6 DNA excretion and serum HHV-6 DNA was undertaken on new patients presenting during the period of the study (August 1992-August 1993) . HHV-6 is known to be excreted in the saliva of those previously infected, and presence of HHV-6 in the serum implies an active viraemia. Saliva samples were collected prospectively from patients presenting with malignancy, from siblings and from volunteer healthy controls. Serum was collected from patients during treatment. Evidence of secretion of the virus in these different sites was sought to assess its activity in these immunocompromised hosts.

Methods developed for this study

Serology

To evaluate serological responses to HHV-6 two systems were developed:
Indirect fluorescent antibody test (IFA): HHV-6, type A was cultured in a lymphoblastoid cell line and slides of infected and uninfected cells prepared for examination with sera under ultra violet illumination.
Enzyme linked immunosorbent assay (ELISA): Antigen from HHV-6 infected and uninfected lymphoblastoid cells was coated onto microtitre plates and binding antibody detected by a colorimetric reaction.
Where volume permitted, all sera were tested by the above methods for HHV-6 IgG, selected samples were tested for HHV-6 IgM by IFA.

DNA Amplification

A nested PCR for HHV-6 was developed for use on saliva and serum. The sequence of DNA chosen for amplification contained a cleavage site for a restriction endonuclease which enabled type A and B HHV-6 to be identified. Any samples which amplified HHV-6 were subsequently subjected to restriction enzyme digestion. Amplification products were stained with ethidium bromide, run on electrophoretic agarose gels and visualised under ultra violet light.

Viral culture and antigen preparation was undertaken with Dr M Ogilvie, at the Department of Medical Microbiology, Medical School, University of Edinburgh, and all other techniques with Dr HA Cubie at the Regional Virus Laboratory, City Hospital, Edinburgh.

The sixth human herpesvirus (HHV-6)

History

Human herpesvirus-6 (HHV-6), originally named human B lymphotrophic virus (HBLV), was discovered in 1986. It was isolated from the the peripheral blood mononuclear cells (PBMC) of patients from the USA and Africa, some of whom were infected with human immunodeficiency virus (HIV) and others undergoing treatment for lymphoid malignancy [Salahuddin 1986; Downing 1987; Tedder 1987]. This double stranded DNA virus (approximately 170,000 bases pairs in length) is the sixth member of the human herpesvirus family. HHV-6 has considerable DNA homology with human cytomegalovirus (CMV) and is the second member of the beta subgroup of human herpesviruses [Efstathiou 1988; Lawrence 1990; Neipel 1991]. HHV-6 preferentially infects human T helper (CD4+) lymphocytes [Becker 1988; Becker 1989].

Initially HHV-6 was described as a virus searching for a disease, until in 1988 it was identified as the causative agent of the childhood fever exanthem subitum (ES), also known as roseola infantum [Yamanishi 1988]. Since then the role of HHV-6 in many other conditions affecting immunocompetent and immunosuppressed individuals has been examined.

Many strains of this virus have now been isolated and on antigenic, genomic and cell culture characteristics these appear to fall into two main subgroups, Types A and B [Ablashi 1991]. The prototype virus isolated from immunocompromised patients was of type A and the great majority of strains isolated from children with exanthem subitum have been type B [Dewhurst 1992; Dewhurst 1993]. Differences in the pathogenicity of these stains have been explored. Since HHV-6 was first grown from the cells of immunocompromised patients the relationship between the virus and the immunocompromised host has been closely examined (see below).

Morphology

Morphologically herpesviruses cannot be distinguished. Ultrastructural studies of HHV-6 demonstrate an enveloped virion with an icosahedral capsid of 162 capsomers. The diameter of the enveloped particle is around 200nm. Within the capsid the viral DNA is coiled into a cylindrical mass. Virus particles mature within the nucleus of an infected cell and here acquire their capsids, the diameter of capsid plus core being around 100nm. Within the cell's cytoplasm the viral particles acquire a distinct tegument of moderate electron density, with an average thickness of 30 nm. Particles acquire their envelope prior to release by a process of exocytosis [Nii 1990 ; Roffman 1990b]. These early ultrastructural studies did not give any time scale to the assembly process of HHV-6 virions.

Culture

The first isolates of HHV-6 came from the peripheral blood mononuclear cells (PBMC's) of immunosuppressed patients, grown in co-culture with either cord blood lymphocytes (CBL's) or healthy donor PBMC's [Salahuddin 1986; Downing 1987 ; Tedder 1987; Becker 1988 ; Agut 1988]. The lymphocyte cultures were activated with phytohaemaglutinin (PHA) prior to addition of the infected patients' cells and after 3-4 days a cytopathic effect could be seen. The large, ballooned cells which appeared were mutinucleate, fragile and some contained inclusion bodies (figure 1a,b,c). Ultrastructural examination of these cells revealed evidence of a herpes virus which by serological and antigenic analysis was not a known member of the human herpesvirus family. Supernatant from some cultures could be used to infect further donor PBMC's or CBL's, but in other cultures this could only be achieved with cellular material, implying that some strains were more cell associated than others [Salahuddin 1986 ; Downing 1987; Tedder 1987; Becker 1988; Becker 1989]. Originally, it appeared that the cells infected with HHV-6 were of B lymphocyte lineage, but all subsequent analysis has shown HHV-6 to preferentially infect lymphocytes of T cell lineage [Ablashi 1988b; Becker 1988; Becker 1989]. Both immature and mature T cells can be infected [Lusso 1987; Lusso 1988]. GS strain, the first strain of HHV-6 type A isolated, grew well in immature T cells from PBMC's, CBL's, bone marrow, thymus and tonsil [Lusso 1988]. Z 29 strain, the first strain of HHV-6 type B isolated, grew well in cord blood T cells and less productively in adult PBMC's [Lopez 1988]. However certain CBL donor cells appeared more permissive to infection than others [Black 1989]. SF strain HHV-6 was isolated from the saliva of a person with HIV and although a B type and genetically most close to Z 29, it grew better in adult PBMC's then CBL's [Levy 1990b]. The CD4 molecule is not the site of attatchment of HHV-6 to the lymphocyte [Lusso 1989b].

Optimisation of culture conditions for HHV-6 is again to some extent strain specific, but it has been demonstrated that HHV-6 replication is encouraged where donor cells are stimulated with PHA and low concentrations of interleukin -2 (IL-2) [Black 1989; Frenkel 1990b; Frenkel 1990c].High concentrations of IL-2 are inhibitory to HHV-6 replication [Roffman 1990]. OK strain HHV-6 was isolated from PBMC's of a patient with exanthem subitum. Polyclonal stimulation of T cells by anti CD-3 monoclonal antibody was found to make PBMC's permissive to infection with OK (B type) strain HHV-6 where PHA stimulation did not. Both anti CD-3 and PHA enhanced infection of CBL's with OK strain by 5-10 and 5-6 fold respectively [Kikuta 1989b]. Centrifugal inoculation of cultures can also enhance infection of PBMC's by up to 100 fold [Pietroboni 1989].

To facilitate culture of HHV-6 in vitro, infection of various transformed and continuous cell lines has been undertaken (table 1a & b). The most productive infections occur in T cell lymphoblastoid lines, and this is strain specific. GS strain HHV-6, grows best in HSB-2 cells, where by 9 days in culture 90% of cells will be infected [Ablashi 1987]. Z 29 strain HHV-6, grows best in MT- 4 cells, where 25% of cells will be infected by 7 days [Black 1989]. Strain specific, low level infection of other tissue cell lines can be achieved, in particular:

neuroblastoma; megakaryocyte; lung epithelium; and bowel (table 1a & b). Similarly with CMV, fibroblasts on coverslip cultures can also be infected by HHV-6 and early viral antigen produced can be detected using monoclonal antibodies [Luka 1990]. Although HHV-6 was originally thought to be a B cell tropic virus only low level infection of B cell lines occurs. Where B cells also carry EBV genome they are more permissive to infection with HHV-6, in some way the presence of EBV may increase receptors for HHV-6. In one study it was shown that infection of EBV-positive cell lines occurs with A type HHV-6 but not B type [Cuomo 1995]. Infection of these EBV-positive cell lines was associated with activation of certain EBV promoter genes indicating possible further interactions between these two herpesviruses.

Legend to figure1a,b,c: Cytospin preparations of HHV-6 infected and uninfected cells, all at x 500 magnification.

1a Cytospin preparation of uninfected lymphoblastoid cells, JJhan strain. Note large nuclei with multiple nucleoli. Geimsa stained.

1b Cytospin preparation of HHV-6 infected lymphoblastoid cells, JJhan strain. Note large ballooned cells,intranuclear and intra-cytoplasmic inclusions and multinucleate syncitia. Geimsa stained.

1c Cytospin preparation of HHV-6 infected lymphoblastoid cells stained with a fluorescent conjugated monoclonal antibody (HA5) to HHV-6 (see below). Note large ballooned cells and multinucleate syncitia with intranuclear and intracytoplasmic fluorescence due to expression HHV-6 antigen.

figure1 a: Cytospin of uninfected lymphoblastoid cells.

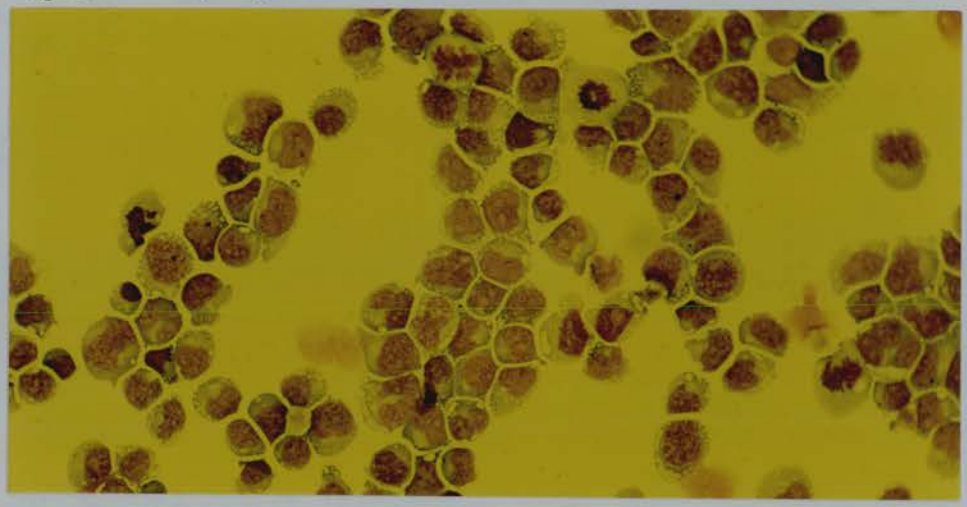


figure 1 b: Cytospin of HHV-6 infected lymphoblastoid cells.

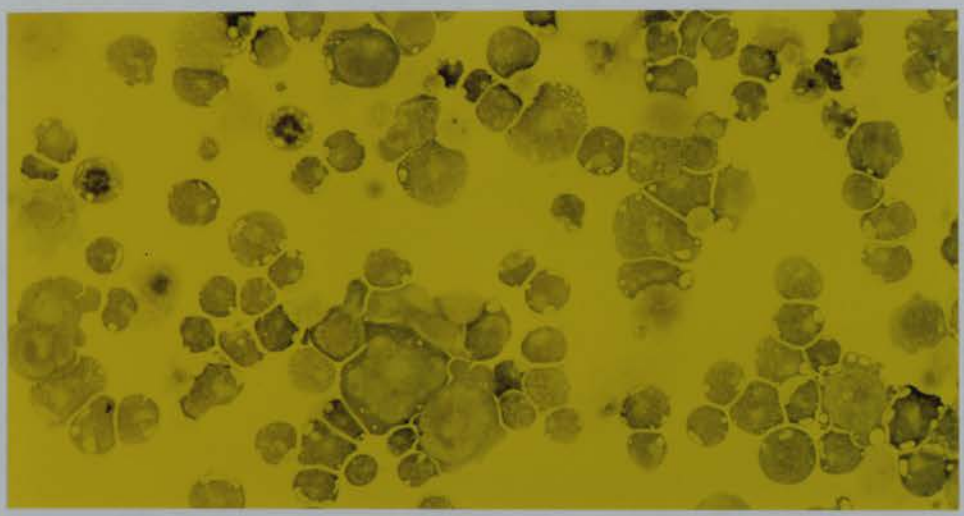
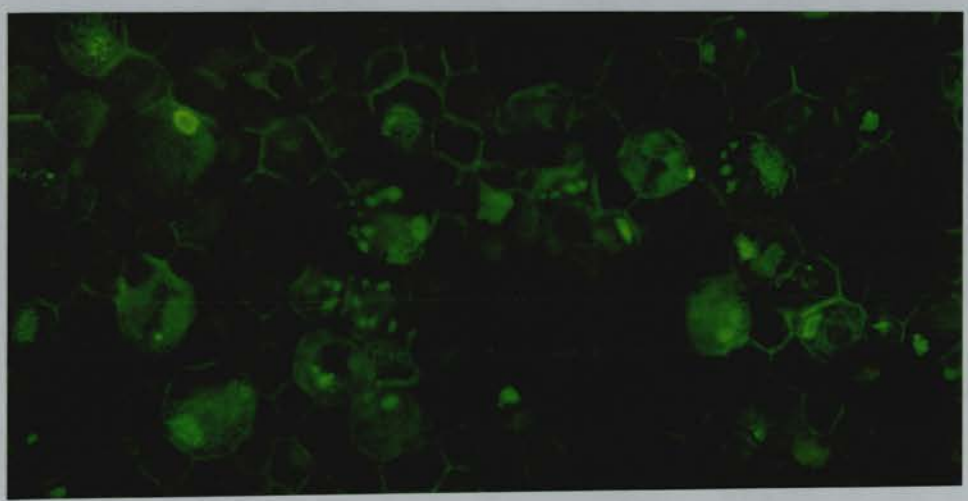


figure 1 c: Cytospin of HHV-6 infected lymphoblastoid cells stained with a fluorescent conjugated monoclonal antibody to HHV-6.



Strain of HHV-6 (type A/B)	Country of origin	Patient source (cells)	Prefered human cell for culture	Prefered cell line	Prefered cell line markers	Other infectable cell lines	Non-infectable cell lines	Authors (year)
GS (A)	USA	AIDS, lymphoproliferative disorders. (PBL)	immature T cells (CD4, CD2, CD5, CD7) (CBL)	HSB-2	CD7, CD38, CD5, CD15, Transferrin receptor.	Lymphoblastoid: T cell (Clone JM2.7 of Jurkat; Clone HUT 78; H9; CCRF-CEM; MOLT-3,); B cell (Clone ET-62 of WIL-2; IM9; BJAB; Ramos). Megakaryocyte (HEL) Glioblastoma (HTB-14).	Fibroblast Epithelial nonhuman primate other animals	Ablashi (1987, 1988) Salahuddin (1986) Lusso (1987)
Z-29 (B)	Zaire	AIDS (PBL)	T cells (CBL)	MT-4	CD4	T cell (Molt-3,Molt-4, HT, HUT 78) Mink lung epithelium (NBL-7)	HEL (embryonic lung) SV40 human kidney (324K) Monocyte (U937) B cell (MRL-13) Neural cells	Lopez (1988) Black (1989) Simmons (1992)
HTLHV (?)	South Africa	AIDS, lymphoproliferative disorders (PBL)	T cells (CD4 (CBL)	T 191	CD4		T-448 (CD8 cell line) B-cell lines	Becker (1988)

Table 1 a:Strains of HHV-6, countries of origin & cells for in vitro culture.

Strain of HHV-6 (type A/B)	Country of origin	Patient source (cells)	Prefered human cell for culture	Prefered cell line	Prefered cell line markers	Other infectable cell lines	Non-infectable cell lines	Authors (year)
AJ (A)	Gambia	AIDS (PBL)	T cells (CBL)	J Jhan cells	T cell	T cell (8166) Myeloma (HFB-1) B cell (EBV, WMPT) Pre-B cell (L4) HEL (embryonic lung) HEB (embryonic glia)	Monocyte (U937, HL60) Erythroleukaemic (K562)	Tedder (1987)
U1102 (A)	Uganda	AIDS (PBL)	T cells (CBL)	J Jhan cells	T cell	T cell (HSB-2, CEM, H9, Jurkat) B cell (Raji, Ramos) Monocyte / macrophage (U937, HL60)		Downing (1987)
SF (B)	USA	AIDS (Saliva)	T cells (PBL)	MT-4	CD4	neuroblastoma bowel transformed cells chimpanzee PBL	B cell lines Monocyte (U937) Fibroblast Melanoma Adrenal Animal Kidney	Levy (1990)
FG-1 (B)	Japan	Exanthem Subitum (PBL)	T cells (CBL)	MT-4	T cell	MO$_2$(U937); B cell (Ragi; LCL-TAS Ramos)		Suga (1989)
HST (B)	Japan	Exanthem Subitum (PBL)	T cells (matureCD4+) (CBL)	MT-4	Tcell	HPB-ALL		Yamanishi (1988)

PBL - peripheral blood lymphocyte, CBL - cord blood lymphocyte

Table 1b:Strains of HHV-6, countries of origin & cells for in vitro culture.

HHV-6 effects on infected cells

Lymphocyte surface markers

Blood samples from infants with exanthem subitum have shown that between $1/10^3$ - $1/10^6$ PBMC's are infected with HHV-6 during primary infection [Takahashi 1989]. Using monoclonal antibodies to lymphocyte surface markers, flow cytometry and cell sorting techniques, HHV-6 infected cells have been identified as mature T cells with helper cell surface markers (CD4+ CD8-, CD3+ CD4+). No HHV-6 was isolated from cells with non T cell, immature T cell or cytotoxic cell markers (CD3-, CD4- CD8-, CD4- CD8+). When the same strain of HHV-6 (HST, B type) was used to infect CBL's in culture the virus was predominantly isolated from cells with similar phenotypes (CD4+ CD8-, CD3+ CD4+, CD4+ CD8+), rarely it was also cultured from CD4- CD8+ cells. Thus it appears in vivo and in vitro that the predominant tropism of HHV-6 is for CD4+, mature T lymphocytes.

This finding was different from that originally reported by Lusso et al who were using the GS strain (A type) of HHV-6 on cells in vitro. They found that HHV-6 infected cells lacked the surface membrane CD3-T cell receptor (TCR) complex and IL-2 receptor and partially co-expressed CD4 and CD8, demonstrating an immature rather than mature T cell phenotype [Lusso 1987; Lusso 1988]. These apparent differences in tropism could be related to the different strains of HHV-6, different conditions of the cultures or other factors. However, in later work Lusso also confirmed that HHV-6 infects T cell of mature phenotype (CD3+ CD4+ CD8-, CD3+ CD4-CD8+) [Lusso 1991a]. Down regulation of CD3-TCR in cells in culture was further examined and shown to be due to virus specific down regulation of transcription of host proteins. Removal of CD3 prevents the T cell from responding to antigens presented and therefore HHV-6 infection may lead to functionally inactive T cells. This effect would obviously be more important in persistent than lytic infection and needs further investigation. It also appears to be specific to HHV-6 in the herpesvirus family [Lusso 1991a].

Infection with HHV-6 has also been shown to up regulate expression of CD4 on the cell surface [Lusso 1991a; Lusso 1991b]. This may have important consequences for the susceptibility of cells to HIV infection. Although HHV-6 preferentially infects CD4+ cells, this is not a receptor for HHV-6 as it is for HIV. Blocking of CD4 by monoclonal antibody prevents infection of cells with HIV but not HHV-6, and the T cell line, CEM, which is chronically infected with HIV with down regulated surface expression of CD4 is still susceptible to infection with HHV-6 [Lusso 1989a].

The possibility that different host cellular changes might be related to the infecting strain of HHV-6 has been examined [Furukawa 1994]. When strains U1102 (A type) and Z 29 (B type) were added to CD4+ T cells in culture, CD3 expression was markedly decreased with A type infection and only slightly decreased with B type. Cytotoxic activity of virus specific CD4+ T cell clones was decreased only after A type infection and could not be restored in the

presence of lectins, implying that HHV-6 infection may have affected production of cytolytic mediators. These in vitro differences in strain infections imply that HHV-6 type A infections may be more immunosuppressive than type B infections and this seems to correlate with the in vivo finding of type A infections occurring more frequently in immunosuppressed patients.

Cytokines

Interferons are natural proteins with anti-viral and anti-neoplastic activities. They are considered to be important for the control and limitation of viral infections. HHV-6 infection of PBMC's from seropositive adults is associated with production of interferon-α (IFN-α) [Kikuta 1990]. Production of this cytokine in vitro can be detected as soon as 12 hours after infection, rises to a peak at 3-5 days and then declines. IFN-α will also be produced when cells are exposed to UV treated HHV-6, so the effect does not depend on infection of the T cells, and addition of exogenous IFN-α will prevent HHV-6 replication. The IFN-α response of infected CBL's was lower than that of the healthy seropositive adults and this may represent some kind of memory in the previously infected. IFN-α was not produced by the HHV-6 infected cells but by other non T cell PBMC's in the culture [Kikuta 1990].
In vitro cultures of HHV-6 have also been shown to produce the monocyte-derived cytokines, interleukin -1 (IL-1) and tumour necrosis factor (TNF-α) [Flammand 1991]. Production of these cytokines depends on interaction of a viral protein with the infected cell, but does not require viral replication. The IL-1, TNF-α cytokine response to HHV-6 infection in vitro was confirmed in another study where the responses to infection with HHV-6, EBV and HSV were compared [Gosselin 1992]. HSV is only a weak enhancer of cytokines and EBV infection increased IL-6 levels where these are not affected by HHV-6. In fact the inhibitory effects of HHV-6 on IL-6 and EBV on TNF-α were dominant to the stimulatory effects. This observation that herpesviruses can selectively affect the regulation of cytokine synthesis is one method by which they may evade or exploit the host immune response. The effect of interferon - γ (INF - γ) on monocytes infected with HHV-6 has also been examined, this cytokine appears to regulate production of interleukin -10 (IL -10) and interleukin -12 (IL-12) by these cells [Li 1997]. IL-12 concentrations are increased and IL -10 concentrations are suppressed. In another in vitro study, INF - γ was found have an effect on secretion of TNF-α, IL-1, and IL-6 by HHV-6 infected monocytes and PBMC's [Arena 1996].
The genes for three interesting HHV-6 glycoproteins have been identified, one which encodes a basic chemokine with a dicysteine "CC" motif (CC-28X-C-15X-C like the chemokines CC-23X-C-13X-C) and another two which encode proteins closely related to chemokine receptors [Gomples 1995b]. How these might interact with the host has not yet been identified. Receptors for some such chemokines are known to serve as co-receptors for HIV infection of CD4+ cells with NSI type HIV strains [Dragic 1996; Wu 1997]. Individuals who are deficient in these receptor molecules are resistant to HIV infection with these strains [Paxton 1996].

HHV-6 inhibition of host immune cell function

In vitro, when the sonicated product of HHV-6 infected cells was added to cultures of PBMC's from HHV-6 seropositive healthy adults there was inhibition of lymphoproliferative responses to mitogens and antigens (PHA, Il-2, Purified Protein Derivative, Mumps) [Hovart 1993]. This inhibition occurred in a dose dependent manner, was not due to cell lysis as cell death was not observed and could be inhibited by addition of rabbit anti-HHV-6 serum. Inhibition of lymphoproliferation was most likely caused by either viral products or viral induced host products rather than a nonspecific effect, but the mechanism by which this occurs is not known. In another study infection of PBMC's with HHV-6 also resulted in suppression of T cell function [Flamand 1995]. There was reduced synthesis of IL-2 and reduced cellular proliferation in the cultures. This inhibitory effect could be achieved with non-infectious UV treated virus. This finding is of interest, as it has been shown that only low levels of IL-2 permit in vitro culture of HHV-6, and higher levels inhibit viral replication [Roffman 1990a]. It appears as if the virus may be modulating the host immune response to allow self replication, but this may also have an inhibitory effect on host immune activity. Infection of CD4+ T cells in culture with both sub types of HHV-6 has been shown to produced apoptosis or programmed cell death [Inoue 1997]. This effect appears to be produced in cells which are themselves uninfected with HHV-6 and is augmented by TNF - α.

Cells rarely infected with HHV-6

Although HHV-6 infection of CD8+ T cells appears to be rare in vivo and in vitro, with high concentrations of HHV-6 it is possible to productively infect CD 8+ cells from PBMC's and transformed T cell lines [Lussob 1991]. Infection of these cells leads to up regulation of CD4 and down regulation of CD3. It is speculated that this could subsequently render them susceptible to HIV infection. Monocytes have also been shown to be rarely infected with HHV-6 in vivo and in vitro [Levy 1990b; Kondo 1991]. It has been suggested that HHV-6 may be found in a latent state in circulating monocytes [Kondo 1991; Luppi 1993b]. In an innovative study of HHV-6 infected monocytes the respiratory burst capacity of the cells was greatly decreased, and this could be of particular importance in persistently infected patients [Burd 1993b]. Natural killer (NK) cells are a subset of non- T non -B lymphocytes with an important primary defence activity against virally infected and malignant cells, and patients deficient in NK cells can suffer severe herpesvirus infections [Biron 1989]. NK cells can be infected with HHV-6, at high concentrations, but only after long term culture in vitro when the cells have been shown to loose their natural cytolytic activity against the virus. If such infection is possible in vivo then this might be a mechanism where HHV-6 could avoid the host immune system. As a result of HHV-6 infection there is de novo expression of CD4 on the surface of the NK cells before cytolysis. Again it can be speculated that this could render them susceptible to HIV infection [Lusso 1993].

It has now been shown that HHV-6 infection of PBMC's leads to up-regulation of NK cell

cytotoxicity. This is mediated by a newly discovered cytokine IL-15 which acts on NK cells and monocytes, and addition of this cytokine to PBMC cultures of HHV-6 severely diminishes expression of the virus [Flamand 1996b]. The exact mechanism whereby NK cells recognise virus infected cells is not completely understood but in vitro without pre-activation a proportion of NK clones will lyse autologous cells infected with HHV-6 [Malnati 1993]. The restriction of NK cell lysis to certain clones appears to depend not only on the NK cells but also on elements of the target cells. The role of possible viral evasion here has still to be elucidated.

γ/δ T cells are a subset of T cells with activity against certain intracellular infections. Cytolytic activity of γ/δ T cells to autologous and heterologous HHV-6 infected cells can be demonstrated. In vitro γ/δ T cells have also been infected with HHV-6 when present in high concentrations, and as for NK cells this induces expression of CD4 at the cell surface [Lusso 1995b]. Whether these cells can be infected with HHV-6 in vivo is not known, nor whether such infection is sufficient to impare host immunity.

Genome of HHV-6

Cross hybridisation of the original probes developed from the prototype GS strain of HHV-6 did not not occur with any of the other five herpesviruses known at that time, which was important evidence that HHV-6 was a new herpesvirus [Josephs 1986; Josephs 1988b; Lopez 1988]. Subsequently, some cross hybridisation with the genome of the more recently discovered HHV-7 has been shown [Frenkel 1990]. Although this did not occur in all fragments examined, it does demonstrate the close genetic relationship of HHV-6 and HHV-7. The complete genomic linkage map for the A type strain of HHV-6, has been derived [Martin 1991b; Gomples 1995b; Nicholas 1996]. The genome is 159,321 base pairs (bp) in length, with a unique long central portion (UL) (143,147 bp) bound at each end by direct repeats (DR Right & DR Left) (8000 bp, approx). The overall base composition of the genome is 43% guanosine & cytosine (G+C), with 41% in the unique region and 58% in the terminal repeats. This simple herpesvirus genome pattern is similar to that of the channel catfish virus and equine CMV, where other human herpesviruses have more complex reiterations and inversions of their genomes [Thomson 1991b]. The genome of over 95% of a B subtype strain of HHV-6 (Z29) has also been mapped and it is closely colinnear with the previously mapped A subtype [Lindquester 1996].

Direct Terminal Repeats

Different overall estimates of the length of HHV-6 relate mostly to variation in the length of the

terminal repeats, with relative stability of the length of the unique central region [Lindquister 1991; Neipel 1991]. The terminal repeats contain simple repeats, unique sequences and regions involved in DNA packaging [Thomson 1994a]. Bounding each direct terminal repeat are arrays of simple repeats similar to human chromosomal telomeric sequences [Martin 1991b; Thomson 1994a; Gomples 1995a]. The function of these telomeric repeats is not certain but it has been suggested that they could in some way protect the viral genome from exonuleases or ligases and that host telomerase could assist in reassembly of viral genome. They might also have some role in maintaining the viral chromosomal structure as they do in host chromosomes, or they may have a role in cleavage and packaging of replicating viral genome [Thomson 1994a; Gomples 1995b]. The replication of HHV-6 occurs by a rolling circle mechanism, where head to tail concatemers are produced and cleaved to unit length genomes as they are packaged into the protein capsid [Martin 1991b].
There are also three short areas of repeats towards the right hand end of the unique long region of the genome, but delineation of these is not precise as they are degenerate [Martin 1991a; Martin 1991b; Thomson 1992; Nicholas 1994a].

Unique Long Region

Although CMV has a larger genome than HHV-6 (230Kbp, compared to 160Kbp), both are densely packed genomes with minimal splicing and the central unique portion of the HHV-6 genome is colinear with that of CMV [Efstathiou 1988; Lawrence 1990]. The degree of relatedness between HHV-6 and CMV is similar to that between VZV and HSV-1, but the CMV genome is considerably longer than that of HHV-6 and has more genes. There is divergence of the genome colinearity with CMV at either end of the HHV-6 genome. Although HHV-6 and CMV share little nucleotide sequence similarity they have a similar gene organisation of "conserved genes" which encode homologous protein products [Littler 1990; Neipel 1991; Teo 1991; Martin 1991a; Gomples 1992; Chou 1992; Efstathiou 1992; Thomson 1992; Nicholas 1994a; Nicholas 1994b]. The homology of HHV-6 proteins with CMV is 67% compared to 21% for the herpesvirus group as a whole [Gomples 1995b].
The genome of HHV-6 contains 119 open reading frames, 8 of which are duplicated in the repeats, 6 span repetitive elements and three are spliced, therefore the genome can encode a possible 102 proteins. The list of all these genes, their known encoded proteins and CMV homologues has been defined [Gomples 1995b]. There is a common core of genes conserved in all herpes viruses essential for viral replication, for HHV-6 this core extends for 86Kbp's in the centre of the unique long (UL) region, and it is the most compact of all the herpesviruses examined [Gomples 1995b]. This core group of genes consists of seven gene blocks (I - VII), and in the β herpesviruses CMV and HHV-6 these are arranged in ascending numerical order. The gene group order for the γ and α herpesvirus is different but group specific. The HHV-6 genome contains at least one origin for DNA replication within the UL region between gene blocks II and III. Interestingly this important area of the genome shows more sequence similarity with that of the α herpesvirus HSV-1 than with that

of CMV [Lawrence 1995]. It has been suggested that HHV-6 may be nearer to a progenitor human herpesvirus, in view of its compact core and more simple origin for DNA replication and that diversion from this pattern has led to evolution of the different subgroups of human herpesvirus [Karlin 1994].

Gene families appear to be a feature of the β herpesviruses and are extensive in CMV, they also exist to a lesser extent in the genome of HHV-6. The largest gene family is the CMV US22 collection of genes which is involved with transformation and transactivation of the genome and has a corresponding family in HHV-6 [Efstathiou 1992; Thomson 1992; Nicholas 1994a; Nicholas 1994b] . Other families include the G-protein coupled receptor and immunoglobulin gene families. These families may have come about by duplications and alterations of tandem genes. There are also HHV-6 genes which do not have detectable herpesvirus homologues and most of these are at the ends of the viral genome. These include genes in the direct terminal repeats (right & left), certain glycoprotein genes, the parvovirus rep homologue [Thomson 1994b] , some of the immediate early proteins and others [Gomples 1995b].

Genes for immediate early proteins & transactivation of HHV-6

Immediate early (IE) proteins control the cascade of gene expression of the viral genome and thus are important in lytic and latent infection. They are the first genes to be transcribed and are essential for the coordinated expression of delayed early and late herpesvirus genes. IE proteins can transactivate a variety of host and viral promoters. Several HHV-6 IE genes have been described which can act as transcriptional activators in vitro (see below, HHV-6 and HIV) [Martin 1991a; Geng 1992; Nicholas 1994a; Thomson 1994b; Thomson 1992; Zhou 1994]. Transcription of at least one gene can occur without production of a corresponding protein [Schiewe 1994]. As regulation of IE gene activation controls viral expression in cells and is therefore crucial to the outcome of infection, understanding the control of activation of IE genes is important. The area of genome to the right of the IE genes contains a complex set of tandem repeats each containing binding sites for cellular transcription factors such as NF-κB and AP2. This IE gene enhancer region is similar to that found in CMV and activation of this region will set in train viral replication [Thomson 1992]. Although major HHV-6 IE genes are homologous and colinear with CMV IE genes, within these groups of genes there are unique genes which occur only in HHV-6 or CMV [Nicholas 1994a; Nicholas 1994b; Gomples 1995b]. These unique genes must contribute to specific biological characteristics of each virus, and may be important elements in the maintenance or interruption of latency in for each virus.

Proteins of HHV-6

Glycoproteins of HHV-6

All human herpesviruses express glycoproteins on their envelope surfaces and on the surfaces of infected cells. Certain of the glycoproteins have homologues throughout the herpesvirus family and others are specific to a sub family or to the individual viruses. Herpesvirus glycoproteins are an important expression of viral pathogenicity. They are required for attachment of virus to cells and thus influence the cell tropism of the virus. They mediate entry of virus to cells by fusion and also spread of virus from cell to cell. In view of their surface expression they are also targets for the host immune response. The glycoproteins gB and gH are conserved throughout the human herpesviruses and neutralising antibody is often produced against them [Manservigi 1991].

The gH glycoprotein is a highly glycosilated protein important in viral-cell fusion, the gene for HHV-6 gH has been identified and lies within the area of highly conserved genes for all herpesviruses [Josephs 1991a]. The organisation of the conserved genes in this area is identical for HHV-6 and CMV, to which the HHV-6 gene is most closely related. In this area the thymidine kinase gene is present in HSV, VZV and EBV, but absent in HHV-6 and CMV [Gomples 1992]. Where gH of a type A and type B strain were examined the overall nucleotide difference was only 0.5%. Expression of HHV-6 type A gH gene in a bacterial fusion protein system demonstrated this to be an immunogenic protein and rabbit antisera produced against the protein could block the infectivity of both types of HHV-6 [Foa-Tomasi 1991; Qian 1993].

In herpesviruses it has been shown that gH combines with another glycoprotein gL to form a stable heterodimer, and this is also the case for HHV-6 [Liu 1993a]. Again the gL of HHV-6 is most closely related to that of CMV. Expression of the HHV-6 gH-gL heterodimer in T lymphoblasts and fibroblasts demonstrated this to be an immunogenic protein which was recognised by HHV-6 immune, CMV negative sera. Monoclonal antibodies have been generated to gH, gL and the gH-gL heterodimer [Liu 1993b]. The monoclonal antibodies to gH, and the gH-gL heterodimer neutralised viral infectivity but that to gL alone did not. The N -terminus of gH has a 230 amino acid domain where interaction with gL occurs, this domain has residues specific for the β herpesviruses and the CMV gH and gL molecules can functionally substitute for the HHV-6 molecules in this complex. Without gL the complex is not expressed at the cell surface. The C- terminal cysteine domain is conserved in all the herpesviruses and is probably important for cell fusion and infectivity [Anderson 1996].

Glycoprotein gB is essential for infectivity and important in attachment and penetration of the host cell membrane and is a prime target for neutralising antibodies, it is the major envelope component with homologues in all known herpesviruses. The HHV-6 gene for gB has been identified and the protein is again most closely related to that of CMV, there is also minimal difference between the A type and B type HHV-6 gB [Chou 1992; Ellinger 1993]. The

degree of similarity between HHV-6 and CMV gB is greatest in the N-terminal portion. This area contains confirmation epitopes recognised by CMV neutralising antibodies from immune human sera and cross reactivity with HHV-6 could well occur (see below).Glycoproteins B and HL are not detected on the plasma membranes of infected cells [Cirone 1994].

The U100 HHV-6 gene encodes part of a glycoprotein complex, gp82-gp105, which is unique to HHV-6. This glycoprotein complex is made up from a multispliced mRNA and the encoding genes can be found at the right end of the unique long region, and in the left and right direct repeats. This is the first example of a human herpesvirus envelope glycoprotein encoded in this way [Pfeiffer 1995; Pfeiffer 1993]. Monoclonal antibodies to this complex neutralise HHV-6 infectivity and the neutralising epitope has been identified. An HHV-6 glycoprotein, gM, has also been identified, this glycoprotein is also conserved within the herpesvirus family but its function remains to be determined. The HHV-6 gM has a high degree of homology with that of CMV [Lawrence 1995].

There are several other less well defined HHV-6 glycoproteins, some which have CMV homologues and some which are unique to HHV-6 [Gomples 1995b]. Some of these have similarities to cellular counterparts which are members of the Immunoglobulin super gene family. Some are similar to G-protein-coupled receptors and only found in the β and γ herpesviruses.

Immediate Early Proteins

Immediate early proteins are expressed by host cell transcription and translation systems without prior viral protein synthesis (see above). They are very important and act as controllers of the "Temporal Cascade" of viral reproduction. It has been demonstrated that within three hours after infection of host cells with HHV-6 nuclear antigens are expressed which are most probably immediate early proteins [Eizuru 1992]. These proteins only appear to elicit a weak host antibody response in certain cases where there is a generally high titre antibody response to HHV-6. The exact function of these proteins has not yet been demonstrated. When considered from genomic examination there is, not surprisingly, homogeneity of HHV-6 genes to those of CMV known to encode immediate early proteins [Gomples 1995b]. The location of these immediate early genes, IE - A and IE - B, lie in two areas on the unique long region of the genome, one at each end of the block of herpes virus conserved genes. Several HHV-6 genes have been described which can act as transcriptional activators and thus turn on the cascade of host protein synthesis under viral control [Martin 1991a; Geng 1992; Nicholas 1994a; Thomson 1994b; Zhou 1994].

DNA Replication Proteins

A series of proteins are required by herpesviruses for viral DNA replication and the majority of these are produced by HHV-6. The first such HHV-6 proteins identified were the DNA

polymerase and DNAase [Bapat 1989; Williams 1989]. The DNA polymerase maps to the HHV-6 genome within block II of the Herpesvirus conserved gene blocks. The HHV-6 DNA polymerase is the smallest of the herpesvirus DNA polymerases and although colinear with CMV it has closer amino acid identity to EBV [Teo 1991; Tsai 1990]. The following HHV-6 DNA replication proteins also have CMV homologues: DNA polymerase processivity factor; single stranded DNA binding protein; and the helicase / primase complex. The HHV-6 origin binding protein does not have a CMV homologue [Gomples 1995b]. HHV-6 produces a homologue to the parvovirus rep protein, a multifunctional protein with a role in priming DNA replication [Thomson 1991a; Agulnick 1994]]. Herpesviruses also produce proteins for DNA repair and nucleotide metabolism, importantly neither CMV nor HHV-6 produce a thymidine kinase which renders them relatively resistant to aciclovir [Williams 1989; Gomples 1995b]. There are also a number of proteins which play a role in the assembly and packaging of newly formed viral DNA and again the HHV-6 proteins have close CMV homologues [Gomples 1995b; Tigue 1996].

Virion Structural Proteins

There are herpesvirus conserved genes for structural proteins of the tegument and capsid and these are also found in HHV-6 [Shiraki 1989 ; Yamamoto 1990]. The HHV-6 major capsid protein is most closely related to that of CMV [Littler, 1990 #199]. The HHV-6 tegument proteins also have significant sequence similarity with this other beta herpesvirus [Josephs 1991a; Neipel 1992].

Antigenic Proteins

The nature and host significance of viral proteins can also be examined by looking at serological responses to these proteins in the natural host and other animals [Josephs 1988b; Saxinger 1988; Balachandran 1989b; Yoshida 1989b; Yamamoto 1990; Balachandran 1991; Chang 1991]. In this way proteins can be identified which are antigenic to the host and might be important in a protective response against the virus. Usually, for such experiments virus is cultured in cells and then the lysate examined for a response after electrophoresis with reactive serum. Not all immunoreactive proteins are necessarily virion derived, some originate from virus directed alterations of cellular metabolism [Black 1991]. Human sera have been shown to immunoprecipitate with both polypeptides and glycoproteins of HHV-6, however the viral glycoproteins have usually been most immunoreactive [Balachandran 1989b; Yamamoto 1990; Okuno 1990b; Balachandran 1991; Okuno 1992]. The glycoprotein complex of HHV-6 gH and gL appears to be a major immunogen for reactive human sera [Liu 1993a]. Monoclonal antibodies to this glycoprotein neutralise HHV-6 and prevent virus induced cell fusion [Liu 1993b]. Human sera containing antibodies to HHV-6 also react strongly with a protein p101 / p100, the homologue of CMV pp150 [Yamamoto 1990; Neipel 1992; Pellet 1993]. In contrast to HSV-1 and similar to CMV the HHV-6 major capsid protein appears only weakly immunoreactive with human sera

[Balachandran 1989b; Yamamoto 1990].

Monoclonals antibodies to early and late HHV-6 proteins have been developed and these have been used to discriminate between ongoing virus activity with continued antibody production to early antigens compared to past infection with development of antibody responses to late antigens, a pattern similar to that seen with EBV [Iyengar 1991].

Strain variation

Two subtypes of HHV-6,"A" and "B", have been described [Ablashi 1993]. Although closely related, they can be distinguished at all levels from the genomic to the serological response. The overall level of genomic similarity between subtypes at most loci examined has been around 94-96%, whereas within subtypes it is 97-100%. At the amino acid level the degree of variability between subtypes is slightly more, 92-96%. [Aubin 1993 ; Gomples 1993]. There is closer subtype similarity in the centre of the genome and more divergence at the ends [Gomples 1995b]. The subtypes of HHV-6 are more closely related than the subtypes of EBV or CMV. Restriction enzyme "finger-printing" of total viral DNA from different isolates has demonstrated polymorphisms which can discriminate between the two subtypes [Kikuta 1989a; Pellet 1990; Ablashi 1991; Aubin 1991; Schirmer 1991].

Amplification of the DNA sequence of the large tegument protein produces a 830 base pair (bp) amplimer for both subtypes of HHV-6 [Aubin 1991; Aubin 1992a]. Digestion of this amplimer with the restriction-endonuclease (RE) Hind III, will produce two fragments for type B strains, but does not digest type A strains. A similar discrimination of the two subtypes can be shown by Hae II digestion of a 380bp amplimer from the major capsid protein [Aubin 1993]. These RE digestion sites have been exploited to differentiate between the subtypes of HHV-6 in clinical samples [Aubin 1994] (see methodology below).Cell culture tropism can also be found between the two subtypes, for example, A type strains grow better in the T-cell line HSB-2 and B type strains grow better in Molt-3 [Wyatt 1990; Ablashi 1991] (table 1).

Panels of monoclonal antibodies raised to the two prototype strains of HHV-6 (GS, type "A" and Z29, type "B") have reproducibly demonstrated that some antibodies react with both strains and that others are strain specific [Wyatt 1990; Ablashi 1991; Chandran 1992; Aubin 1993; Campadelli-fiume 1993; Takeda 1996; Takeda 1997]. An HHV-6 subtype A specific neutralising epitope on glycoprotein B has been identified with monoclonal antibody 87-y-13 [Takeda 1996]. An HHV-6 subtype B specific neutralising epitope on glycoprotein H has been identified with monoclonal antibody OHV3 [Takeda 1997].

Reaction of human sera to different strains has demonstrated that most show a similar titre of antibody to both subtypes, but a minority can show a 2-4 fold difference in titre. This would imply that it is possible to have serological response to both strains [Chandran 1992].

On a clinical level, the overwhelming majority of isolates from children with ES have been B type viruses [Schirmer 1991; Aubin 1991; Ablashi 1991; Dewhurst 1992; Diluca 1992; Aubin 1993]. Immunocompromised patients and those with malignancy have been found to have

both subtypes and sometimes both together [Ablashi 1991; Aubin 1993; Gomples 1993; Luppi 1993a]. The clinical significance of infection with either subtype is not yet elucidated.

Latency and dissemination of HHV-6

In common with all the herpesviruses after primary infection HHV-6 persists in the host in a latent state. Latency allows competent viral genome to remain within a particular cell type, in a nonreplicative state, without generation of antigenic proteins. Recognised RNA transcripts have been found in cells latently infected with herpesviruses and these may play a role in preventing initiation of lytic cycles of replication [Stevens 1989]. At certain stimuli, viral replication will be reinitiated and reactivation of infection can occur. Reactivation from latency may be at an asymptomatic level and fail to progress in the presence of an active immune response. In the immunocompromised host by contrast, reactivation from latency can lead to severe symptomatic infections with a number of the herpesviruses (e.g. varicella zoster, and Epstein Barr virus).

HHV-6, known to be a lymphotrophic virus in primary infection, appears to establish latent infection in a proportion of circulating mononuclear cells. HHV-6 DNA can be found in the blood lymphocytes and monocytes of healthy adults [Kondo 1991; Sandhoff 1991; Cone 1993a; Luppi 1993a; Aubin 1994; Cuende 1994; Di Luca 1994]. The proportion of persons with detectable HHV-6 genome and the proportion of infected cells has varied in different studies, perhaps relating to use of different amplification techniques. HHV-6 genome has been detected in PBMC's from 5% - 90% of healthy adults [Aubin 1994; Cone 1993a] . In one study, 10 - 4,000 HHV-6 genomes were detected per 10^6 PBMC's examined, implying that between $1/10^5$ to $1/ 10^3$ cells could be HHV-6 infected [Cone 1993a]. In a study of previously infected children, a median of 1606 HHV-6 genomes per 10^6 PBMC's were detected [Clark 1997]. Other workers have suggested that HHV-6 positive cells are less frequent (< $1/10^5$ PBMC's) [Jarrett 1990; Sandhoff 1991; Cuende 1994 ; Rajcani 1994]. Non-diseased lymph nodes from patients with carcinoma of the head and neck have also been shown to contain HHV-6 DNA. The quantity of DNA present ranged from the detection limit of the test, 10 viral genomes / µg of DNA to 10^3 viral genomes / µg of DNA. This was not found to be increased in nodes examined from patients with AIDS or Hodgkin's disease [Secchiero 1995b].

HHV-6 can frequently be found in the saliva of healthy adults and children, and this may be the mode of transmission of the virus [Levy 1990a]. HHV-6 genome can also be found in salivary and bronchial glands which may serve as another site of latency for the virus [Fox 1990a; Krueger 1990; Di Luca 1995]. HHV-7 also appears to persist in the salivary glands and is secreted in saliva [Sada 1996].

In vitro models have been developed to study reactivation of HHV-6 from latency [Kondo

1991; Katsafanas 1996]. HHV-6 could be reactivated from monocyte culture after treatment with phorbol ester [Kondo 1991]. Lymphocyte cultures activated with T cells first reactivated HHV-7 and after HHV-6. It was suggested that the HHV-7 may have some transacting functions in the reactivation of HHV-6 [Katsafanas 1996]. A cell line derived from a Burkitt's lymphoma containing latent HHV-6 DNA sequences and no EBV has been established [Bandobashi 1997]. It will be possible also to use this cell line to study the mechanisms of HHV-6 latency and reactivation.

HHV-6 has been found in a number of other tissues in the body, in addition to those already mentioned, including the central nervous system, the bone marrow, and the kidneys. Whether the virus can reactivate from these sites and cause later symptoms has been disputed.

Natural transmission of HHV-6

The sero-epidemiology of HHV-6 implies that most infants are infected with this virus within the first two years of life (see chapter 2). Although HHV-6 DNA can be found in peripheral blood mononuclear cells of most seropositive persons direct viral transmission via blood is unlikely to be a common route of infection. HHV-6 is often secreted asymptomatically in the saliva of the seropositive and this would seem to be a plausible mode of transmission. [Pietroboni 1988a; Gopal 1990; Harnett 1990; Jarrett 1990; Levy 1990a; Cone 1993a; Di Luca 1995a; Aberle 1996; Hall 1998]. This corresponds with the finding of persistence of HHV-6 genome in the salivary glands [Fox 1990a; Krueger 1990; Di Luca 1995a]. Viral DNA has been detected in saliva as well as the virus cultured from saliva, and the virus has been found to be both cell free and cell associated. In longitudinal surveys of healthy individuals persistent shedding of HHV-6 in saliva has been demonstrated [Cone 1993a; Hall 1998]. Presence of virus in saliva did not correlate with serum antibody levels. Quantification of HHV-6 DNA has revealed that in some persons up to 2×10^5 HHV-6 particles per ml of saliva are secreted. In one small study of infants with primary HHV-6 infection and their mothers, one of the three mothers was found to be persistently secreting HHV-6 DNA in the saliva, and all three mothers had HHV-6 DNA in peripheral blood lymphocytes [Suga1995]. Direct sequence analysis of PCR amplified HHV-6 DNA from 4 mother-infant pairs where the infant was found to have primary HHV-6 infection revealed possible mother to infant transmission in 3 cases [van Loon 1995]. The maternal and infant PBMC strains of HHV-6 were identical, but interestingly, in one case the maternal saliva strain of HHV-6 was different from her PBMC strain and the PBMC strain was found in the infant. A further study also demonstrated close similaritly between HHV-6 strains identified within families compared to other family groups [Mukai 1994].

In a series of 52 electively aborted foetuses from mothers with HIV one case of a congenital infection with HHV-6 in a 26 week gestation foetus was found, the foetus was clinically normal but, virus was amplified from all the major organs [Aubin 1992a]. A possible link with spontaneous abortions and HHV-6 infection has also been suggested [Ando 1992].

Serological evidence for possible intrauterine transmission of HHV-6, with IgM in cord blood samples has been demonstrated in one study, where 2 / 799 (0.28%) samples were IgM antibody positive [Dunne 1992]. HHV-6 DNA could not be amplified from these samples, and no clinical data on the infants was given. Thus far, no clinical syndrome associated with congenital HHV-6 infection has been demonstrated, as is well known for the closely related virus, CMV. As several of the human herpesviruses (HSV 1 & 2, CMV, EBV) are known to replicate in the female reproductive tract, evidence of HHV-6 in the birth canal has also been sought. In one study, HHV-6 DNA could be amplified from 19% (14/72) of cervical swabs from women in late pregnancy, and all were subtype B [Okuno 1995]. PCR amplification of vaginal secretions from non-pregnant women demonstrated HHV-6 DNA in 10% (3 / 29) [Leach 1994]. In another study the virus could not be cultured from 50 endocervical swabs or 30 male urethral swabs, but these samples had been stored in liquid nitrogen and were not cultured fresh [Harnett 1990]. In a study of vertical transmission of HHV-6, virus could be amplified from vaginal swabs taken during the first trimester in 25% of 110 pregnant Japanese women [Maeda 1997]. No saliva and PBMC's collected from their infants at birth and one month of age amplified HHV-6, implying no evidence of vertical transmission, although the maternal samples were collected very early in pregnancy and might not represent the birth canal at delivery. As regards postnatal infection, examination of 120 randomly selected, breast milk samples for HHV-6 by PCR did not demonstrate any HHV-6 DNA [Dunne 1993]. There is no difference in the seroprevalence of antibodies to HHV-6 in breasted or bottle fed babies at 12 -23 months of age, again implying that breast milk is unlikely to be a significant source of this infection [Kusuhara 1997].

Interaction between HHV-6 and other viruses

HHV-6 and other Herpesviruses

Attempts have been made to understand the nature of interactions between HHV-6 and other herpesviruses in vitro and in vivo. In most studies it has been difficult to tease out not only the roles of the different viruses but also that of the host immune response. As mentioned above secretion of certain cytokines after infection with one of the herpesvirus family may alter the cellular environment in a negative or positive way for other herpesviruses.

HHV-6 and the other β herpesviruses, HHV-7 & CMV

In terms of genome, HHV-6 is most closely related to these two viruses and all three infect lymphocytes. Interactions between these viruses at a genomic or transcriptional level can therefore be implied, but there is little current evidence. In vitro infection of cells with HHV-6 and HHV-7 can be achieved and both have different effects on expression of cell surface

markers [Furukawa 1994]. Such experiments with CMV have not been undertaken. Further study on the cellular effects of combined infection could be undertaken. As far as the host response is concerned, there are cross over epitopes between HHV-6 and HHV-7 and between HHV-6 and CMV for antibody production [Adler 1993 ; Wyatt 1991; Wyatt 1992 ; Foa-Tomasi 1994; Nakagawa 1997]. There are also cross over epitopes between the three viruses for cytotoxic responses [Yasukawa 1993] . In the clinical situation, primary infection with HHV-6 usually occurs first followed by HHV-7 and then CMV. Therefore, in most infants any cross reaction with other herpesviruses is not seen with primary HHV-6 infection [Asano 1990a]. An anemnestic serological response to HHV-6 can be found with primary CMV infection, as well as evidence of virus reactivation [Chou 1990; Irving 1990a; Linde 1990; Ward 1991]. In one study of liver transplant patients, seroconversion to HHV-6 post transplant was associated with an increased risk of later CMV disease [Dockrel 1997]. Whether this was simply a serological phenomenon or a result of direct viral interaction remains to be fully elucidated. Primary HHV-7 infection may reactivate HHV-6, and both viruses can be found in blood and saliva [Asano 1995]. The viral or host interactions which allow these responses to occur are not well understood. In the immunocompromised host, reactivation of more than one herpesvirus infection can occur symptomatically or asymptomatically. In this situation the inadequacy of the normal host immune response to the virus enables reactivation to occur, but whether there are viral interactions which also affect this is not well understood. (See different Host sections for particular immunocompromised groups).

HHV-6 and the γ herpesviruses: EBV & HHV-8

The natural target cell for EBV infection is the CD21+ B lymphocyte, not usually permissive to HHV-6 infection. However, in vitro T cell lines which have been infected with HHV-6 can express CD21 and could therefore be susceptible to EBV infection [Schonnebeck 1991]. B cell lines which have been EBV transformed can become infected with HHV-6 with variable efficiency [Ablashi 1988a]. Such EBV genome-positive cell lines, when infected with HHV-6 can demonstrate EBV reactivation, with evidence of production of both early and late structural EBV proteins [Flamand 1993]. This effect of HHV-6 on latent EBV could not be produced by UV light - irradiated or heat inactivated HHV-6, implying that infectious virus particles are required. The mechanism by which HHV-6 activates the EBV lytic cycle has been investigated [Flamand 1996a]. Activation of EBV from latent to lytic cycle follows expression of the immediate - early EBV Zebra gene, and HHV-6 can up regulate Zebra gene transcription through a cyclic AMP- responsive element located within the Zebra gene promoter. This effect of HHV-6 has been found to be due to subtype A [Cuomo 1995].
In clinical terms these findings imply that HHV-6 activation can lead to increased production of EBV. This is of most importance in the immunocompromised who are at risk of EBV polyclonal activation with subsequent clonal activation and lymphoproliferative malignancy. Thus although HHV-6 activation may not be significant to the host its effects on EBV activity

could very well be. The interactions between these two viruses in the host deserves further investigation.

Interactions between HHV-6 and HHV-8 are only beginning to be investigated, HHV-8 can be cultured in transformed lymphoblastoid cell lines [Renne 1996]. HHV-8 infection is most prevalent in Africa. In one study of infants in Zambia presenting with first fever, infection with HHV-6 and HHV-8 was sought [Kasolo 1997]. Levels of viral DNA concordant with primary infection were detected in 30% of infants for HHV-6 and in 8% for HHV-8. HHV-6 subtype A was found in 44% of these African infant samples, very different from almost universal subtype B infection in studies of first fevers from Japan, Europe and the USA. Whether there might be an interaction between HHV-6 and HHV-8 which predisposes to development of later Kaposi's sarcoma is under investigation. It is also of interest to note that EBV associated Burkitts lymphoma is also prevalent in sub-Saharan Africa. Perhaps there could there be an interesting link here with the increased prevalence of HHV-6 sub type A in children and activation of EBV.

HHV-6 and the α herpesviruses: HSV 1&2 and VZV

The main target cells for infection of the α herpesviruses are epithelium and neurones. Although HHV-6 appears to be tropic for the central nervous system, major interactions between these groups of viruses have not so far been demonstrated. However in vitro, HHV-6 encodes a transactivator region which can activate heterologous promoter-chloramphenicol acetyltransferase (CAT) constructs of HSV-1 and HIV [Campbell 1991]. Therefore it is possible that there could be interaction of activity of these two viruses.

HHV-6 and other viruses

HHV-6 and HIV

Since some of the first isolates of HHV-6 came from patients with HIV and as HIV and HHV-6 preferentially infect the same host cells, the relationship between these two viruses has been closely examined. The main question being, whether the ubiquitous virus, HHV-6, acts as a possible co-factor up regulating replication of HIV and thus progression of immunodeficiency [Ablashi 1995]. In early culture studies it appeared that replication of HHV-6 was so quickly cytopathic to host cells that it tended to suppress growth of HIV [Pietroboni 1988a; Agut 1989a]. Using different strains of HHV-6 at lower concentrations and PBMC's rather than transformed T cell lines it has conversely been shown that HHV-6 can suppress HIV replication and prolonged survival of CD4+ cells can be maintained [Levy 1990a; Carrigan 1990].

Subsequently it was demonstrated that dual infection could lead to up regulation of HIV replication and transactivation of the HIV promoter region by HHV-6 [Lusso 1989a; Ensoli 1989; Horvat 1989]. In most of the experimental systems described the T cell lines

investigated were transfected with plasmids containing the HIV Long terminal repeat (LTR) region ligated upstream to the indicator gene chloramphenicol acetyl transferase (CAT). Evidence of activation and production of the CAT gene product could then be sought by measurement of acetylated chloramphenicol in a biochemical system [Horvat 1989]. Several groups of investigators have examined the relationship of HHV-6 to the HIV promoter region. A fragment of the HHV-6 clone pZVB70, can act via an NF-$_{\kappa}$B site to activate the HIV promoter [Horvat 1991; Geng 1992]. This protein had homology to the CMV early US22 gene family and may be an early regulatory protein of HHV-6. Another HHV-6 transactivating protein described by Zhou et al also makes use of an NF -$_{\kappa}$B site [Zhou 1994]. This protein P41, is conserved among all the HHV-6 strains examined and the amino acid sequence shows homology with the CMV UL44 gene product coding for the ICP36 family of early-late class phosphoproteins. Another HHV-6 protein has been identified which acts by attachment to a different transactivation response element, Sp1[Wang 1994]. This protein B115 (115 amino acids) comes from the HHV-6 gene segment ZHV14 which is capable of neoplastically transforming NIH 3T3 cells [Razzaque 1990]. Another protein from a Sal1-L fragment of HHV-6 also appeared to have HIV transactivating properties but does not require an Sp1 site [Kashanchi 1994]. In one study it was demonstrated that subtype A HHV-6 strain may have more up regulatory effects on HIV [Knox 1996].

The interaction between HHV-6 and HIV is not necessarily going to be one way, it has also been shown that the HIV retroviral transactivator protein (tat) can act synergistically with HHV-6 to increase transactivation of HIV, but that tat has an inhibitory effect on HHV-6 replication [Di Luca 1991]. Another group has found that the HIV tat gene activates HHV-6 expression and enhances replication of HHV-6 [Sieczkowski 1995]. An HHV-6 functional transformation suppression gene has also been described which suppresses HIV LTR expression [Araujo 1995]. This gene has homology with the adeno- associated virus type 2 Rep 68/78 gene. All of these experimental systems are far from the physiological situation in terms of the cells, the viruses and the many factors which can affect the interactions which occur in vivo. It is likely that a balance of positive and negative effects on activation of HHV-6 and HIV is constantly swinging, and in different situations the effect of one virus or another will be ascendant. Within this it is important to remember that in the host the daily rate of turn over of CD4+ cells infected with HIV is extremely high and the number of cells co-infected with HHV-6 is not actually known [Ho 1995; Wei 1995]. A more complete understanding of these CD4+ dynamics in vivo would help to better elucidate the relationship of HHV-6 and HIV. Double infection with HIV and HHV-6 can also occur in monocytes in vivo and in vitro, the importance of the interactions of the viruses within these cells also requires further study [Kempf 1997].

Human T Lymphotrophic Virus-1 (HTLV-1)

This is another virus which has a tropism for CD4+ lymphocytes. HTLV-1 can cause transformation of these cells and is associated with the development of adult T cell leukaemia. HTLV-1 transformed T cell lines (CD4+ and CD8+) can be infected with HHV-6 in

vitro and both viruses can simultaneously replicate [Ablashi 1992]. HHV-6 encodes a transactivator region which can activate heterologous promoter-CAT constructs of HTLV-1 and HIV [Martin 1991a; Campbell 1991]. Interactions of these two viruses at a clinical level have not been described.

Adenovirus

HHV-6 has acquired an adeno-associated virus type-2 (AAV-2) rep gene [Thomson 1991a]. This gene has an essential role in AAV-2 DNA replication. It can regulate homologous and heterologous gene expression and inhibits cell transformation. In vitro HHV-6 and AAV-2 can both infect CD4+ T cells so exchange of genetic material between these viruses is conceivable [Thomson 1994b]. The acquisition of this gene by HHV-6 may have important effects on the host cell and the HHV-6 life cycle, although these have not yet been demonstrated in vivo.

Measles virus

It has been reported from Japan that concurrent measles and HHV-6 infection can occur in infancy [Suga 1990a]. In a further prospective study of 50 infants with acute measles infection, all but 5 of whom had a prior antibody response to HHV-6, it was found that the measles infection led to rising titres of antibody to HHV-6 in 40% of cases [Suga,1992a]. In three cases HHV-6 was also cultured from the blood. Measles infection is well know to be strongly suppressive to the cell mediated arm of the host immune response, whether this is why reactivation of HHV-6 can occur, or whether there is a more direct interaction between the viruses which can both infect lymphocytes is not known.

HHV-6 and other hosts

Antibodies to HHV-6 have been detected in serum from eight species of monkeys [Higashi 1989]. It is also possible to experimentally infect monkeys (Cynomolgus and African Green) with HHV-6. In most cases the infection is asymptomatic but a rash has been described [Yalcin 1992]. In vitro, chimpanzee T lymphocytes have been productively infected with both HIV-1 and HHV-6 [Lusso 1990].

The Host and HHV-6

The Serological Response to HHV-6

Methods used to assess the antibody response to HHV-6

The first method used to test sera for an antibody response to HHV-6 was the indirect immunofluorescence assay (IFA) [Salahuddin 1986]. This was adapted from the assay first designed to study EBV antibody responses [Henle 1966]. Results of IFA for HHV-6 from different groups have not always been concordant, this may be for a variety of different methodological reasons, as it is difficult to standardise the nature of antigen presentation by infected cells. Thus the use of different cell lines, different strains of virus and different ages of cultures may affect results. The dilution of human serum tested is also very important and studies where a dilution of 1: 10 has been used have given a much higher seroprevalence than those using more dilute sera (table 1). The IFA test can be adapted to assess for avidity of IgG produced to HHV-6 [Ward 1993a]. Low avidity IgG antibody is produced after primary infection and this evolves to high avidity antibody after six months or so. In this way primary infection can be distinguished from reactivation [Ward 1993b].

Other serological methods have been used to examine sera for an antibody response to HHV-6, including a neutralising antibody test (NT) [Yoshikawa 1990], anticomplimentary antibody test (ACIF) [Robert 1990], circular-immunoassay (CIA) [Coyle 1992], and radio-immunoassay (RIA) [Coyle 1992]. The most sensitive test of antibody have been the enzyme linked immunosorbent tests (ELISA) and neutralising antibody tests (NT) (table 1,2). (For further description of these techniques, chapter 5 - Methods for Clinical Detection of HHV-6).

Seroprevalence of antibodies to HHV-6

Many seroprevalence studies of IgG antibodies to HHV-6 have now been reported from countries all around the world [Tedder 1987; Krueger 1987b; Ablashi 1988; Andre 1988; Briggs 1988; Brown 1988a; Krueger 1988a; Linde 1988 ; Pietroboni 1988a; Saxinger 1988; Balachandra 1989; Okuno 1989; Yoshikawa 1989; Asano 1990a; Dahl 1990; Enders 1990; Farr 1990; Fox 1990 ; Levy 1990a; Robert 1990; Yanagi 1990; Yoshikawa 1990; Soriano 1991; Ranger 1991; Wahren 1991; Yadav 1991; Coyle 1992; Levine 1992a; Huang 1993; Parker 1993; Ward 1993a; Kangro 1994; Wilborn 1994a; Cleghorn 1995] (table 2, 3). In early studies using IFA the test reported a wide range of seroprevalence in different populations, however where more sensitive tests, such as the ELISA, have been used over 90% of children and adults have been found to be HHV-6 antibody positive. Most studies have been carried out in the USA, Europe and Japan, and in all these areas similar high seroprevalence rates have been found. There are some more isolated populations which show a lower

prevalence of seropositivity to HHV-6 [Ranger 1991 ; Yadav 1991]. In some cohorts the titres of antibody detected have been higher in women than men [Levine 1992b].Some studies have demonstrated that seropositivity can wain with advancing age in adulthood [Andre 1988; Brown 1988a; Levy 1990a; Yanagi 1990; Parker 1993]. All these seroprevalence studies have been undertaken using either responses to fixed HHV-6 infected cells or crude lysates of HHV-6 infected cells, and different strains of A and B type virus have been used, no apparent differences in response to the different strains have been demonstrated (table 2). In general, it would appear that a serological response to HHV-6 is widespread in the human population and implies that this is a ubiquitous infection.

Acquisition of antibodies to HHV-6

There is positive placental transfer of IgG antibody to HHV-6 to the newborn and higher titres of antibody have been found in the cord blood than maternal serum [Yoshikawa 1989; Yoshikawa 1990]. Seroprevalence wanes during the first months of life to low levels and the majority of children then acquire antibody within the first 2 years of life [Okuno 1989; Yoshikawa 1989; Farr 1990; Yanagi 1990; Yoshikawa 1990; Huang 1992a; Huang 1993; Ward 1993a]. The highest geometric means titres of IgG antibody to HHV-6 are found in this young age group [Yoshikawa 1989; Yanagi 1990; Yoshikawa 1990; Ward 1993a] . Neutralising antibody to HHV-6 is produced within 3-5 days of presentation with fever and persists long term, the appearance of neutralising antibody is associated with disappearance of the virus from the blood [Asano 1989a]. In children with primary infection, HHV-6 neutralising antibody appears earlier and to a higher titre than IgG antibody detected by IFA , although both IgG and IgM will be detected by this technique [Suga 1990b]. There is no difference in the seroprevalence of antibodies to HHV-6 in breast fed or bottle fed babies at 12 -23 months of age, implying that breast milk is not a significant source of this infection [Kusuhara 1997].

Sero-epidemiological studies of percentage HHV-6 IgG antibody prevalence at different ages in different populations.

IFA - indirect immunofluorescent antibody test, NT - neutralising antibody test, ACIF - anticomplement immunofluorescence assay, ELISA-enzyme linked immunosorbent antibody test, RIA-radio immunoassay, CIA-circular immunoassay. NA - information not available from article quoted. CPE - cytopathic effect. * Studies where a fall off in antibody titres was found with advancing age in adulthood. ** Studies which did not show an adult age effect.

Method	HHV-6 strain	Serum dilution	Country	Sample size	< 1mth	4-6mths	10-12mths	3-5yrs	Adult	Author	Year
IFA	GS /A	1:10	USA	1135	-	-	-	-	63	Krueger	1988
IFA	SF/ B	NA	USA, UK,China, Scandanavia, Caribbean	600	-	36	85	95	35-95	*Levy	1990
IFA	AJ /A	1:50	UK	96	-	-	-	-	76	Fox	1990
IFA	AJ /A	1:50	UK	460	41	7	63	60-65	52	Briggs	1988
IFA	GS /A	1:10	Malasia	234	-	-	-	-	58-80	Yadav	1991
IFA	Z29 / B	1:10	Malasia	234	-	-	-	-	49-70	Yadav	1991
IFA	NA	NA	Taiwan	221	32	83-100	100	-	-	Huang	1993
IFA	AJ /A	1:10	UK	517	100	57-28	63-68	86-93	98	Ward	1993
IFA	FG-1 /B	1:10	Japan	301	87	6-50	70-86	69	76	Yoshikawa	1989
IFA	GS /A	NA	USA, Africa, Malasia	158	-	-	-	-	87	Levine	1992
IFA	Z29 / B	1:10	Japan	325	-	-	80	92.5	55-100	*Yanagi	1990
IFA	GS /A	NA	USA	287	-	-	64	94-100	50-68	*Brown	1988
IFA	AJ /A	1:50	UK	66	-	-	-	-	18	Tedder	1987
IFA	ST.W/ B, U1102 /A	1:16	Germany	1105	71.9	23.2	79.5	79.5	66	Enders	1990
IFA	GS /A	1:10	Sweden	180	-	60	60	84	88	Linde	1988
IFA	GS /A	1:20	USA, Canada, Europe, West Africa, Malasia	1095	-	-	-	-	9-52	Ablashi	1988
IFA	Australian strain	NA	Australia	338	93	65-71	70-81	93	94	Pietroboni	1988
IFA	Australian strain	1:10	Australia	141	85	20-45	65-68	-	-	Farr	1990
IFA	NA	NA	Germany	500	-	-	-	61-66	52-90	*Andre	1988
IFA	NA	1:40	Spain	102	-	-	-	-	35	Soriano	1991
IFA	GS /A	1:20	France, Africa, Caribbean	550	-	-	-	-	20-92	Ranger	1991
IFA	Z29 / B	1:20	Thailand	457	23.1	15.4-34.8	50	-	40-45	Balachandra	1989
NT	FG-1 /B	dil to prevent CPE	Japan	540	97	60	90	100	87-100	**Yoshikawa	1990
ELISA	GS /A	Multiple	Sweden	403	-	-	-	-	90	Dahl	1990
ELISA	FG-1 /B	1:200	Japan	207	100	57	67	94	80-100	Asano	1990
ELISA	GS /A	1:400	USA	500	-	-	-	77	80-97	Saxinger	1988
ELISA	GS /A	1:100	Canada	696	-	70	7	90-100	85-95	*Parker	1993
ELISA	NA	1:100	Germany	112	-	-	-	-	96.4	Wilborn	1994
RIA	? /A	NA	UK, Hong Kong	593	72-80	65-85	-	76-92	80-86	Kangro	1994
RIA / CIA	AJ /A	neat / 1:15	UK	96	-	-	-	-	71	Coyle	1992
ACIF	GS /A	1:20	France	214	-	-	-	-	30	Robert	1990
ACIF	Z29 / B	1:10	Japan	179	52	5-14	83	100	70-83	Okuno	1989

table 1: Sero-epidemiological studies of HHV-6 IgG antibody prevalence at different ages in different populations.

Sum of seroprevalence studies of percentage HHV-6 IgG antibody seropositivity at different ages and by different methods.

Method	< 1 month	4-6 months	10-12 months	3-5 years	Adult
IFA n = 22	23-100	7-100	50-100	60-100	9-100
ACIF n = 2	52	5-14	83	100	70-83
NT n = 1	97	60	90	100	87-100
ELISA n = 5	100	57-70	7-67	77-100	80-100
RIA n = 2	72-80	65-85	-	76-92	71-86
CIA n = 1	-	-	-	-	71

IFA - indirect immunofluorescent antibody test
ELISA - enzyme linked immunosorbent antibody test
ACIF - anticomplement immunofluorescence assay
n = the number of studies reported
NT- neutralising antibody test
RIA - radio immunoassay
CIA-circular immunoassay
(See the other serology table for individual study details and references)

table 2: **Sum of seroprevalence studies of HHV-6 IgG antibody seropositivity at different ages and by different methods.**

Cross reactivity of the serological response to HHV-6

When infants have their primary infection with HHV-6 there does not appear to be any cross reactivity of their antibody response to the other herpes viruses, including EBV, CMV, VZV, and HSV [Okuno 1989; Asano 1990a]. Where the IgG antibody responses of healthy blood donors to HHV-6 have been examined this has also been found to be the case [Salahuddin 1986; Downing 1987; Tedder 1987; Briggs 1988; Lopez 1988; Saxinger 1988; Buchbinder 1989; Fox, 1990].

Older patients experiencing primary CMV infection have usually already been infected with HHV-6, and rises in the IgG and IgM antibody titre to HHV-6 commonly occur simultaneously with the response to CMV. This has been shown in immunocompetent and immunosuppressed individuals [Irving 1988; Larcher 1988; Linde 1988; Morris 1988; Chou 1990; Enders 1990; Fox 1990; Irving 1990a; Irving 1990b; Sutherland 1991; Ward 1991 ; Adler 1993]. Similar IgG HHV-6 antibody responses have rarely been seen with EBV and VZV infection [Morris 1988; Linde 1988].

This HHV-6 antibody response to infection by CMV, the most closely genetically related herpesvirus, could demonstrate either, reactivation of latent HHV-6 infection consequent to CMV infection, production of cross reactive antibody, polyclonal activation of antibody, or a combination of the above. Using selective absorption of sera with cells infected with CMV or HHV-6 some groups did not find antibody cross reactivity and concluded that genuine dual viral activation / reactivation was occurring [Irving 1990a; Ward 1991]. Other groups have been able to demonstrate depletion of cross reactive antibodies by absorption studies[Sutherland 1991; Adler 1993]. These divergent results may be due to the complexities of absorption studies, but also to the possibility that in different circumstances different responses or combinations of responses may be occurring.

The most detailed study of Adler et al confirmed that children with primary HHV-6 infection do not make a serological response to CMV [Adler 1993]. They were also able to study primary CMV infection and a live CMV vaccination response in adult women. In both groups a more than fourfold rise in antibody titre to HHV-6 occurred. Absorption of the sera with CMV antigens reduced the HHV-6 titres two-fourfold, but did not abolish them. Immunoblotting was undertaken to identify HHV-6 proteins which reacted with CMV antibodies. In some hosts antibodies which reacted with CMV glycoprotein B also reacted with a 116-KDa protein of HHV-6, which is the likely HHV-6 homologue of glycoprotein B. The immunoblotting provides additional evidence that cross-reactive antibody is produced but does not completely exclude the possibility that reactivation can occur. However, the brisk rate of the antibody response might be more in favour of cross reaction than viral reactivation. Further elucidation of this relationship would require quantitative assessment of HHV-6 and CMV viraemia during the infection.

In conclusion it is important when considering antibody responses to HHV-6 to be aware of the CMV serostatus of any individual.

The Cytotoxic response to HHV-6

T cells of healthy adults seropositive to HHV-6 will proliferate when exposed to HHV-6 antigens in PBMC cultures, in contrast to a lack of response from cord blood lymphocytes [Yakushijin 1991]. HHV-6 specific CD4+ clones were identified which proliferated in response to HHV-6 antigens, but not HSV. Thus it appears that a T cell cytotoxic immune response to HHV-6 is generally present in the healthy adult population. In a further study the nature of this T cell response was examined [Yakushijin 1992]. The HHV-6 active T cell clones were shown to respond to both types of HHV-6 (B type - strain HST, A type - strain GS). The cytotoxic activity of some clones was inhibited by addition of monoclonal antibodies to HLA-Dr, implying that the response was HLA class II restricted. Other clones which were not inhibited by antibodies to HLA-Dr could lyse autologous and heterologously infected cells as well as the NK sensitive cell line K562. The third group of clones did not have cytotoxic activity. All three groups produced INF-γ and the first two groups expressed the cytotoxic protein perforin. It therefore appears that CD4+ T cell response of the healthy host produces a range of effector activities against HHV-6 and is not just a helper response for production of antibody by B cells. Loss of such effector CD4 cells in HIV infection might contribute to the widespread infection with HHV-6 which has been shown in some such patients. (see section, "patients with abnormal immunity and HHV-6").

The cytotoxic T cell response to HHV-6 appears to be strain specific in some individuals. In a large study the majority of T cell clones generated produced a proliferative response to both A and B type HHV-6, but a small percentage were strain specific [Yasukawa 1993]. In this group of healthy adults some had a memory response to B type virus, some to A type and some to both types, but the numbers were too small to make any epidemiological conclusions. Conversely, a small number of clones produced proliferative responses to HHV-6 and HHV-7 and a very small number to HHV-6, HHV-7 and CMV, indicating cross reactive epitopes between these three related viruses which are recognised by host CD4 cells.

The cytotoxic responses of children or immunosuppressed patients to HHV-6 have not yet been investigated.

Clinical Infection with HHV-6

Primary HHV-6 infection - often the "First Febrile Illness".

The clinical syndrome of exanthem subitum (ES), also known as roseola infantum, was first described in 1913 and in greater detail in the 1950's when an association with an infectious pathogen was suggested by the fact that the infection could be passed from one infant to an other [Zahorsky 1913; Kempe 1950; Hellstrom 1951]. ES was noted to be a condition of infants, in whom several days of high fever and malaise were followed by defervescence of the fever and appearance of a rash. The rash was erythematous, maculo-papular, usually over

the head and trunk and could last for a few hours to several days. These observant clinicians also documented that this condition was associated with febrile convulsions.
In 1988 Yamanishi et al, identified HHV-6 as the cause of ES [Yamanishi 1988]. In a series of infants primary infection was demonstrated: by seroconversion with production of IgM and IgG antibody to HHV-6; by culture of virus from peripheral blood lymphocytes; and later by using PCR, HHV-6 genome was amplified from the PBMC's. Subsequent studies confirmed Yamanishi's findings [Knowles 1988; Ueda 1989; Yoshida 1989a; Irving 1990c; Yoshiyama 1990; Okuno 1991; Segondy 1992; Kanegane 1993; Okada 1993]. A relationship between the blood viral load of HHV-6 and the severity of the infection in terms of days of fever and malaise has been shown [Asano 1991a]. It was also found that primary HHV-6 infection could produce fever without rash, or rash without fever [Suga 1989; Asano 1989b]. More recently quantitative PCR assays for HHV-6 have been developed and HHV-6 can be amplified from plasma as well as cells [Huang 1992a; Secchiero 1995a; Clark 1996]. Seccherio et al amplified HHV-6 DNA from the plasma of 6 / 7 children with acute ES and suggested this method could be used to detect acute infection [Secchiero 1995a]. In this study, the infants had from 6×10^2 - 6×10^3 genome copies / ml of plasma. A quantitative examination of the PBMC load of virus, demonstrated that children with primary HHV-6 infection had a median 24,213 HHV-6 genomes per 10^6 PBMC's compared to 1,606 in children who have been previously infected [Clark 1997]. In this study, in half the children with primary infection, HHV-6 was also amplified acutely from the serum.
After the discovery of HHV-6 as the cause of ES many sero-epidemiological studies soon confirmed that an IgG response to HHV-6 was found in up to 100% of children tested, and the peak period for seroconversion occurred in the second half of the first year of life (table 2,3). HHV-7 can also cause the classical syndrome of ES [Hidaka 1994; Tanaka 1994; Torigoe 1995; Tanaka-Taya 1996]. This virus, like HHV-6, is secreted in the saliva [Frenkel 1990a]. Most children also have a serological response to HHV-7, although it appears to develop slightly later than that to HHV-6 [Clark 1993; Yoshikawa 1993; Cermelli 1996; Huang 1997].

Clinical Features of Primary HHV-6 infection

The largest Japanese study of the clinical features of primary HHV-6 infection, where infection was diagnosed in 176 out of 688 children presenting to hospital with fever, rash or both, tended to confirm the classical syndrome of ES [Asano 1994]. This study found 94% of infants were infected by one year of age with the mean age of infection being 7.3 months (3 weeks-18 months) and for the majority of infants this was their first febrile illness. Virtually all children (98%) had high fever to a maximum of 39.4^{o}c, lasting a mean of 4.1 days. A maculo-papular rash appeared on the face and trunk in 98% of cases, most commonly at the resolution of the fever. The rash lasted a mean of 3.8 days and there was no desquamation. Mild diarrhoea occurred in 68% of cases and erythematous papules were seen in the pharynx

in 65%. Cervical lymphadenopathy was seen in 31% of cases and 50% had a cough. A bulging anterior fontanelle was present in 26% of the children and convulsions occurred in 8% (14 / 176). The convulsions were all of short duration and occurred during the febrile stage of the illness, before the rash had developed. The mean age of infants having convulsions was 10.9 months (range 5-17 months).

The most exhaustive studies of primary HHV-6 in children however, have been carried out in the USA. The first of two very large studies, examined 243 children under 2 years of age presenting consecutively to hospital with fever ($\geq 38^{o}c$) for evidence of HHV-6 infection by culture, PCR and antibody response [Pruksananonda 1992]. Primary HHV-6 infection was identified in 34 cases (14%), with a mean age of 9.5 months. Features of this group included, high fever (mean $39.7^{o}c$) and inflamed tympanic membranes. Three cases had a rash after the fever defervesced and 3 had a rash with the fever. One child had a febrile fit. The mean white blood count on presentation was $8.9 \times 10^{9}/L$ with 39% lymphocytes and 51% neutrophils. All but one of the strains of HHV-6 cultured were sub-type B and the other culture included subtypes A and B [Dewhurst 1993].

A further much larger study examined 2587 children presenting under three years of age for adverse effects of HHV-6 primary infection [Hall 1994]. There were 586 infants with acute non febrile illness and 356 healthy controls, none of them had HHV-6 primary infection. Of 1653 infants with acute febrile illness, 160 had HHV-6 primary infection (9.7%). The mean age of this group was 9.4 months (range 2 weeks - 25 months). Importantly,where only infants aged 6-12 months were considered then 21% (75 / 365) of febrile illnesses were caused by HHV-6.

In only 17% of the 160 cases was the correct clinical diagnosis made, 30% were diagnosed as fever due to otitis media and 29% as fever of uncertain cause. The incidence of infection did not vary with the season. The majority had high fever($> 39^{o}c$), and 15% remained febrile for more than six days. Only 11% had a rash at defervescence and 6% had a rash at presentation. Seizures occurred in 13% (21/160), compared with a 9% seizure rate in the 1394 febrile children without HHV-6 infection ($p = 0.18$). HHV-6 primary infection accounted for a third of first febrile seizures, median age 14 months. Where primary infection with HHV-6 occurred, in the second year of life, this was associated with febrile seizures in 30% of cases. Some fits were prolonged or recurrent. Cerebro-spinal fluid (CSF) collected from 29 children with primary HHV-6 infection, including 7 with convulsions, was examined. The cell count, protein and glucose concentrations were normal in all specimens and no HHV-6 could be cultured. HHV-6 genome was amplified from CSF by PCR in 7 cases, including 2 with convulsions.

In an Italian study of 56 infants presenting to hospital with fever, a viral infection was confirmed in 61%, and 40% were due to HHV-6 [Portolani 1993]. In two thirds of cases the HHV-6 was a primary infection and in one third was considered to be reactivation. In the UK, Ward et al examined a retrospective collection of sera, submitted for viral diagnosis, from 248

children for anti-HHV-6 IgG antibody avidity, where low avidity antibody implies recent infection [Ward 1994] . Within this study, a group of 25 children had presented with febrile seizures and in one case clinical ES was diagnosed at the time. Five of this group had low avidity anti-HHV-6 IgG implying primary infection, whereas 9 were seronegative on acute phase serum and no convalescent sample was obtained. The remaining 11 children had high avidity antibodies to HHV-6, almost certainly excluding primary HHV-6 as a cause of the infection and seizures. This study would also tend to support the possibility that primary HHV-6 can be a cause for febrile seizures. Again, in the majority of the cases primary HHV-6 infection was not considered in the differential diagnosis. In a study of 25 children from Finland where a clinical diagnosis of ES was made, this was due to HHV-6 in all but 2 cases, implying that when the diagnosis is considered it is usually correct [Linnavuori 1992]. In this cohort a third of the cases also had convulsions.

In the only PCR study of infants presenting in Africa with first fever, infection with HHV-6 was found in 30% [Kasolo 1997]. Levels of viral DNA appropriate to primary infection were detected, but unlike the almost universal subtype B infections detected in studies of first fevers from Japan, Europe and the USA, HHV-6 subtype A was found in 44% of these African infant samples. As mentioned previously, the significance of this finding deserves further investigation.

In addition to the fact that the non-specific findings make clinical diagnosis of ES difficult, the virological diagnosis is also not straightforward for most clinicians. Culture of HHV-6 from PBMC's is a specialised technique, not universally available. A few laboratories offer HHV-6 PCR of CSF and plasma, again as special tests. Serology, although an indirect method of diagnosis, is a little more widely available and primary infection can be diagnosed in paired samples by seroconversion, presence of IgM, or low avidity early IgG antibodies. Paired serum and CSF antibody levels could also be used to diagnose CNS infection. However, in the majority of straightforward cases with brisk recovery, convalescent serum samples are never obtained.

It remains the case that children with this infection are still most often diagnosed as having otitis media or a "virus" with fever. They may or may not get a rash, and if they do, they have probably left hospital or the GP's surgery by that time. Indeed, it is possible that many of these infants are unneccesarily prescribed antibiotics and then if the rash appears are erroneously described as "allergic to penicillin". Despite widespread use of MMR vaccine, many children with ES are still clinically diagnosed as having measles or rubella [Black 1996; Tait 1996].

Unusual features possibly associated with primary HHV-6 infection

All the possible associations with primary HHV-6 infection described below are case reports, or series of small numbers, but they demonstrate that HHV-6 may have serious effects on the skin, the bone marrow, the gut and the immune system.

A case of vesicular rash with ES, where HHV-6 was amplified from the skin lesions and the throat and seroconversion to HHV-6 occurred has been reported [Yoshida 1995]. There was no evidence of HSV or VZV infection in this child. A case of Gianotti-Crosti syndrome with a papular rash has also been described in an eight month old girl with primary HHV-6 infection [Yasumoto 1996].

As with other members of the herpesvirus family, cases of haemophagocytic syndrome in association with primary HHV-6 have also been reported [Huang 1990; Chen 1995; Portolani 1997]. In two cases of transient erythroblastopaenia of childhood, a rare condition where there are decreased red cell precursors in the marrow, HHV-6 DNA was amplified from the bone marrow [Penchansky 1997]. HHV-6 has been demonstrated in vitro to suppress erythroid and granulocyte- macrophage colony forming units [Burd 1993a].

A case of progressive immunodeficiency with fatal pneumonitis has been described, where the authors suggest that HHV-6 infection was the cause of T cell depletion and consequent immunosuppression, as well as the cause of the pneumonitis [Knox 1995a]. In this case the infant was found to be infected with A type HHV-6.

Several cases of intussusception in association with primary HHV-6 infection have been reported [Asano 1991b]. Whether this is just coincidental or whether the virus infection leads to lymphoid swelling in the gut and an increased risk of intussusception needs further study.

Langerhans cell histiocytosis (LCH) is a rare disease characterised by Langerhans cell infiltration of skin, bone and in the severe form other organs. In one study tissue from 47% (14 /30) of lesions examined by PCR amplified HHV-6. Negative controls in the study included: tissue from other lymphocytic and histiocytic benign and malignant conditions [Leahy 1993]. In another study of LCH no evidence of HHV-6 was found by hybridisation [McClain 1994]. Further investigation is warranted.

There is no good evidence to link primary HHV-6 infection with Kawasaki disease [Okano 1989; Hagiwara 1992; Hagiwara 1993]. No evidence of HHV-6 activity was found in a pathological study of sudden infant death syndrome cases [Coumbe 1990].

HHV-6 and the Central Nervous System (CNS)

Early clinical evidence described a relationship between ES and febrile seizures and this has been confirmed by the above mentioned large studies and others [Yamanishi 1992; Barone 1995; Bertolani 1996]. Although HHV-6 genome can be identified in the CSF, the lack of inflammatory cellular changes makes it difficult to understand the mechanism by which this virus might affect the CNS. In an extension of the large American study of primary HHV-6 infection, CSF and PBMC's from children who had experienced primary HHV-6 infection were examined for persistence of HHV-6 DNA. HHV-6 was shown to persist in the CSF and PBMC's after acute infection and in some patients was detected only in CSF [Caserta 1993; Caserta 1994]. In a follow up study it was demonstrated that although more than 98% of HHV-6 amplified from PBMC's in these children was type B, of the HHV-6 found in CSF 14% of

samples were typeA [Hall 1998]. Whether this is of pathogenic significance is not known. It has also been suggested that children with recurrent febrile convulsions may be having reactivation of HHV-6 in the central nervous system [Kondo 1993]. HHV-7 infection has also been demonstrated to cause febrile seizures [Torigoe 1996].

Since the discovery of HHV-6, there have been several case reports of more serious CNS complications including fatal encephalitis with primary HHV-6 infection [Ishiguro 1990; Huang 1991; Asano 1992; Yoshikawa 1992a; Jones 1994; McCullers 1995; Yanagihara 1995; Webb 1997; Kamei 1997; Koshiniemi 1997]. HHV-6 genome and antibodies to HHV-6 have both been found in the CSF in these cases; inflammatory changes have been seen on CT and SPECT scans; and abnormal EEG's have been reported. In only one of these cases was brain tissue examined for evidence of HHV-6 infection, and in that case a needle biopsy of brain was negative for HHV-6 antigens at a late stage, 21 days after presentation [Asano 1992]. In a fatal case of dual infection with HHV-6 and VZV, evidence of dissemination of both viruses to the brain tissue as well as other tissues was seen at post mortem [Ueda 1996].

In a small study of autopsy brain specimens, HHV-6 DNA was found in frontal cortex and / or basal ganglia of immunocompetent adults [Luppi 1994]. The authors suggest that the high frequency of HHV-6 DNA in the CNS implies that HHV-6 can invade the brain and remain latent with the possibility of subsequent reactivation. This finding has been confirmed by other groups, who have found not only HHV-6, but also HSV, VZV, CMV and EBV in the brain tissue of "control" patients [Luppi 1995; Challoner 1995; Sanders 1996; Merelli 1997]. It has also been demonstrated in vitro, that HHV-6 can infect human foetal astrocytes as well as glial cells (derived from the monocytic lineage) [Ablashi 1988a; He 1996]. Thus it appears that HHV-6 genome may frequently be found in the brain, and whether the virus reactivates from this site or not remains controversial.

HHV-6 infection, whether primary or reactivation has been associated with some cases of acute demyelination in the CNS. Whether this is as a direct result of viral activity or as a result of an auto-destructive host immune response is not known. Fulminating demyelination has been described in the apparently immunocompetent as well as the immunosuppressed [Knox 1995b; Mackenzie 1995; Carrigan 1996; Novoa 1997].

Multiple sclerosis (MS) is a chronic condition characterised by plaques of demyelination and an association with HHV-6 in the CNS has been suggested, but remains unconfirmed. HHV-6 antigens and DNA have been demonstrated in nuclei of oligodendrocytes (myelin producing cells) and in particular in those close to the myelin destructive plaques, the pathological lesions of MS [Challoner 1995]. In a subsequent large postmortem PCR study of neural tissue from MS patients and controls HSV (MS:Controls 37% : 28%), HHV-6 (MS:Controls 57% : 43%) , VZV (MS:Controls 43% : 32%), EBV (MS:Controls 27% : 38%), and CMV (MS:Controls 16% : 22%) were all found in normal and plaque tissue, making explanation of an association only with HHV-6 more difficult [Sanders 1996]. Higher levels of serum anti-HHV-6 antibodies have been found in MS patients than controls [Sola 1993; Wilborn 1994b].

In one study IgM to an HHV-6 early antigen (p41/38) was more commonly found in patients with relapsing-remitting MS compared to those with chronic progressive MS, patients with other neurological diseases, or healthy controls [Soldan 1997]. In that study HHV-6 DNA was also found in the serum of 43% of MS patients and none of the other groups. HHV-6 DNA has also been found in the CSF of a number of MS patients [Wilborn 1994b]. However, other groups have not been able to confirm either the serological or CSF findings in MS patients [Martin 1997; Nielsen 1997]. There is an abnormal, auto destructive immune response in MS, and whether this is auto-reactive secondary to a viral infection such as HHV-6 still remains to be proved. It has been suggested that an immune response in a host of a particular genetic make up to a viral antigen which mimics a host myelin protein could trigger later T cell activation against the host protein. As a number of the human herpesviruses can be found in the CNS perhaps more than just HHV-6 could be implicated in this possible pathophysiology [Steinman 1997].

HHV-6 in the CNS of the immunosuppressed

Whether HHV-6 is a significant CNS pathogen for children or adults immunosuppressed iatrogenically, congenitally or by infection is unknown. In a preliminary study of 5 adult patients dying with AIDS, HHV-6 was demonstrated by PCR in different areas of the brains of all patients but in no brain tissue from 2 patients with accidental deaths [Corbellino 1993]. A case of fulminant HHV-6 encephalitis has also been reported in an infant with HIV infection [Knox 1995c]. CNS demylination associated with HHV-6 infection has also been reported in adults with AIDS [Knox 1995b]. This was associated with active HHV-6 infection, identified by immunohistochemical techniques [Knox 1995b]. HHV-6 infected cells were seen at the edges of areas of demyelinisation and not elsewhere, implying an ongoing active destructive process. A similar pattern of demyelinisation has also been described in a patient immunosuppressed after a bone marrow transplant [Drobyski 1994].

HHV-6 associated clinical syndromes described in adults

Several cases of infectious mononucleosis like illness in adults have been described in association with HHV-6 infection. In a number of these cases activity of other herpesviruses such as EBV and CMV was also found, so it is hard to be certain whether the symptoms were due to one or more of these herpesviruses [Niederman 1988; Kirchesch 1988; Steeper 1989 ; Bertram 1991; Akashi 1993 #136]. In the majority the only evidence presented of HHV-6 activity was serological and not molecular. Since reactivation of HHV-6 is a well known phenomenon and cross reactive antibodies are also known to occur the clinical significance of these cases for an infection like HHV-6, known to occur ubiquitously in infancy, is uncertain.

Chronic fatigue syndrome is a common condition, occurring in adults and older children. The onset of this condition is often heralded by a viral type prodrome with fever, malaise and 'flu like" symptoms. Many viral aetiologies have been sought and in some cases it may be

possible that HHV-6 has a role to play. A number of studies have examined HHV-6 activity in these patients, evidence of active serological responses to HHV-6, increased culture of the virus from lymphocytes and an increased proportion of HHV-6 sub type A have all been found [Krueger 1987a; Wakefield 1988; Josephs 1991b; Marshall 1991; Buchwald 1992; Di-Luca 1995b; Patnaik 1995; Sairenji 1995]. However, it has not been possible to identify HHV-6 as a single aetiological factor.

It has been suggested that HHV-6 may have a role to play in a number of connective tissue disorders. Since HHV-6 is known to remain latent within the salivary glands its relationship to Sjogrens syndrome has been examined [Biberfeld 1988]. In the most comprehensive study however, no evidence of any difference from controls in serological response to HHV-6, nor presence of HHV-6 in PBMC's or salivary glands was found [Ranger Rogez 1995].

HHV-6 is commonly found in lymph node tissue and whether it is pathogenic or not in Kikuchi's disease (Histiocytic Necrotising Lymphadenitis) is not certain, but the virus has been found in lymph nodes undergoing this process [Eizuru 1989; Hoffman 1991; Sumiyoshi 1993a; Hollingsworth 1994]. In patients with Systemic Lupus Erythematosis (SLE) raised titres of antibody to HHV-6, as well as an increased ability to culture HHV-6 from PBMC's has been shown [Krueger 1991]. However, from the study reported it is impossible to ascertain whether this is causal or secondary to more general immunosuppression in these patients.

In all of the clinical situations mentioned in this section it is possible that HHV-6 activity may be occurring, perhaps as a result of an abnormal or diminished host immune response, whether innate or iatrogenic. However, in most cases it has not been possible to prove any prime aetiological role for this virus.

Patients with abnormal immunity and HHV-6

Patients with lymphoproliferative malignancy

As certain herpesvirus infections are well known to be problematic from some immunosuppressed patients, and as HHV-6 was first isolated from such patients, the serological response to HHV-6 has been examined in these groups.

Early serological studies using the IFA test reported increased seroprevalence and increased titre of IgG antibody to HHV-6 in patients with Hodgkin's disease, acute myeloid leukaemia, African Burkitt's lymphoma, and other non-Hodgkin's lymphomas [Ablashi 1988b; Biberfeld 1988; Clark 1990]. However the control seroprevalence to HHV-6 in these studies was lower than might be now expected (10-55%). A case controlled study of 50 patients with acute lymphoblastic leukaemia (mean age 17 years, range 1-52 years) where antibody levels were assessed by IFA and ELISA demonstrated no difference in seroprevalence or titre of antibody between patients and controls (94% were seropositive) [Levine 1992b]. The

authors of this study suggested that the previous differences found may have related more to age differences in the populations tested than disease. This group also found no difference in pre-treatment HHV-6 antibody seroprevalence or titre in patients with Hodgkin's disease, but in follow up where titres to HHV-6 increased over time the Hodgkin's disease was more likely to relapse [Levine 1992c].Using monoclonal antibodies, an ELISA was developed which distinguished antibody responses to immunodominant early and late HHV-6 antigens, enabling a possible differentiation between active and latent infection [Iyengar 1991]. In a control population, 56-96% were seropositive to the late antigen and 10-30% to the early antigen. Patients with African Burkitt's lymphoma and Hodgkin's disease had a similar response to the late antigen but a greatly increased response to the early antigen in terms of seroprevalence and titre (63-97% were positive). This was considered to represent possible increased viral replication or immune activation in such patients. In a serological study of 121 German children with leukaemia, with age and gender matched controls, there was no difference in seroprevalence or titres of antibody to HHV-6 [Schlehofer 1996]. However, antibodies to EBV were found more frequently in children with leukaemia who were less than six years of age. Serological studies cannot be expected to demonstrate aetiological links between HHV-6 and lymphoid malignancies, however it does appear that in some patients the serological response to HHV-6 may be more active and this could be due to increased viral activity or immune dysregulation. In a study of patients with various myelodysplastic syndromes, elevation of titres of antibody to EBV, HHV-6 and CMV was associated with expression of viral antigens in the bone marrow, implying active viral replication [Krueger 1994a]. The authors suggested that the cytokine milieu created by the presence of these viruses could contribute to development of dysregulation of host cellular controls.

Herpes viruses in addition to being active in patients with malignancy may also play a part in the oncogenic process leading to the development of malignancy, as is well known for Epstein- Barr virus (EBV) [Gledhill 1991; Levine 1992d]. The potential for HHV-6 to be an oncogenic virus has also been explored, in-vitro HHV-6 DNA can neoplastically transform cells of murine and human origin which will then go on to form murine tumours [Razzaque 1990; Puri 1991; Razzaque 1993]. Sequences of HHV-6 DNA known to cause transformation in vitro have also been identified in some human lymphomas. HHV-6 DNA can be present without production of viral antigen, implying that expression of all viral proteins is not required for transformation [Razzaque 1996]. Recently, a possible HHV-6 oncogene has been identified. An HHV-6 derived ORF-1 protein has been shown to bind to the human tumour suppressor protein p53 at the specific DNA binding domain, and functionally inactivates it. Interestingly, analysis of several human tumours, by PCR has shown ORF-1 DNA sequences in some angioimmunoblastic lymphadenopathies, Hodgkins and non-Hodgkins lymphomas and glioblastomas [Kashanchi 1997].

Tissue from many human lymphoproliferative and other malignancies has been examined for evidence of HHV-6 genome and the results remain controversial. Early studies using

Southern blotting and HHV-6 DNA probes identified HHV-6 DNA in a small proportion of tissues examined: 4 /30, 7/50, 2/177 respectively [Ablashi 1988b; Josephs 1988a ; Jarrett 1988]. Where the more sensitive technique of PCR has been used HHV-6 positive specimens have been detected frequently in both malignant and reactive lymphoid tissues [Borisch 1991; Shiroky 1992; Sumiyoshi 1993b]. However, in other studies the number of HHV-6 positive samples remains much lower (<10%) [Torelli 1991; Di Luca 1994].

A possible connection with Hodgkin's disease (HD) was identified where 29% (13/45) of samples were PCR positive for HHV-6, 38% of samples were also positive for EBV, but there was no correlation between presence of the two viruses[Di Luca 1994]. Where lymph nodes and PBMCs have been examined a small number of patients (2 /64 with non-Hodgkin's lymphoma (NHL), and 3/55 with HD) as well as having detectable HHV-6 in the lymph nodes also had a higher copy number of HHV-6 in circulating cells. In half the cases this was complete viral genome and in the rest fragmented [Torelli 1995]. In all these cases the complete viral genome was found to be attached to human chromosome 17 at the same site (near the P53 gene). The authors suggest that these patients may demonstrate an unusual type of HHV-6 viral latency with more viral replication and more cells affected. In a larger study of 73 HD, 15 NHL, and 19 reactive lymph nodes, over 2/3 of all the lymph node tissue was positive for HHV-6 by PCR and in-situ hybridisation, although not by southern blotting, implying low copy numbers of virus [Valente 1996]. HHV-6 was only seen in lymphocytes and not in Hodgkins or Reed-Sternberg cells, and there was no expression of viral antigens by immunocytochemistry. There was no relationship between presence of HHV-6 DNA and histological type of HD, clinical parameters, or outcome of disease and the authors concluded that a specific pathogenic role for HHV-6 was unlikely. In a much larger study of 243 lymph node and salivary gland biopsies, HHV-6 DNA was detected by PCR in 39% of NHL, 52% of HD, 64% of non-neoplastic lymph nodes, 23% of tumour metastases, and 50% of salivary glands. The presence of HHV-6 correlated only with the age of the patient and not with the diagnosis, present in 54% of those under 60 years and 35% of those over 60 years [Hallas 1996]. In a very large case control study of herpesvirus serology in HD (n = 494), a sub sample of nodular sclerosing cases was examined for antibody titres to EBV and HHV-6. Increased titres to both viruses were found in patients compared with controls, the highest HHV-6 titres were found in cases where the Reed-Sternberg cells were PCR negative for EBV, the significance of this is unknown [Alexander 1995].

In contrast to most studies, Krueger et al, demonstrated the presence of HHV-6 antigens in over 3/4 of HD tissues examined [Krueger 1994b]. They also detected HHV-6 antigen expression most frequently in Hodgkins Disease and Reed-Sternberg cells and less often in lymphoid cells. They suggested that viral replication may affect cellular cytokine release in such a way as to encourage dysregulation of host cell proliferation, rather than as a direct oncogenic effect. Whether HHV-6 may have a particular role in childhood HD is not known, but reactivation of HHV-6 in such patients has been shown [Maeda 1993; Trovato 1994].

HHV-6 preferentially infects T cells and one study of childhood T cell leukaemia

demonstrated bone marrow from 14 /16 cases to be PCR positive for HHV-6 [Luka 1991]. In situ PCR was positive in 20-58% of cells, and 2-7% of cells were expressing HHV-6 early antigen, but no late antigen. HHV-6 has also been associated with a rare adult form of T cell leukaemia, being found only in the malignant cells [Hanson 1991; Braun 1995]. A case of angioimmunoblastic lymphadenopathy with dysproteinaemia (AILD) associated with active HHV-6 infection has been reported in a patient who had been treated successfully for acute myeloid leukaemia. The patient developed pericarditis, polyclonal gammaglobulinaemia, and died of fulminant hepatitis. At autopsy, all the major organs were infiltrated with lymphoma and HHV-6 DNA (sub type B) was present in involved tissue. The authors suggest that in some way reactivation of HHV-6 may have triggered the development of AILD [Daibata 1997]. In an earlier study, HHV-6 sequences were found in tissue from 7 /12 patients with AILD, and a causitive role for HHV-6 was also suggested [Luppi 1993c].

Tissue from certain carcinomas (oral, salivary gland, larynx, breast, and cervix) has also been examined for presence of HHV-6 by in-situ hybridisation and PCR [Arivananthan 1997]. Not surprisingly HHV-6 was found in over 70% of salivary gland and oral tissue, but only 33% of cervical and in no breast tissue. Again, a possible cofactor role in carcinogenesis was suggested for the virus. In vitro HHV-6 infection of human papilloma virus (HPV) infected cervical epithelial cell lines enhanced expression of the HPV RNA's coding for viral oncoproteins [Chen 1994a]. In a study of human cervical carcinoma tissue HHV-6 was found in a small number (6/72) and in association with HPV-16 in 4 cases. None of 30 normal cervical tissues was positive for HHV-6 DNA. The authors suggested that HHV-6 could in some cases act as a cofactor with HPV for development of cervical malignancy [Chen 1994b].

Transplant patients

Serological responses to infections are often difficult to interpret in patients undergoing severe immunosuppression for transplantation. In these cases it is often more helpful to examine for evidence of direct viral activity. Most groups of transplant patients have been examined for HHV-6 activity. None of the cohorts studied have been of very large numbers and some of the findings have been conflicting.

Bone marrow transplantation and HHV-6

The majority of bone marrow transplant (BMT) patients are seropositive for HHV-6 prior to transplant, and HHV-6 activity after transplant can be demonstrated in 18-88% of patients by culture of virus from PBMC's or bone marrow, by greater than fourfold rises in antibody titre, and by amplification of virus from saliva and urine. In studies with multiple sampling over longer periods of time the highest HHV-6 positive rates are seen [Yoshikawa 1991; Drobyski 1993b; Carrigan 1994; Wilborn 1994c; Kadakia 1996]. Early studies of children demonstrated that some of those with positive cultures for HHV-6 and rises in neutralising antibody titres also experienced fevers and rashes. In one child the rash was associated with acute graft versus host disease (GVHD)[Asano 1991c; Yoshikawa 1991; Fugita 1996]. In

some cohorts of patients an association between HHV-6 activity early after transplant and acute GVHD has been demonstrated [Wilborn 1994c; Appleton 1995], but this has not been found in others [Kadakia 1996].

One of the most important effects of HHV-6 in BMT patients is the finding that HHV-6 activity can suppress the bone marrow. This correlates with the situation in natural primary infection where leukopaenia can be seen. In vitro HHV-6 can suppress bone marrow culture colony formation [Knox 1992; Burd 1993a; Isomura 1997]. HHV-6 can infect the marrow monocytes, although cell free virus could not be found, so this may not be a very productive infection. Severe inhibition of all the colony forming units and stromal growth was seen, and this effect could be reversed by addition of a neutralising monoclonal antibody specific for interferon -α [Knox 1992]. Further experiments demonstrated that the inhibitory effect on marrow macrophages did not require the presence of infectious virus, and was produced by some soluble mediator (s) [Burd 1993a]. In another in vitro study marrow progenitor cells from cord blood were directly infected with both variants of HHV-6 [Isomura 1997]. Variant B HHV-6 was shown to have a more suppressive effect on granulocyte / macrophage colony forming units than variant A, but both had an equal suppressive effect on erythroid colony forming units. The authors suggest that as HHV-6 was frequently amplified by PCR from single colonies, the suppressive effect was related to presence of virus rather than indirect effects via accessory cells of the marrow. Further investigation of these mechanisms is warranted.

Clinically, in BMT patients bone marrow suppression can also be found with activity of HHV-6, [Drobyski 1993b; Carrigan 1994; Wang 1996]. In one study 16 adult patients were followed for the first 100 days after BMT for evidence of HHV-6 activity [Drobyski 1993b]. In 5 /16 patients unexplained post-transplant marrow suppression was found, this was more frequent in those who had concurrent HHV-6 viraemia (4 / 6) than in those from whom HHV-6 was not isolated (1 / 10, $p < 0.05$). HHV-6 was also isolated from the marrow of the 4 patients during the period of suppression, and by in vitro colony forming assays was shown to be suppressive for granulocyte / macrophage colony forming units and erythroid colony forming units. All the HHV-6 isolates were variant B. In a further cohort of 15 adult patients similar findings were obtained by the same group [Carrigan 1994].

The activity of HHV-6, HHV-7, EBV and CMV in a group of 37 allogeneic BMT recipients was studied before and after transplant by PCR of PBMC's [Wang 1996]. Prior to BMT, HHV-6 DNA was detected in 8 (22%) patients and HHV-7, EBV, and CMV DNA were detected in 21 (57%), 10 (27%), and 1 (3%) patient, respectively. After BMT, HHV-6 DNA was detected in 26 (70%), HHV-7 in 21 (57%), EBV in 28 (76%), and CMV in 21 (57%) patients. Thirty-two (87%) patients were positive with more than one virus. HHV-6, HHV-7, and EBV DNA were found earlier than CMV DNA in most patients after BMT. The proportions of HHV-6 positive samples during the first 3 months after BMT were higher in the patients with either delayed granulocyte engraftment ($P = .04$) or delayed platelet engraftment ($P = .001$). The HHV-6 DNA in samples from the patients with delayed engraftment was variant B. The detection of any lymphotrophic herpesvirus was not related to the development of acute GVHD. In this

study, high-dose aciclovir prophylaxis significantly reduced the proportion of HHV-6 positive samples and tended to lower HHV-6 DNA levels. The authors suggest that since HHV-6 variant B can inhibit marrow engraftment, high-dose aciclovir may be beneficial to engraftment after BMT by preventing HHV-6 reactivation. No relation between the proportions of HHV-7-, EBV-, and CMV-positive samples in the first 3 months and engraftment was found.
The great majority of strains (97%) of HHV-6 isolated from BMT patients have been type B [Drobyski 1993a; Gompels 1993; Wilborn 1994c; Frenkel 1994; Kadakia 1996; Wang 1996]. A case of late graft failure after BMT has been reported where both variant A and B HHV-6 were found, and no other attributable cause, the authors suggested that the graft failure may have been due to the presence of A variant HHV-6 [Rosenfeld 1995]. Restriction enzyme analysis of three HHV-6 variant B isolates obtained from a child, one prior to and two post BMT, demonstrated that the strain isolated before transplant was identical to one of the strains found after transplant. In this case there was reactivation of an autologous strain and also the possibility of new infection either from the donor or else where [Yoshikawa 1992b]. Although it is possible that HHV-6 could be more pathogenic in children than adults, very few studies of the activity of HHV-6 after BMT have been undertaken in paediatric cohorts. It would seem prudent to observe BMT patients closely for all the herpesviruses as they can have a wide range of deleterious effects. Although HHV-6 may be important for causing marrow suppression, its role in GVHD and rejection remains uncertain.

Liver transplantation and HHV-6

In vitro, it has been demonstrated that HHV-6 can productively infect the hepatoma cell line, hep G2 [Inagi 1996]. The presence of HHV-6 induced activation of the inflammatory cytokine IL-8, but not IL-1β. This effect was only seen with infectious particles of HHV-6, and the authors suggest that as HHV-6 can directly infect liver and this could result in organ dysfunction in vivo. Indeed, there are case reports of primary infection with HHV-6 in previously normal hosts which have been associated with fulminant hepatitis [Asano 1990b; Tajiri 1990; Sobue 1991; Dubedat 1989]. In one child, not only liver dysfunction but also persistent anaemia and granulocytopaenia in association with persistent active HHV-6 infection was found [Takikawa 1992].
Small series of liver transplant recipients have been investigated for evidence of HHV-6 activity. The first case of primary infection after liver transplant was reported with associated fever, convulsions and hepatic dysfunction [Ward 1989]. In a similar case post liver transplant, with fever, hepatitis, cytopaenia, and encephalopathy, HHV-6 DNA was found in blood and bone marrow at high copy number [Singh 1995]. This patient responded favourably to a three week course of gancyclovir. A further 4 cases of HHV-6 infection in liver transplant recipients were reported where liver dysfunction was associated with cytopaenia, and in one case pneumonitis [Singh 1997]. In a prospective cohort of 46 patients, where all were HHV-6 seropositive, buffy coat samples were examined before and after liver transplant by PCR [Schmidt 1996]. Pre-transplant 9.5% were HHV-6 positive, comparable with 12.5% of blood

donor controls, after transplant 28.3% were positive (but this did not achieve statistical significance). All but one of the amplified strains were B type. No correlation was found between the HHV-6 result and clinical problems and the authors of this study concluded that HHV-6 did not reactivate and cause problems in their patients.
Serological studies of liver transplant recipients have often been difficult to interpret as responses to HHV-6 and CMV are not easy to tease apart [Ward 1991; Ward 1993b; Sutherland 1991; Chou 1990]. In some cases it appears that dual responses occur and in others cross reactive antibody may be produced, in most no strong correlations with symptomatology have been shown. In a very large study of 247 liver transplant recipients, HHV-6 seroconversion was identified as a significant risk factor for development of symptomatic CMV infection in the first 90 days after transplant ($P < 0.001$) [Dockrell 1997]. As the authors point out the exact significance of this finding is difficult to explain and further study of direct virus activity by molecular techniques is required. In another study which examined 32 recipients of either liver or kidney transplants, HHV-6 infection or reactivation of infection occurred in 31% [Herbein 1996]. The findings of this study were that, severe clinical disease only occurred in patients with concomitant HHV-6 and CMV activity. There was no correlation between HHV-6 activity and graft rejection.
From the available evidence, it would appear that HHV-6 can occasionally cause hepatitic problems in the host, whether immunocompetent or not. HHV-6 may also interact in some way (s) with CMV to cause disease in the immunocompromised. There is not enough evidence to say whether HHV-6 may participate in liver rejection.

Renal transplantation and HHV-6

HHV-6 has been found in renal tissue and HHV-6 DNA has been found more frequently in the blood of post transplant patients than controls [Asano 1989c; Okuno 1990a; Wrzos 1990 ; Kikuta 1991; Hoshino 1995]. Several studies have indicated that HHV-6 can reactivate and cause febrile illnesses after renal transplantation [Morris 1989; Gudnason 1991; Yoshikawa 1992c]. In a prospective examination of HHV-6 infection in 65 renal transplant recipients all donors and recipients had neutralising antibody and virus could not be isolated from PBMC's prior to transplant, but HHV-6 could be cultured from 3 transplanted kidneys. [Yoshikawa 1992c]. After transplant 14% of patients were viraemic, 2-4 weeks after transplantation, and 41% had significant rises in antibody titre. There was no correlation with organ rejection or clinical symptoms. The authors suggested that the kidney may act as a site for latent HHV-6 infection, with reactivation after transplant, but they also point out that most patients also received blood transfusions.
In a small study a strong association with HHV-6 reactivation and renal rejection was found [Okuno 1990a]. However, this may have had more to do with the level of immunosuppression in rejecting recipients. A study of 37 paediatric transplant recipients was undertaken to examine the role of herpesviruses in rejection, six of eight rejection episodes were associated with herpesviruses (HHV-6 - n = 6, EBV + HHV-6 - n =1) [Acott 1996]. In this

study treatment of rejection with immunosuppressives without anti viral therapy led to a poor outcome, and the authors suggest that this implies that viral activity may be important in the process of rejection. In a much larger study, renal tissue from 105 kidneys from 72 recipients was examined for HHV-6 and any clinical effects on renal transplants [Hoshino 1995]. HHV-6 antigen in the tubular epithelia was detected in 63 (61.2%) specimens, and a higher incidence of the antigen was noted in specimens of accelerated rejection, acute rejection, and cyclosporine nephropathy. The antigen was present and absent an almost equal number of times in chronic rejection, intra-operative and routine protocol biopsies. These results indicate that overall HHV-6 infection is common in renal allografts and might be reactivated in acute rejection or cyclosporine nephropathy. The presence of HHV-6 antigen, however, does not necessarily correlate with a poor prognosis for the renal graft.
Although it does appear that HHV-6 can reactivate post renal transplantation, it is still uncertain whether it has a significant role in kidney rejection.

Heart transplantation and HHV-6

There is very little information about HHV-6 in heart transplantation. One case report of a 44 year old heart recipient has been described where the patient presented with gastroduodenitis, pancreatitis, and hepatitis [Randhawa 1997]. HHV-6 variant A was amplified from biopsies of multiple sites and the inflammatory infiltrate contained many giant cells.

The Lung and HHV-6

The first report of HHV-6 as a possible respiratory pathogen was from a severely ill previously immunocompetent man with legionella infection [Russler 1991]. HHV-6 was isolated from PBMC,s and was found by immunohistochemstry in lung macrophages and lymphocytes on lung biopsy. The patient did not improve with antibiotic therapy alone, but only when steroid therapy was added. The authors suggest that HHV-6 may have contributed to lung inflammation by induction of cytokines which could have been counteracted by the anti-inflammatory steroid therapy. It was demonstrated in vitro that culture of HHV-6 could be inhibited in a dose dependent manner by addition of steroid. Further to this case, an association of HHV-6 infection with pneumonitis was found in two BMT patients with isolation of virus from the PBMC's and also within abnormal lung tissue [Carrigan 1991]. The HHV-6 infected cells were predominantly intra-alveolar macrophages present in large numbers, with some lymphocytes also seen. There was no evidence of epithelial involvement with HHV-6, suggesting more of an inflammatory than destructive process caused by HHV-6. Co-infection with other respiratory pathogens could be exacerbated by HHV-6 induced inflammation. These two patients also had evidence of poor function of their bone marrow, possibly also related to HHV-6 activity [Knox 1994].
In a small postmortem study of the lung tissue of 8 immunocompromised patients who died of pneumonitis, HHV-6 was found in the macrophages and pneumocytes of 6 / 8 [Pitalia 1993]. In 6 patients evidence of more than one virus was found (HHV-6 / CMV / Adeno virus). This

study was not able to address whether these viruses were pathogens or not. An investigation of lung biopsies, by a quantitative PCR for HHV-6, from BMT patients with pneumonitis and immunocompetent patients with other problems demonstrated all seropositive patients to be PCR positive, however there was a group of BMT patients with greatly elevated HHV-6 DNA levels (10 - 500 x) [Cone 1993b]. This group of pneumonitis patients with high levels of HHV-6 DNA had a decreased risk of mortality from pneumonitis, an increased risk of GVHD, no other microbiological pathogens found in the lungs, and frequently pneumonitis was associated with falling antibody levels to HHV-6. Although this study suggests that idiopathic pneumonitis in BMT patients may be caused by HHV-6, and may tend to have good outcome, further studies have yet to confirm this. Variant A and B HHV-6 were found together in the majority of lung tissues from these patients, variant B alone occurred not infrequently, and variant A alone rarely [Cone 1996]. In this study the controls included patients undergoing lung resection for other reasons and victims of accidental death, the HHV-6 variant findings were similar among controls and patients. In a study of 33 children (aged 2 months -16 years) who died with pneumonitis, active HHV-6 infection was demonstrated by immunohistochemical staining in the post mortem lung tissues of four: a BMT recipient with concomitant adeno virus infection; a patient with hepatitis of unknown aetiology; a patient with congenital anomalies; and a patient with congenital immunodeficiency [Hammerling 1996].

In summary, HHV-6 can definitely infect or reactivate in immunosuppressed transplant patients, but the broader significance of its pathogenic role remains to be fully elucidated. It appears that in many cases activation is not associated with obvious symptomatology, but in others with more devastating organ failures. If HHV-6 can be shown in larger cohorts to contribute to organ disease, GVHD or donated organ failure then treatment of reactivations becomes an important issue. The study which showed that high dose aciclovir inhibited HHV-6 activity is important in that respect even though HHV-6 does not appear to have a thymidine kinase gene [Wang 1996].

HIV infected patients

HHV-6 activity in HIV infected patients

Most serological studies of HHV-6 in HIV infected people have been of caucasian, male, homosexuals and there is very little information about children or heterosexually infected adults. In the majority, no difference in seroprevalence or antibody titre to HHV-6 between patients or controls has been shown [Brown 1988b; Enders 1990; Fox 1988; Soriano 1991]. Seroprevalence and titre of HHV-6 antibody may even decline with progression of immune dysfunction to AIDS [Krueger 1988a; Spira 1990]. In the only study of children almost all were found to be HHV-6 IgG seropositive and 10/25 were IgM positive. The IgM positive children had a higher prevalence of pneumonitis [Nigro 1995].

HHV-6 in the blood of HIV infected patients

Where attempts have been made to correlate HHV-6 activity with progression of HIV disease some interesting findings have emerged. A close correlation of HHV-6 genome equivalents from PBMC's with CD4+ cell count in HIV infected men was demonstrated [Fairfax 1994]. All patients with a CD4+ count > 400 x 10^6/l had detectable HHV-6 genome (median -800, range 10-1500, genomes /10^6 cells), but only 58% of men with a CD4+ count< 400 x 10^6/l had detectable HHV-6 genome (median -20, range 0-300, genomes /10^6 cells). There was no correlation between HHV-6 PCR results and antibody titres or stage of disease. Dolcetti et al also demonstrated a correlation between CD4+ count and amplification of HHV-6 by PCR in PBMC's from HIV infected patients, 29% of healthy blood donors amplifed HHV-6 compared to 11% of asymptomatic HIV infected and 5% of AIDS patients [Dolcetti 1996]. A similar positive correlation between presence of HHV-6 in PBMC's and high CD 4+ count was found in another study, where a correlation between low CD4+ count and presence of CMV was also seen [Fabio 1997]. In this cohort however HHV-6 DNA was only detected in 6% (15 / 247) of patients, and the maximum median HHV-6 viral load was 400 genomes / ml of blood (range 400 - 7,180,000). Conversely, in another cohort there was no relationship between presence of HHV-6 and CD4+ count, and 21% of HIV infected and only 3.3% of controls were HHV-6 positive in this group [Blazquez 1995].

HHV-6 in the saliva of HIV infected patients

Although Fairfax et al, found a positive correlation between HHV-6 in the blood and CD4+ count , HHV-6 DNA was found in 97% of their patients' saliva, and did not correlate with CD4+ count or stage of HIV disease [Fairfax 1994]. In a study where saliva and urine from HIV infected patients were examined, CMV (61%), HHV-6 (43%), and HHV-7 (63%) were frequently detected in patients' saliva, and no correlation with CD4 count was found [Gautheret-Dejean 1997]. HHV-6 and HHV-7 were rarely found in urine and presence of CMV in urine correlated with immune deficiency. In a study where salivary gland tissue was also examined, HHV-6 was frequently amplified from the gland tissue (63%), but rarely found in the saliva (3%), and no difference was found between healthy controls and HIV infected patients [Di Luca 1995a].

HHV-6 in the lymph nodes of HIV infected patients

As HHV-6 and HIV are both CD4+ lymphotrophic viruses, it would seem important to examine the role of HHV-6 in the lymph nodes as well as the blood. Corbellino et al, first detected HHV-6 by PCR in the lymph nodes of the majority of patients with AIDS, compared to only half their HIV uninfected controls [Corbellino 1993]. Immunohistochemical examination of 9 patients who had died of AIDS infections also revealed evidence of HHV-6 activity in all their

lymph nodes, compared to CMV activity in only 3 /9 [Knox 1994a]. In 5 of these AIDS patients without known active HHV-6 related disease at death, the lymph nodes contained 5 -122 HHV-6 infected cells / cm^2 of tissues, compared with none in 4 healthy individuals and 1,823 HHV-6 infected cells / cm^2 of tissues in a post BMT patient who died of HHV-6 pneumonitis [Knox 1995c]. In another quantitative PCR study of postmortem lymph nodes from AIDS patients, all the samples from patients (5 / 5) and controls (5 / 5) contained HHV-6 DNA [Secchiero 1995b]. There was no difference in the number of HHV-6 copies per μg /DNA in patients or controls (median value 230, range <10 - 1,150 genome copies / μg of DNA). In a further postmortem quantitative PCR study of lymph nodes from AIDS patients, using a slightly different technique, HHV-6 was amplified from the lymph nodes of 6/7 patients (median value 170, range 5 - 1044 genome copies / μg of DNA) and 2/3 controls (range 5 - 165 genome copies / μg of DNA) [Clark 1996].

Examination of lymph node biopsies from HIV infected patients during life, may give more information on the possible interactions of these lymphotrophic viruses during earlier stages of disease. In an immunohistochemical study of lymph node biopsies from 10 HIV-infected patients, with CD4 + counts > 200 / mm^3, (8 with follicular hyperplasia, 1 with follicular involution, and 1 with reactive lymphadenitis) all contained HHV-6 infected cells, but the virus was seen in 0 /7 biopsies from HIV uninfected patients [Knox 1996]. In this small cohort, variant A was predominantly found, and in an in vitro experiment it was shown that variant A can massively up regulate HIV replication from latency. The authors therefore suggest that HHV-6 could catalyse the progression of HIV infection. In a PCR amplification study of lymph nodes from HIV infected patients an association between presence of HHV-6 DNA and early histological phases of lymphadenopathy syndrome (LAS) was noted, but no relationship with malignancy [Dolcetti 1996]. HHV-6 DNA was found in 65% (13 /20) of LAS biopsies, and only 20% (2 /10) of HIV unrelated lymphadenopathies ($p = 0.02$). The presence of HHV-6 correlated closely with a histological pattern of follicular hyperplasia, 81% (13 /16), although HHV-6 prevalence was decreased in the PBMC's of LAS patients. In this group HHV-6 variant B was the most prevalent sub-type. This study demonstrated HHV-6 more commonly in the PBMC's of healthy controls and yet HHV-6 more commonly in the lymph nodes of patients, a paradox not easily explained.

All the studies described are of very small numbers and none are longitudinal. There is therefore very incomplete information on the activity of HHV-6 in the lymphoid tissue of HIV infected patients. It would be an oversimplification to suggest that the presence of HHV-6 within populations of PBMC's is simply a proportional representation of activity within the lymphoid system. Indeed the activity of both of these viruses may differ greatly in different parts of the lymphoid system, such as the gut and respiratory associated lymphoid tissue. Improved understanding of the activity of HHV-6 and HIV within the lymphoid system in vivo is hampered by access to tissue, although fine needle aspiration techniques have greatly improved. There is little or no data for children in this area.

HHV-6 and clinical disease in HIV infected patients

In view of the possible associations of HHV-6 with diseases such as pneumonitis and bone marrow suppression in the transplant patients and because HHV-6 and HIV preferentially infect the same cells histopathological examination of tissue from HIV infected patients for HHV-6 has been undertaken. In small studies of autopsy material from adults, an increased prevalence of HHV-6 was found in all tissues from AIDS patients compared with immunocompetent controls (brain, lymphoreticular system, bone marrow, gut, lung, skeletal muscle, heart, liver, kidney, adrenal gland, pancreas and thyroid) [Corbellino 1993; Knox 1994a; Clark 1996]. Using a sensitive quantitative PCR, the highest burden of HHV-6 in controls and patients was found in the lungs, kidneys and lymphoreticular system [Clark 1996]. The median HHV-6 level in AIDS patients was 56 copies / μg DNA (range 0 - 43,321) compared to 10 copies / μg DNA (range 0 - 423) in controls. Although an increased burden of HHV-6 has been demonstrated here, no relationship to disease was sought in this small group. In vivo, there is very little information about active HHV-6 infection and its effect on HIV disease. One long term study of two patients demonstrated reactivation of HHV-6 to be associated with a decline in the CD4+ count which persisted, this effect would need to be confirmed in a much larger study [Iuliano 1997].

Respiratory problems are common in patients with HIV infection, but in a PCR study of bronchioalveolar lavage in 34 adult patients, although CMV was commonly demonstrated, no HHV-6 was amplified [Portolani 1996]. Retinitis, another common problem in patients with advanced HIV disease, is most commonly caused by CMV. HHV-6 can also be found in the retinal tissue of patients with HIV and even within areas of retinitis, but usually in association with CMV [Qavi 1994; Fillet 1996]. It seems unlikely that HHV-6 acts as a direct pathogen in the eye, but it could be a predisposing factor to development of retinitis.

HHV-6 has also been demonstrated in the CNS of some patients with HIV, in a preliminary study of 5 adult patients dying with AIDS, HHV-6 was demonstrated by PCR in different areas of the brains of all patients but in no brain tissue from 2 patients with accidental deaths [Corbellino 1993]. CNS demylination associated with HHV-6 infection has also been reported in adults with AIDS [Knox 1995c]. This was associated with active HHV-6 infection, identified by immunohistochemical techniques [Knox 1995c]. HHV-6 infected cells were seen at the edges of areas of demyelinisation and not elsewhere, implying an ongoing active destructive process. A similar pattern of demyelinisation has also been described in a patient immunosuppressed after a bone marrow transplant [Drobyski 1994]. As CNS infection is not uncommon in patients with HIV a large PCR study for virus associated diseases, including HHV-6 was undertaken [Cinque 1996]. Samples of CSF from 500 symptomatic adult HIV infected patients were examined, and in 219 patients the CSF findings were compared with histological examination. DNA of one or more viruses was detected in 36% of samples (HSV-1 2%, HSV-2 1%, VZV 3%, CMV 16%, EBV 12%, HHV-6 2%, JC Virus 9%). HHV-6 was

detected in 7 CSF samples (variant A 4, B 3) and in two patients the CSF was repeatedly positive. HHV-6 was not found by immunocytochemistry in any patients, but could be amplified from the frontal cortex of 1 / 7. In the majority of cases HHV-6 was found in association with CMV and a possible interaction between the two viruses was suggested. However, from this study of HIV infected adults there was no evidence that HHV-6 is a major CNS pathogen. A case of fulminant HHV-6 encephalitis in an infant is the only specific paediatric case reported of HHV-6 related CNS disease in HIV infected children [Knox 1995b]. In this case, immunohistochemstry demonstrated infection of all cell types and the predominant variant was type A.

Thus, although there are reports of possible associations of HHV-6 with disease in different systems in the HIV infected, the number of cases reported remains small and many studies are poorly controlled. In particular there is very little information for children with HIV, who might be expected to be more likely to have clinical problems with primary infection with HHV-6 when they already have HIV on board.

HHV-6 and malignancy in HIV infected patients

The incidence of lymphoid malignancy is increased in patients with HIV, which may be related to both the immunodeficiency and also the cell tropism of HIV. There is a well recognised relationship between HIV associated lymphoid malignancy and presence of EBV. The relationship of HHV-6 to malignancy in HIV infected patients has been examined in mainly small studies and obtaining adequate controls is not easy. In the largest and best controlled studies no obvious relationship between HHV-6 and development of lymphoid malignancy has been demonstrated [Di Luca 1994; Fillet 1995; Dolcetti 1996]. Using a nested PCR for HHV-6, Fillet examined lymph node and malignant tissue from patients with non-Hodgkin's lymphoma with or without HIV infection, as well as lymph nodes from patients with follicular hyperplasia, with or without HIV infection. With this system PBMC's from 2% (2/95) of healthy blood donors and 15% (3/20) of HIV infected patients amplified HHV-6. Around half of all the tissues examined amplified HHV-6 and there was no significant difference in any of the groups. Most commonly both types A and B were found. The authors note that the number of DNA copies in each sample was low (< 1 copy / 750 cells) and therefore most tumour cells did not carry HHV-6. The increased level of HHV-6 in lymph nodes compared to PBMC's could implicate the nodes as a possible site of latency or persistent infection and further in situ studies are required to examine this more closely. Dolcetti et al found no difference in amplification of HHV-6 by PCR from lymphoid malignancies from patients with or without HIV, around 30% of Hodgkin's lymphomas and 0-6% of non-Hodgkin's lymphomas were HHV-6 positive [Dolcetti 1996]. Type B strain HHV-6 was amplified more frequently than type A. The authors suggest that HHV- 6 is unlikely to be involved with development of malignancy in these patients.

Malignancies are less common in children with HIV than adults, but any possible role for HHV-6 in paediatric HIV associated lymphoproliferative disease has not so far been demonstrated.

The herpesviruses and paediatric oncology patients

Background

In considering the role of HHV-6 as a possible pathogen in paediatric oncology patients, it is useful to review the pathogenicity of the other human herpesviruses in this group of patients. It is important to remember that the interaction between the virus and the host depends not only on the activity of the virus but also on the level of immunity of the host. In this respect paediatric oncology patients are not homogeneous, some children will be more immunosuppressed than others, not only by therapy but also by their underlying condition. As a result of more severely ablative and longer courses of chemotherapy which particularly affect lymphocyte number and function, the pathogenicity of the herpesviruses in this patient group has increased with time, although better anti-viral therapies are now available. Susceptibility to infection with the herpesviruses depends on several factors. The younger the child with a malignancy the less likely the child is to have met and become infected with members of the herpesvirus family. Such a naive child will be at risk of primary infection during a period of immunosuppression which may be severe. A child however, who has already been infected with herpesviruses will harbour the persistent / latent virus and during immunosuppression, will be susceptible to reactivation of this virus as well as new re-infections. This child, depending on the degree of immunosuppression, may also have some persistent protective host immunity against the virus, so that the infection may conversely be less severe.

Serological studies of children from different parts of the world demonstrate that in most places acquisition of HHV-6 occurs within the first two years, but that acquisition of HSV, VZV, CMV and EBV can occur at different rates. The acquisition may relate to different home circumstances, child care practices, levels of crowding and breast feeding. In general, it appears that acquisition of the herpesviruses occurs earlier in childhood in less developed communities [Farr 1990; Shen 1992; Huang 1993; Kangro 1994]. Local knowledge of the patterns of acquisition of the herpesviruses is important in the monitoring of immunosuppressed children, indeed at presentation, it is important to ascertain the antibody status of each child.

table 1: Herpesvirus seroprevalence in children of varying ages in London and HongKong.

Taken from Kangro 1994. (593 samples tested).

	% seropositive at different ages					
	Cord		0.5 - 4yrs		4.1 - 12yrs	
	L	HK	L	HK	L	HK
HSV	70	85	29	52	37	57
VZV	95	90	23	32	52	83
CMV	34	95	25	37	20	51
EBV	60	100	34	55	61	95
HHV-6	72	80	65	85	76	92

L = London HK = HongKong

Host responses against Herpesviruses

Control of human herpesvirus infections depends on the integrity of natural or innate defence mechanisms as well as specific immunity, which may be cell mediated or via antibody. In the host with an altered level of immunity as well as monitoring the immune response it is also possible to monitor the activity of the virus. Evidence of persistent and increased viral activity can be sought. This may be directly by examining for virus by culture, antigen detection or genome detection, or indirectly by monitoring antibody levels. Where the host is capable of mounting an antibody response rising or high titres of antibody may be found, implying increased activity of persistent virus.

The host-virus interaction for some of the herpesviruses, including EBV, HHV-8 and possibly HHV-6, is important not just for protection of the host from reactivation of the virus but also from development of malignant transformation related to infection. Immune suppression facilitates development of lymphoid and other malignancies in patients with EBV and HHV-8, the role of HHV-6 remains controversial (see above).

The natural defence systems that are active against the herpesviruses are particularly important for containment of primary infection and to moderate reactivation prior to the onset of the specific immune responses. These defences are also often less effective in paediatric oncology patients. An intact mucosa and skin is more protective than one which is breached by mucositis, rashes or other lesions. Non specific killer cells are also known to be active against virus infected cells, and these will also be diminished in number in those on chemotherapy. Monocytes and macrophages will ingest and destroy antibody coated virus,

natural killer (NK) cells also lyse virus infected and tumour cells, the activity of both these cells is increased in the presence of the cytokines, interferon (INF) α, β, and γ. Other patients with decreased numbers and activity of NK cells, such as premature and term infants, Wiskott-Aldrich and Chediak Higashi immune-deficients, have increased susceptibility to herpesvirus infections. Null cells are large granular lymphocytes which produce INF-α , in newborns and patients with HIV these cells produce less of this important antiviral cytokine. INF-α and INF-β are produced in response to virus infection and INF-γ is produced by T cells when re-exposed to known viral antigens [Lopez 1994].

Specific immunity to the herpesviruses is very important for clearance of primary infection as well as containment of persistent virus. Cell mediated immunity (CMI) is the prime arm of the immune system against the herpesviruses. In general, the antigens which activate the cycle of CMI are viral surface glycoproteins, the very proteins which the virus uses for attachment and entry to host cells. These antigens are taken up and processed by antigen presenting cells and are then presented on the cell surface within the groove of the appropriate major histocompatibility (MHC) molecules. From here they can interact with the appropriate T cells to initiate the antiviral response. Against HSV the CMI response is more commonly a CD4+ cell response, and MHC class 2 restricted, against the others it is more commonly a CD8+ cell response and MHC class 1 restricted. In healthy adults, between 1/4000 - 1/ 8000 circulating CD4+ cells recognise and proliferate to HSV antigens, this high level of circulating memory cells to HSV represents the host vigilance required to keep this virus from reactivating. Persons with more frequent recurrences of HSV have less proliferative responses to HSV antigens. Proliferation of the T cells is associated with production of cytokines, including interleukin -2 (IL-2), and these are important for activation of macrophages and lymphocytes [Lopez 1994] (figure 1).

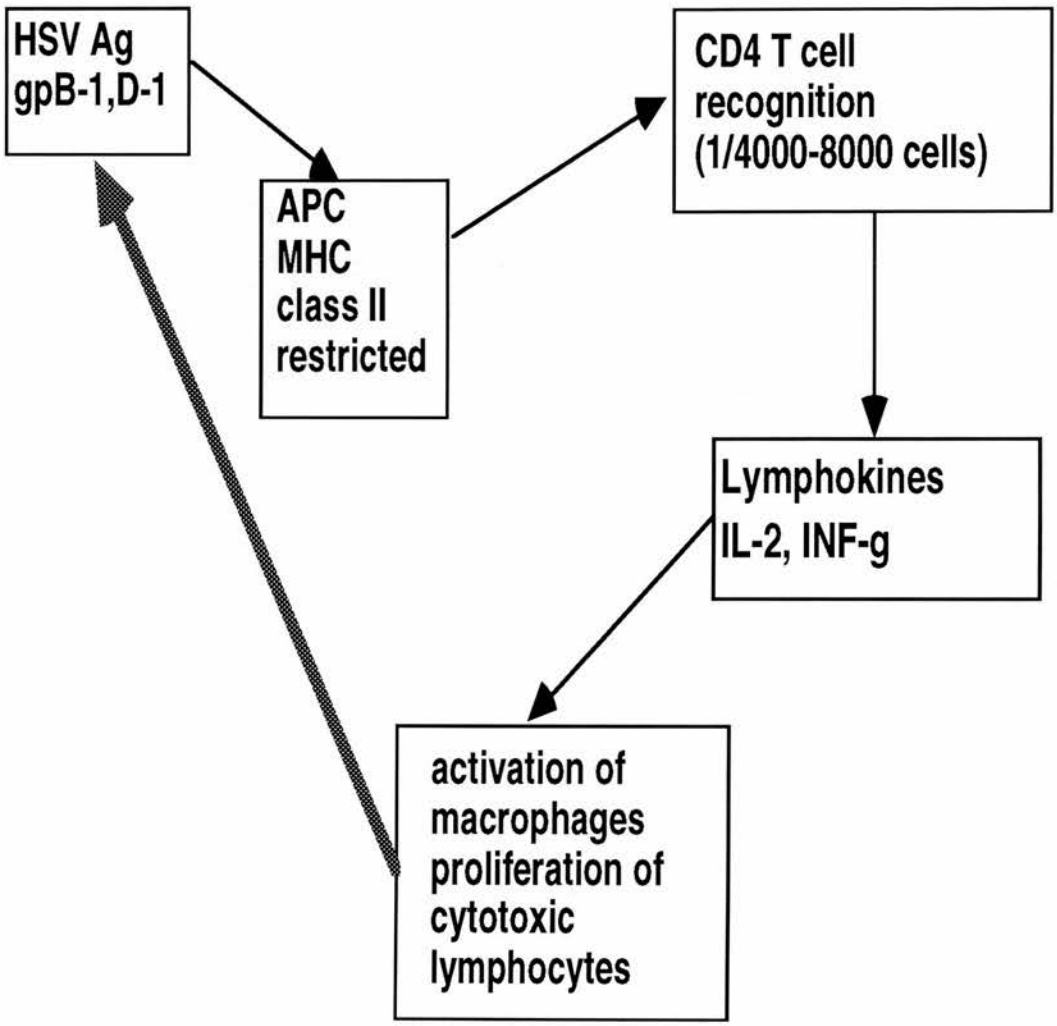


Figure 1: Diagram of cell mediated immune responses to HSV.

Humoral immunity to the herpesviruses is complex. The first type of antibody to appear at seroconversion is antibody to surface glycoproteins, against those used by the virus for adhesion and penetration of cells. This antibody is recognised by cells which mediated antibody dependent cell mediated cytotoxic responses (ADCC), such as macrophages and killer cells. Neutralising antibody functions primarily against cell free virus, and is formed against the surface glycoproteins and prevents viral adherence to and penetration of cells. Early antibody responses tend to be of low avidity, where later antibody is of high avidity, complement fixing antibody appears during convalescence. Anamnestic antibody responses are very common and occur particularly for infections with viruses in the same class, eg VZV and HSV, or CMV and HHV-6. These can be of different antibody classes including IgG and IgM. High circulating antibody levels, are often associated with high recurrence rates of infection where maintenance of latency has been poor [Lopez 1994].

Herpes Simplex 1 & 2

In a large study of over 1000 asymptomatic oral surgery patients, HSV DNA was detected in saliva by PCR. Overall viral DNA was detected in 4.7% of patients, but more commonly in those: under ten years and over sixty years; with inflammatory diseases; with malignancy; and with trauma, implying that those with impaired immunity or damaged mucosal surface were more likely to be shedding the virus [Tateishi 1994]. Other studies of secretion of HSV in the immunocompromised have shown this to depend on the underlying disease as well as the degree of immunity. In paediatric patients, HSV-2 is rarely a problem, where for adult patients, especially those with HIV infection, this can be a major cause of genital symptoms. Asymptomatic shedding in the immunocompromised can progress to local cutaneous disease which may be atypical and extensive. In some patients oral mucositis may be as much caused by HSV and other infections (eg candida) as by the effects of chemotherapy and obvious vesicular / ulcerated lesions are not necessarily seen [Carrega 1994]. Secondary viraemia can also occur with dissemination and development of organ disease, in particular of the central nervous system, respiratory tract, skin, and gut. In organ transplant patients, those with the highest pre-transplant titres of antibody to HSV are at most risk of recurrence [Schubert 1990]. In most transplant programs patients are treated prophylactically with aciclovir to suppress reactivating virus [Gluckman 1983].

Varicella Zoster virus

VZV infection can be lethal in patients with poor CMI, including the new born, those with congenital immunodeficiency, acquired immunodeficiency, malignancy, on steroids or other immunosuppressive therapy. Recovery from primary infection depends on making an adequate T cell response, and this is more important than the antibody response. In the normal host, VZV antibody can be detected on the first day of the rash. VZV surface glycoproteins (pg 1, gp 111, p 170) stimulate the CD8+ T cell mediated immune response as well as activation of CD4+ cells which help B cells to produce antibody. High titre antibody is

well as activation of CD4+ cells which help B cells to produce antibody. High titre antibody is not universally protective against infection and recurrence of infection as zoster can occur with good antibody titres. A cytotoxic T cell response to VZV appears after 2-3 days of rash, and this is necessary to terminate the cell associated viraemia. In the immune, VZV memory cells are found to occur in 1 / 10,000 - 1 / 30,000 circulating T cells. The number wains with age, but is boosted in adults and children in contact with cases of VZV. A decrease in VZV memory T cells is associated with an increased risk of reactivation and occurrence of herpes zoster [Arvin 1996].

A very large follow up study of over a thousand paediatric patients treated with BMT between 1989 and 1994 identified 216 cases of VZV [Han 1994]. The cases occurred from 4 days to 10.8 years after transplant, but 86% were within the first eighteen months after BMT. Most were cases of dermatomal zoster (62%), but one third were complicated cases with CNS infection, disseminated infection, and visceral infection. All the serious infections were within 7 months of BMT, but only 2 patients died of pneumonitis. The incidence of VZV was similar in allogeneic and autologous BMT. Patients who were VZV seropositive prior to BMT had more frequent, earlier, and often more complicated or disseminated infections. Age over 10 years and irradiation pre transplant were significant, independent risk factors for higher rates of VZV infection. Using mathematical modelling the authors found that: VZV seropositive patients aged over 10 years and irradiation pre transplant had a 44% incidence of infection by 3 years where, VZV seronegative patients aged under 10 years without irradiation pre transplant had a 0% incidence of infection by 3 years. Aciclovir given for prophylaxis against HSV or CMV did not affect the timing of VZV infection.

A paediatric study of 294 children with lymphoblastic leukaemia demonstrated 14% (41) to have episodes of VZV [Rowland 1995]. Twenty patients (49%) had received prophylaxis after their contact with varicella zoster immunoglobulin (VZIG) and all recognised to have VZV were treated with aciclovir. The majority (70%) had only skin lesions and none of them died. Visceral involvement occurred in 13 (30%) and 5 died. Risk factors for progressive infection included age over 6 years, intensive immunosuppression at the time of presentation and lymphopaenia. A very worrying symptom was severe abdominal and / or back pain, which could occur up to several days before any rash, this was noted in 4 / 5 fatal VZV cases. Two of the fatal cases had received VZIG. Disseminated intravascular coagulation and liver dysfunction are commonly found in fatal cases of VZV [Chen 1994].

In a study of the regeneration of the immune response to VZV after autograft in 41 children, the nadir of the T cell response even to T cell mitogens was at 3 months after transplant and gradually improved over the next 6-12 months. In patients with previous VZV infection, a proliferative T cell response to VZV was seen in 45% at 6 months and 67% at 12 months [Takaue 1994]. NK cell activity and ADCC as well as cytotoxic activity against VZV have also been shown to be decreased in children with leukaemia and this persisted for up to one year after completion of therapy [Ihara 1994]. Although VZV immunisation could decrease the frequent exogenous exposure of immunosuppressed children to this virus, this is not

currently part of the schedule in the UK.

Epstein-Barr Virus

It is difficult to study the immune response to EBV in most natural infections in children as these are usually asymptomatic. Infectious mononucleosis (IM), can be studied, but may represent a particular type of host response to EBV which is not necessarily "normal" . During an acute episode of infectious mononucleosis around 10% of circulating B cells are infected with EBV. The EBV epitopes presented on B cells which are recognised by CD8+ T cells include: Epstein-Barr nuclear antigen (EBNA) 2; 3A; 3B; 3C; and latent membrane protein 2 (LMP 2). B cells which only express EBNA 1 are not recognised by the cytotoxic cells. Most of the "atypical lymphocytes" seen in IM are NK cells or CD8+ T cells which are active against the EBV infection and important to sustain long term immunity [Lopez 1994]. However in IM, the cytotoxic T cell response is not EBV specific, as there is polyclonal stimulation of multiple MHC class 1 alloreactive CD8+ cytotoxic precursors. Lysis of allogeneic target cells occurs, and this is what leads to the symptomatology of IM. In the normal host, ultimately the cytotoxic response against the virus is effective and the EBV infection is brought under control, but even in the immune individual 1 in 10^6 circulating B cells are latently infected with EBV. Removal of an active CD8+ response will allow reactivation of EBV and polyclonal B cell activation can occur. This activation can then proceed to become clonal and lymphoproliferative malignancy can result. In conditions where the host has a low cytotoxic T cell response, such as post transplant, during HIV infection, and in congenital immunodeficiencies, increased numbers of EBV positive circulating B cells occur and there is an increased risk of EBV associated lymphoproliferative diseases [Lopez 1994].
There is a recognised pattern of normal antibody response to EBV which evolves for several months after primary infection. High titres of IgM and IgG antibody to viral capsid antigen (VCA) are present during the acute illness and convalescence, after this IgM is lost and VCA IgG persists at a lower titre life long. Heterophile antibody which gives the positive monospot test (rarely present in young children), and antibody to early antigen (EA) persist for approximately as long as VCA IgM. Titres of IgG antibody to EBNA 1 gradually increase during convalescence to reach a steady level after recovery and as with IgG to VCA are maintained life long [Epstein 1973; Henle 1987]. Persistence of high titres of antibody and the early antibody responses can be found in those with altered immunity and increased virus exposure.

lymphoproliferative diseases and Epstein-Barr Virus

This virus is associated with a number of different lymphoproliferative diseases. The first human malignancy demonstrated to be associated with a virus infection was Burkitt's lymphoma (BL) [Burkitt 1958]. EBV can be isolated from more than 95% of BL cells grown in culture, and most cells contain multiple copies. BL is common in equatorial Africa, particularly

in areas holo-endemic for falciparum malaria infection. The majority of BL affects the jaw and approximately half the abdominal viscera. There is a tight childhood age distribution, and it has been demonstrated that children who go on to develop BL have been infected with EBV for many months. Children who develop the tumour tend to have higher titres of IgG VCA antibody to the virus than those who do not. It has been suggested that children who are continuously reinfected with malaria have consequent polyclonal B cell proliferation, and this leads to an expanded number of EBV infected B cells. The T cell immune response is suppressed by a combination of infections, malnutrition and possible other host factors, thus allowing further expansion of EBV positive B cells and consequently an increased risk of development of cells with cytogenic abnormalities and malignant clonal potential [Liewbowitz 1994].

Development of malignant clones also depends on evading the cytotoxic T cell response. The majority of BL are clonally identical with the chromosomal translocation t8:14. This translocation leads to the placement of the c-myc oncogene sequence within the IgG heavy chain constant region. C-myc expression is up-regulated by EBV which leads to proliferation of cells with several growth advantages. EBNA 1 is the only EBV antigen expressed and this epitope is not recognised by cytotoxic T cells. Presence of EBV latent membrane protein 1 (LMP1) upregulates Bcl 2 expression which allows EBV infected cells to escape apoptotic cell death as they traverse lymph node germinal centres. Eventually out of this dysregulated proliferation a dominant malignant clone of cells will occur. BL grows extremely rapidly and is highly sensitive to chemotherapy.

In patients who are immunosuppressed long term for iatrogenic reasons such as organ transplantation, post transplant lymphoproliferative disease (PTLD) can occur [Wu 1996]. Loss of cytotoxic T cell responses leads to polyclonal B cell proliferation which can then progress to malignant monoclonal lymphoma, however even the polyclonal disease itself can also be very severe and symptomatic.

PTLD occurs in 2% of liver and kidney transplants and 5-13% of heart / heart-lung transplants. The paediatric BMT patients with the greatest risk of infection are those who have received T cell depleted grafts (14%) [Gerritsen 1996]. PTLD is more common in paediatric patients and the risk is greatest in those who are EBV seronegative and are infected from a seropositive donor at a time of maximal immunosuppression [Ho 1988]. It is possible to monitor the load of EBV infected cells in patients post transplant to assess the risk of PTLD [Kenagy 1995]. A study of recovery of EBV cytotoxic T cell precursors in patients post BMT demonstrated that after six months cytotoxic cells were recovered in 70%, this time period correlates with the maximal susceptibility to PTLD [Lucas 1996]. Various treatments have been tried for PTLD, but the most effective is restoration of T cell immunity to EBV. This can either be via reducing the host's level of total immunosuppression, or if this is not possible, by transfer of donor T cell clones which are cytotoxic for EBV infected cells [Lucas 1996].

Some types of Hodgkin's Disease (HD) lymphomas may be caused by EBV, the HD

associated Reed-Sternberg cell is often EBV positive [Weiss 1989]. EBV is strongly associated with nasopharygeal carcinoma where all the tumour cells are infected with EBV. This tumour is common in the Chinese of the Far East and many possible reasons for this susceptibility have been suggested [Desgranges 1975]. Nasal T cell lymphoma, also common in Asia, is also associated in some cases with EBV infection of malignant cells [Tomita 1997].

Particularly in adult patients immunosuppressed by HIV and rarely in children, infection with EBV causes a variety of different problems. There is an increased incidence of lymphoma in these patients, especially in the central nervous system, where 80% contain EBV genome [Levine 1990]. Lymphomas in other sites are less frequently EBV positive. EBV is associated with a condition known as oral hairy leukoplakia where the virus is infecting oral epithelium and leads to ridged overgrowth of the epithelium often under the tongue, this can respond to treatment with aciclovir [Niedobitek 1991]. EBV has also been associated with leiomyosarcoma of the gut, a rare tumour which is more common in the HIV infected [Timmons 1995]. Finally, EBV may be associated with a condition known as lymphoid interstitial pneumonitis, this is found in 30% of children with HIV and has been associated with a better long term prognosis for survival [Katz 1987].

A rare congenital defect in the host response to EBV has been described which is usually x-linked and affects boys [Seemayer 1995]. This is often known as Duncan's syndrome and a number of affected kindreds are described, the genetic defect of the condition is close to being defined and may give important general clues to the nature of the host response to EBV. Boys affected by this condition cannot contain primary EBV infection and may die from fatal primary infectious mononucleosis. If they survive the primary infection then they can go on to develop: a more chronic lymphoproliferative state; immunodeficiency with agammaglobulinaemia; and ultimately lymphoma.

Cytomegalovirus

As with EBV, it is also difficult to study the response to primary CMV infection in most persons as the infection is usually asymptomatic. Some patients do suffer an IM type illness, but again they may not have the "best" immune response to the virus and can take several months to mount a T cell response to CMV antigens. Individuals vaccinated with the live attenuated CMV vaccine take 2-4 weeks to produce a T cell proliferative response to CMV and this persists long term. In immune adults, 1/4,000 - 1 / 40,000 PBMC,s is a memory T cell for CMV. Lack of an active T cell response to CMV leads to reactivation of infection and development of CMV viraemia. After congenital infection the immunologically immature foetus has a prolonged failure to develop T cell proliferation to CMV antigens [Lopez 1994]. In the immunocompromised the frequency and severity of CMV infection depends on the nature and duration of the immunosuppression. In transplant patients the serostatus of the donor and the type of transplant are also important. After BMT most CMV infections usually happen within 1-4 months. The patient can develop a severe IM type illness, pneumonitis (

which has up to 40% mortality) , chorioretinits, hepatitis, gut infection and CNS infections [Arabia 1993; Enright 1993; Foot 1993; Mellon 1993; Einsele 1994; Ljungman 1994; Nagle 1994]. In all cases the greatest risk of CMV disease occurring is where the host is seronegative and the donor is positive, and a primary infection can occur during maximal immunosuppression. The risk of symptomatic infection is also increased: where there has been previous organ damage, eg to lungs or liver; where there is graft versus host disease; where there is liver rejection; where there is use of non-CMV-screened blood products; where T cell depleted BMT is administered; where there is prolonged lymphopaenia; and where irradiation is used as part of the conditioning regimen [Bostrom 1990; Miller 1991; Einsele 1993; Atkinson 1995; Bacigalupo 1995; Cakaloglu 1995; Falagas 1996].

Delay in diagnosis of CMV activity in the immunocompromised host can lead to severe symptomatic disease. Where culture and antigen detection techniques are used for diagnosis, these are often only positive by the time the patient is symptomatic, so to demonstrate evidence of viral activity earlier PCR is used. Earlier treatment of CMV before development of symptomatic disease decreases the morbidity and mortality from this infection. It also leads to use of shorter courses of gancyclovir, shorter episodes of neutropaenia, and a decreased incidence of secondary non-viral infection [Link 1993; Schmidt 1994; Einsele 1995; Boeckh 1996].

Prophylaxis against CMV infection in the immunocompromised includes use of CMV-negative blood products [Winston 1993]. Treatment with aciclovir is advocated by some and considered ineffective by others [Nicol 1993]. Gancyclovir, a toxic antiviral drug, is usually given for treatment but in some studies has been used prophylactically, although an adequate dose is important [von-Bueltzingsloewen 1993; Enright 1993; Goodrich 1993]. CMV specific intravenous immunoglobulin has also been used, without proven success [Boland 1993; Ljungman 1994].

Treatment of active CMV disease depends on gancyclovir and / or foscarnet. CMV disease is associated with an increased risk of other secondary infections, including fungal sepsis, as well as an increased risk of auto-reactivity [Ljungman 1992; Roberts 1993].

Monitoring of the host with PCR for CMV during immunosuppression and pre-emptive treatment of any rise in blood viraemia with gancyclovir is currently the accepted practice to control CMV disease in immunosuppressed patients.

Human Herpesvirus 7

This virus like HHV-6 also infects T cells but, unlike HHV-6 it uses the CD4+receptor to enter cells [Lusso 1994a]. Although not so extensively studied as the other herpesviruses, there is very little evidence that it causes problems for the immunosuppressed [Wang 1996; Fabio 1997]. There is also no current evidence that it has any relationship to development of malignancy in the host.

Human Herpesvirus 8

The DNA of HHV-8, also known as Kaposi's sarcoma associated herpesvirus, was first detected in lesions of Kaposi's sarcoma (KS) [Chang 1994]. KS occurs endemically in Africa and in certain Mediterranean populations, but since the arrival of HIV the incidence of this tumour has increased in Africa, the USA and Europe [Beral 1991]. Epidemiologically, KS in HIV infected patients is most strongly associated with homo-sexually acquired HIV and is uncommon in those who have acquired HIV via blood transfusion. KS has also been shown to occur in other immunosuppressed patients [Beral 1991; Beral 1990]. HHV-8 DNA has also been demonstrated in association with lymphoproliferative conditions, including body-cavity-based lymphomas and Castleman's disease [Cesarman 1995; Dupin 1995]. Although KS is rare in children with HIV in the USA and Europe its incidence has been rapidly increasing in African children [Serraino 1996; Ziegler 1996].

Serological studies , have demonstrated that over 90% of HIV uninfected patients with endemic KS are HHV-8 antibody positive, where 60-100% of HIV infected patients with KS are antibody positive. Where adults with HIV but no clinical KS have been tested, between 20-50% have been found to have antibodies to HHV-8, the higher seroprevalence being in Africans. Indeed among African adults with neither HIV nor KS, 6-43% have been found to be HHV-8 antibody positive. Very low seroprevalence rates of 0-5%, have been found in blood donor populations and haemophiliacs in Europe and the USA [Gao 1996a; Gao 1996b; Kedes 1996; Lennette 1996; Simpson 1996].

The largest group of children examined for antibodies to HHV-8 has been in the USA (n = 263), none had antibody to latent antigen and 3.8 % had antibody to the lytic antigen tested. HHV-8 seropositivity was 0.9%(1 /115) in children aged from 1-5 years, 6.5% (7/108) those aged 6-15 years, and 18% (7 /40) in young people aged 16-20 years (12). Seroprevalence to HHV-8 was 0% in 24 UK children presenting with rash and fever (11). In a study of 51 HIV infected children from the USA, median age 7 years (range 0.5-18), HHV-8 seroprevalence was 0% [Blauvelt 1997].

Recent studies from Zambia and Japan have identified the possible clinical illness of HHV-8 seroconversion in children [Kasolo 1997, Kikuta 1997]. In the Zambian study, 53 HIV uninfected infants (median age 10 months, range 2-12) presenting to hospital with their first fever had blood was examined by PCR for HHV-8. HHV-8 DNA was detected in 8%, with 300-18,000 copies of HHV-8 per cell, consistent with primary infection. In two cases HHV-6 DNA was also found in the blood. None of the mothers of these children were HIV infected. The authors suggest that in the African setting of endemic KS and HIV, infection with HHV-8 may be relatively common in childhood and that subsequent development of KS may have more to do with decline in host immunity and increase in HHV-8 viral load, rather than denovo sexual transmission. In the Japanese study peripheral blood lymphocytes from 56 children, with acute febrile illness were examined for HHV-8 DNA by PCR. The children were aged 2 months to 6 years and none had HIV or KS. HHV-8 DNA was detected in 38% of those less

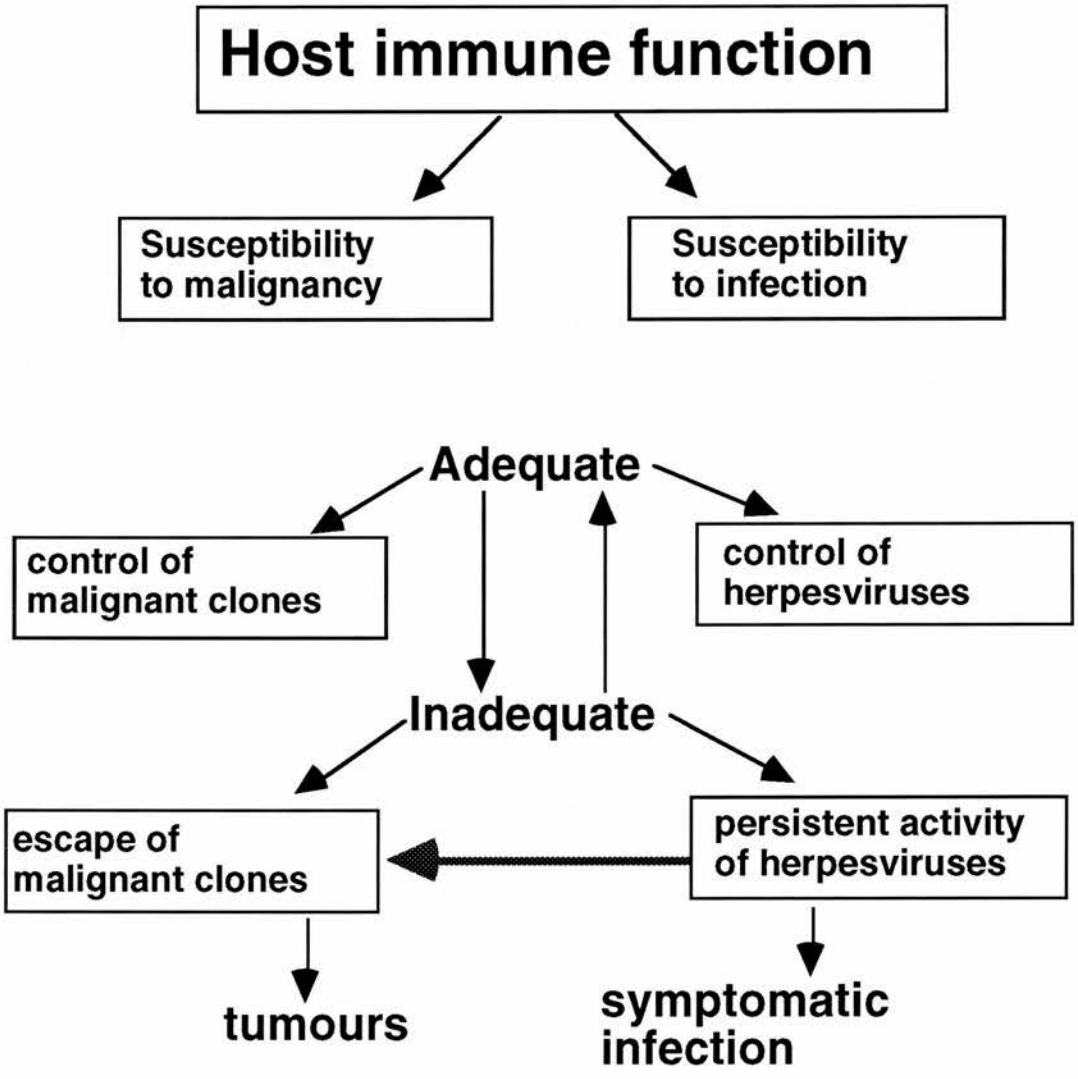
than two years, in 87% of those over two years, and in 80% of healthy adult controls. These results imply that infection with HHV-8 is widespread in Japan. A non-sexual mode of infection is most likely in a population where there is a high rate of infection in childhood, eg via saliva. There have been no studies to date of HHV-8 seroprevalence in paediatric oncology patients.

Conclusions

This limited review demonstrates that the different herpesviruses have differing degrees of pathogenicity for the immunosuppressed child. The nature of infections depends not only on whether they are primary or recurrent, but also on viral escape from host immunity due to immunosuppression, which is usually iatrogenic (figure 2).

The most severe problems are seen in the most severely T cell depleted patients. The cohort of paediatric oncology patients in this study do not in the main reach such severe levels of T cell depletion. However, many of the patients on treatment for leukaemia were intermittently lymphopaenic for about two years.

figure 2: **The balance of host immune function and herpesvirus activity.** **see over page 62**


Host immune function
Susceptibility to malignancy
Susceptibility to infection
Adequate
control of malignant clones
control of herpesviruses
Inadequate
escape of malignant clones
persistent activity of herpesviruses
tumours
symptomatic infection

The Immune response of paediatric oncology patients

Prior to immunosuppressive therapy

Immunodeficiency or immunosuppression in paediatric oncology patients depends on a variety of factors relating to the host and the underlying malignant condition. There may have been defects in the host immune response which have allowed a malignancy to develop which may also make the child more vulnerable to certain infections. These are most noticeable in children with severe congenital immunodeficiencies such as Wiskott-Aldrich syndrome, ataxia telangiectasia and common variable immunodeficiency where susceptibility to severe infections is associated with a greatly increased risk of lymphoproliferative malignancy [Kersey 1988; Rosen 1997]. However, more subtle cellular control abnormalities are likely to be involved in the development of most childhood leukaemias and lymphomas. Genetic predisposition may play a part in the host's susceptibility to these changes, but interestingly, the infections and responses to infections which the host has made may also be important in moulding these responses. Children with solid tumours may or may not have defects of cellular control which have allowed tumour development and could also increase susceptibility to infections. Patients with malignancy, even after successful treatment for their initial disease, have an increased risk of further malignancy. This partly relates to the cytotoxic drug regimens administered but also to host response factors [Lowsky 1994].
Patients with leukaemias and lymphomas, in particular Hodgkins disease have abnormal T cell responses at presentation with disease. Tests of T cell function demonstrate them often to be anergic with poor proliferative responses, therefore increasing their risk for more severe viral and intracellular infections (eg TB, Leishmania, salmonella etc). Children presenting with malignancy may have more or less subtle defects in their immune responses which can increase their susceptibility to infections even before commencing immunosuppressive therapy. Unfortunately this area has been very difficult to investigate and more information in many areas could be sought.

During and after immunosuppressive therapy

When immunosuppressive therapy has been commenced to treat a malignancy obvious deficits in the host immunity develop. Treatment is aimed at fast dividing cells and affects both normal and tumour tissues. Working from the simplest protective mechanisms to the more complicated, treatment affects mucosal integrity and therefore increases the capacity for organisms to penetrate the host, especially from the alimentary and respiratory tracts. Total numbers of leukocytes are reduced which leads to deficiencies at varying levels. There are greatly reduced phagocytes (neutrophils and monocytes) necessary to remove

organisms. This makes the host especially susceptible to bacterial and fungal infections. The cytokine milieu required to attract cells to a site of infection and amplify the host response is also affected by the paucity of cells, thus local infections can quickly spread and become systemic. The function of existing phagocytic cells is also affected by poor opsonisation and complement activation.

Overwhelming bacterial infections are the acute risk of chemotherapy. The risk of more severe viral infections in these patients increases with the length of the period of induced lymphopaenia. Fatal varicella zoster or measles infections most often occur after 18 -24 months of treatment for leukaemia, when the course of chemotherapy is almost over, and neutropaenia is rarely a risk but lymphopaenia has been prolonged. Lack of T and B cells is the crux of this susceptibility. Deficiency of T cells which can recognise viral antigens presented and then interact either to help B cells to proliferate to produce antibody or to proliferate themselves to produce a cellular cytotoxic response, enables viruses to escape immune control and progress, in some cases, to severe viraemia. The most lengthy periods of immunosuppression and lymphopaenia are seen in the treatment of leukaemia and lymphoma and are the price which has to be paid for eradication of malignant clones of cells. This degree of immunodeficiency however, it not so severe as the bone marrow ablative therapy which is required for transplantation. BMT patients are susceptible to bacterial / fungal infections early on until phagocytic reconstitution occurs. Lymphopaenia and lymphocyte dysregulation is more prolonged and severe. In all these patients, a degree of re-education of the cellular immune system has to occur and for as long as this persists the host is more vulnerable to infection. This period is particularly protracted and severe in patients who have received T cell depleted donor grafts to decrease the risk of graft versus host disease in less well matched transplants. In certain circumstances T cell clones specific for viral infections have been selected from the donor T cell pool to treat severely infected recipients as well as recipients with PTLD [Ilan 1994; Riddell 1994; Emanuel 1997].

Recovery of immune function after cessation of therapy

Analysis of recovery of immune function after therapy is important as this is required for return of the host to a normal state of immunity. It also demonstrates the immune deficits which are induced by chemotherapy. Studies have examined the cellular recovery as well as functional recovery in terms of response to vaccination.

Lymphocyte numbers and function

Most of the data comes from patients recovering from BMT. After T cell depleted grafts, the total lymphocyte count, total T cells and CD4+ cells can remain depressed for up to 2-3 years. CD8+ cells normalise more quickly, within 18 months, giving an inverse CD4+: CD8+ ratio for a year or so.The percentage or number of NK cells can be elevated early after BMT. B cells can also be depressed for 12 -18 months after the graft. Younger patients tend to have higher total lymphocyte counts after transplant, but presence of graft versus host disease or

CMV disease can delay the recovery [Milosevits 1995; Kook 1996]. In vitro proliferative responses to T cell mitogens normalise within 12 months of transplant and mixed lymphocyte culture responses within 9 months. Again, presence of graft versus host disease or CMV delay these responses. Recovery of proliferative immune function is correlated with a rise in the total lymphocyte count, although the CD4+ : CD8+ ratio can still be abnormal [Kook 1997].

Delay in reconstitution of CD4+ cells renders longer term susceptibility, particularly to viral infection , and the method of regeneration of CD4 cells is not well understood, it may be thymus dependent or thymus independent. Naive CD4+ cells which express the surface glycoprotein isoform CD45RA are likely to have been generated by a thymus dependent pathway. In a study of 15 patients aged 1-24 years recovering from intensive chemotherapy, expression of the CD4+ isoforms was serially measured [Mackall 1995]. Over the first six months after BMT, the younger patients recovered the CD4+ T cell count most quickly, and this correlated quantitatively with the presence of CD4+CD45RA+ cells in the blood. The presence of CD4+CD45RA+ cells was also correlated with thymic enlargement after stopping chemotherapy. In addition, patients with radiographic evidence of thymic rebound had significantly higher ratios of circulating CD45RA : CD45RO, ie naive : memory cells. The authors of this study concluded that rapid T cell regeneration requires residual thymic function, which is more often present in young children.

The thymic influence does not seem to affect regeneration of the CD8+ cells [Koehne 1997]. It has been shown in adults that regenerating, non-thymus dependent CD4+CD45RO+ cells have increased expression of HLA-DR, indicating activation of the population. However this also renders them more prone to apoptosis, thus during recovery this CD4+ cell population appears to be more vulnerable to cell death [Hakim 1997].

Gamma-delta ($\gamma\delta$) T cells appear to regenerate quickly after BMT, and are often elevated in patients with viral infections, so may be active in responding to infections when other cell types are still depleted [Cela 1996]. Recovery of B cells appears to follow an ontogenic pattern with deficiency of certain adhesion molecules as see on some cord blood B cells, the significance of this requires further study [Parra 1996].

Recovery of B cells and immunoglobulin production after chemotherapy for acute leukaemia in children has been studied in a small group of children [Alanko 1991]. At the end of therapy the blood lymphocyte counts of all children were reduced, the mean value being 60% of the lower limit of normal. The reconstitution of lymphocytes was parallel to that of total leukocytes and all but one child reach a normal level by one year. In 12 / 14 patients the number of B cells was normal at cessation of therapy, however in terms of function, most children had low levels of total IgG at cessation of therapy. In most, at cessation of therapy, the IgG1 IgG3 and IgG4 levels were normal but, in half IgG2 was decreased. In most children IgM and IgA levels were in the low normal range at cessation of therapy. Recovery of antibody usually occurred within the first year, but some children showed poor recovery, and they also had an increased risk of relapse and infection.

Specific antibody responses

In a small Dutch study of 49 children previously immunised to diphtheria, pertussis, tetanus and polio and treated successfully for leukaemia with relatively mild therapy (only 2 or 3 drugs in remission), antibody responses to the vaccines were measured [van der Does-van den Berg 1981]. At cessation of chemotherapy the antibody titres were generally lower than those of healthy children, but in most, not below levels considered to be protective. One year later, there was no spontaneous rise in antibody levels, but after revaccination, responses equivalent to those of normal children were seen. In another study diphtheria-pertussis-tetanus vaccine was administered to 27 children with various malignancies in clinical remission on maintenance therapy (17 leukaemias, 5 lymphomas, 5 solid tumours) [Kung 1984]. After 4 weeks only one child had no response to the two antigens tested (diphtheria and tetanus). Two children did not respond to tetanus but did to diphtheria. Most children made good antibody responses to both antigens regardless of the disease or therapy. The study was too small to make comments about the reasons why three of the children had poor responses.

A study of responses to Haemophilus influenzae type b (Hib) vaccine in Hib vaccine naive older children, either during maintenance chemotherapy or after completing chemotherapy was undertaken in a group of 27 children [Weisman 1987]. Only 37% of children had a pre-vaccine level of antibody considered protective. There was a generally good response to vaccine in this group, and 85% of children had protective levels after this intervention. The best response was in children with solid tumors who all achieved protective levels, compared to only 81% of children with leukaemia.

In a more recent study of 13 children treated for acute lymphoblastic leukaemia (ALL) responses to measles, mumps, rubella, polio, Hib, and pneumococcus were examined 2-5 years after completing therapy [Smith 1995]. More aggressive chemotherapy was given to this group of children. Compared with age-matched control subjects, the long term survivors of ALL had significantly lower baseline titres of protective antibody to measles and polio and more than half had no protective antibody to one or more previously administered vaccines or related prior childhood infections. Most produced protective responses of specific antibody after re-immunisation Some were repeatedly unable to make protective antibodies or mount a normal antibody response, despite natural infection and / or re-vaccination, demonstrating persisting functional immune defects.

Examination of immune status in these 13 children demonstrated them all to have normal absolute numbers of CD4+ and CD8+ T cells, although three had a reversed CD4+ : CD8+ cell ratio. Lymphocyte proliferation to the mitogen Phytohaemaglutinin (PHA) was normal in all but one child and the delayed hypersensitivity response to varied antigens was normal in all the children. Total haemolytic complement was normal in all cases and quantitative serum immunoglobulins were normal for 8 children (2 had low IgG4 levels for age; 2 had increased IgG4 levels for age; 1 had low IgG1; and 1 had low total IgG).

4) Immune responses of paed onc patients **EGH Lyall**

Two children with poor responses to specific antibody after vaccination or true infection were treated with IVIG for a year and 6 months later revaccinated, even after this they did not make specific antibody responses to all vaccines. They appeared to represent a sub group of children with persistently abnormal immunity after treatment. This study comprised a very small group of children but demonstrated that recovery after therapy was variable over time, these investigations need to be repeated in a larger group of patients.

A live attenuated vaccine against varicella zoster has been used to protect leukaemic patients in remission and on maintenance therapy [Gershon 1989]. The vaccine appeared to remain effective even when detectable antibody levels wained, implying a persisting active T cell response. With recovery of immune function reappearance of an anamnestic antibody to VZV was often seen. In this study it appeared that immune function against this herpesvirus was well maintained despite chemotherapy, however these studies were carried out at time when chemotherapy regimes were less intense than currently.

Conclusions

Children presenting with malignancy may have more or less subtle defects in their immune responses which have increased their susceptibility to infection as well as malignant escape even before commencing immunosuppressive therapy. During treatment and for a variable time after stopping treatment lymphopaenia and lymphocyte dysregulation are the most important risk factors for severe viral infection, and T cell lymphopaenia carries the greatest risk for the host for new or recurrent viral infections. The more protracted and severe the duration of lymphopaenia the greater the risk. In all these patients for immune recovery, a degree of ontogenic re-eduction of the cellular immune system has to occur and for as long as this takes the host continues to be more vulnerable to viruses. Monitoring of immune function during treatment and after cessation is important as when function is seen to return then prophylactic therapies can be discontinued and re-immunisation schedules can be initiated.

Methods for the clinical detection of HHV-6

Detection of HHV-6 antibodies

Indirect immunofluoresence assay (IFA)

The first method used to test sera for an antibody response to HHV-6 was the indirect immunofluorescence assay (IFA) [Salahuddin 1986; Downing 1987; Tedder 1987]. This was adapted from the assay first designed to study EBV infected cells [Henle 1966]. In this assay a counted number of HHV-6 infected lymphocytes, either from cord blood or T cell lymphoblastoid cell lines are spotted on to wells within slides and fixed. The cell layer is then incubated with dilutions of human sera and after washing, with an appropriate dilution of fluorescein conjugated anti-human IgG. After further washing and preparation, the slide can then be visualised under ultraviolet light where reactive sera will show a recognised pattern of immunofluorescence. Testing of different dilutions of sera can ascertain the titre of reactive antibody. Sera can also be tested against uninfected cells as negative controls as some sera show non specific reactions with the cells themselves. This system can also be adapted to assay sera for IgM by first absorbing out IgG antibody and using a fluorescein conjugated, anti-human IgM. Results of IFA for HHV-6 from different groups have not always been concordant. This may be for a variety for different methodological reasons, as it is difficult to standardise the nature of antigen presentation by infected cells. Thus the use of different cell lines, different strains of virus and different ages of cells at harvest may affect results. When HHV-6 infected cells are maintained for long periods in culture this can also decrease the level of viral antigen expression [Yadav 1991]. Prior to making slides it is therefore important to assess HHV-6 replication and this can be done by examining for cytopathic effect in cultures, or by use of monoclonal antibodies to stain for viral antigens.

Enzyme linked immunosorbent assay (ELISA)

Most early ELISA assays to detect antibodies to HHV-6 were noncompetitive and developed by similar methods to those already existing for other herpesviruses, such as CMV [Chou 1988]. HHV-6 was grown in cell culture to a maximal harvest, usually around 5-7 days. In the majority of assays subtype A HHV-6 cultured in T lymphoblastoid cells has been used. Preparation of viral antigen was either by separation of virus from cellular material by density gradient centrifugation or more commonly, by preparation of a more crude infected cellular lysate [Saxinger 1988; Asano 1990a; Chou 1990; Dahl 1990]. Yield of viral antigen from the cellular lysate is increased by glycine extraction of the infected cells followed by ultrasonic disruption [Kettering 1977]. Soluble viral antigen can then be adsorbed to the wells of the solid phase, and for protein antigens this should be at a concentration between 1-10 μg / ml [Wreghitt 1990]. The antigen can be stored frozen until required. Control antigen from

uninfected cells is also prepared in the same way.
Dilution of the serum applied to the antigen is required to reduce non-specific staining, and the optimal incubation time has to be ascertained. Washing with buffer solution is critical to ELISA between each stage to reduce nonspecific reactions and again for every ELISA the optimal regimen has to be derived.
The detection phase of the ELISA depends on a further incubation with an enzyme labelled anti-human IgG antibody, followed by application of the enzyme substrate which leads to the development of a colour reaction. This reaction is stopped after a certain time by a change in pH and the resulting colour density can be measured optically in a spectrophotometer. For any ELISA all the optimal times of incubation, concentrations of reagents, temperatures etc, have to be ascertained. Using well characterised control positive and negative sera, it is necessary to demonstrate that the system can perform repeatedly within as tight as possible a coefficient of variation, usually < 10%, to be considered robust enough for use with clinical samples [Wreghitt 1990]. Interpretation of the results obtained can be by a variety of methods, and a cut off value for positive and negative results can be calculated, results can also be expressed as a ratio (or index) when samples are tested against infected and uninfected control antigen.
ELISA assays for HHV-6 IgG antibodies have been at least as sensitive, if not more so, than IFA, they are also much easier to carry out when large numbers of samples are to be tested [Asano 1990a; Chou 1990; Dahl 1990 ; Cermelli 1994]. A competitive ELISA for HHV-6 IgG (using HHV-6 rabbit polyclonal antibodies and HHV-6 cellular antigen) has also been developed and it appears to have greater sensitivity for antibody than IFA [Nielsen 1996]. In a competitive ELISA the antigen is adsorbed to the plate as before, but a comparison is then made between a well to which sample and enzyme labelled antibody are added and a well to which only enzyme labelled antibody is added. After addition of the substrate, the difference in amount of product occurring between the two wells equates to the amount of antibody present in the clinical sample [Wreghitt 1990]. As well as increased sensitivity this technique can also give a clearer distinction between positive and negative samples. Commercial ELISA tests for antibodies to HHV-6 have now also been evaluated [Sloots 1996].
Earlier ELISA assays were not satisfactory to test for HHV-6 IgM antibodies, but an efficient non-competative assay for this class of antibodies has now also been developed [Parker 1993].

Anticompliment immunofluorescence test (ACIF)

Slides with HHV-6 infected cells are prepared in the same way as for IFA. Diluted patient sera are then incubated on the slides and subsequently washed with PBS. Diluted guinea pig serum is then spotted on the slides as a source of complement and incubated for a similar time, then subsequently washed. The slides are then mounted with fluorescein conjugated goat anti-guinea pig C3 with a further similar incubation and wash. After mounting the slides are examined for fluorescence under ultra violet illumination [Okuno 1989]. In a comparison

of this technique with IFA , no difference in sensitivity was found, although the authors suggested a more distinct pattern of immunofluoresence was seen with ACIF [Robert 1990]. However, as this technique is equally labour intensive to IFA , less sensitive than ELISA, and cannot discriminate between classes of antibody it has not been widely adopted for clinical purposes.

Radio-immunoassay (RIA) and Circular-immunoassay (CIA)

A competitive radio-immunoassay and a circular-immunoassay for HHV-6 were developed and found to be more sensitive than IFA for anti-HHV-6 IgG [Coyle 1992]. Crude viral infected cellular antigen was used for both. RIA is obviously a less desirable test for clinical use because of the requirement for radio-labelling. The CIA follows a similar protocol to ELISA except that the antigen is fixed to Zetaprobe membrane rather than within wells of a microtitre plate.

Tests for HHV-6 neutralising antibodies

Serial dilutions of test serum from infants with ES were added to cord blood cell cultures inoculated with $10^{2.5}$ tissue culture infective doses $_{50}$ / 0.1 ml of cell free virus. After incubation for 7 days the neutralising antibody titre was determined as the reciprocal of the dilution of serum which completely prevented cytopathic effect and syncytium production [Asano 1989a]. Neutralising antibody is produced within 3-5 days of presentation with fever and persisted for up to 6 months, the appearance of neutralising antibody is associated with disappearance of virus from the blood [Asano 1989a]. Neutralising antibody appears earlier and to a higher titre than IgG antibody detected by IFA in children with ES although both anti-HHV-6 IgG and IgM will be detected by this test [Suga 1990b; Suga 1992b]. Sero-epidemiological investigation for neutralising antibodies to HHV-6 in different age groups give very similar results over all to those obtained by ELISA, implying that this may be a more sensitive test than IFA [Yoshikawa 1990]. Although a more refined monolayer culture method has been developed to detect HHV-6 neutralising antibodies this remains a highly labour intensive test and is not therefore applicable to routine clinical use [Asada 1989].

Detection of HHV-6 by virus culture

Due to the cellular tropism of HHV-6 for T lymphocytes, culture of this virus from clinical samples depends on having either a ready source of cord blood lymphocytes, adult peripheral blood mononuclear cells or T lymphoblastoid cell lines available. According to the subtype of HHV-6, growth in certain cell types is preferential (see above). Culture is facilitated by activation of the cells with IL-2, PHA, or anti-CD3. Detection of virus in the culture can either be by evidence of the ballooned cells and / or syncitia of the cytopathic effect, by electron microscopy or by using monoclonal antibodies to HHV-6 antigens. The culture

method is expensive in terms of time and resources and although specific may not be as sensitive as either molecular or antigen detection methods.

Detection of HHV-6 antigens

Recently an antigen capture assay method for detection of HHV-6 antigens (gp 116 / 64 / 54) has been developed which can be used in the clinical setting as well as to monitor cell cultures for virus isolation and quantification [Marsh 1996]. A dot blot assay method for detection of HHV-6 antigens has also been developed and used to evaluate efficacy of antiviral drugs against this virus [Yoshida 1996].

Detection of HHV-6 Genome

Polymerase Chain Reaction (PCR)

The principles of amplification of genomic material by the polymerase chain reaction (PCR) are briefly described. The technique was first used in 1985 for identification of genetic variation in b-globin gene sequences associated with sickle cell anaemia and has been continuously under development since [Saiki 1985]. For the chain reaction amplification of a DNA fragment certain conditions and substances are required: template DNA extracted from the sample for analysis; a thermostable DNA polymerase; buffer solution; nucleotide bases; primer oligonucleotides; and a thermal cycling heating block.

Extraction of DNA from samples requires digestion of proteins followed by precipitation of the DNA and there are different methods for this process. The process of DNA extraction from certain clinical samples is more difficult than others as substances which are inhibitory to PCR can occur, eg in saliva. Where small amounts of viral DNA are sought within clinical samples then great care maybe required at this stage not to loose the rare viral template during the extraction process.

DNA polymerase from the organism *Thermus aquaticus* (Taq) is heat stable at a wide temperature range. This enables the enzyme to continue assembling new genomic material from the original template despite cycles in temperature which would inactivate most enzymes after the first round of amplification. There are stringent requirements for optimal enzyme activity in terms of buffer solution and magnesium concentration, and for any particular reaction these need to be optimised. Nucleotide bases in appropriate concentration must be added to allow the polymerase to build new copies of the template and the oligonucleotide primers are crucial to the commencement of the amplification process as they identify the area of attachment and amplification.

During the cycling process the template double DNA strand is first denatured at 94 - 98 ^{o}c for up to a minute. This allows the primers to bind to the separated single DNA strands once the temperature is lowered to an annealing temperature of around 50-60 ^{o}c for most reactions,

again for up to a minute. The extension temperature is 72 ^{o}c when new nucleotide bases are added to the primers along the template, again for up to a minute, but allowing enough time for extension of the entire sequence. The timings of the different parts of the cycle should always be optimised for any particular reaction. The number of cycles undertaken is often 30-40 allowing exponential increase in the original number of template sequences.
The expected amplified genome product is usually detected after being run on an electrophoretic gel. Detection methods include demonstration of the expected length of amplimer under ultra violet light with ethidium bromide intercalation, or hybridisation with a labelled oligonucleotide probe, or production of a specific restriction enzyme digest pattern. The original 9000bp probe for HHV-6 developed from a fragment named pZVH 14 from a Bam H1 restriction enzyme digest of the GS strain (subtype A) was demonstrated at that time not to react with any other known herpesviruses [Josephs 1986] . This fragment contains the open reading frame (ORF) for the large tegument protein gene [Josephs 1991a]. Many clinical investigations for HHV-6 genome have amplified sequences of genome from within this region [Buchbinder 1988; Jarrett 1990; Collandre 1991; Aubin 1991; Aubin 1992a; Torelli1991; Di Luca 1994]. Other subtype A, HHV-6 sequences have also been amplified from conserved, specific regions identified within the U1102 strain of the virus [Downing 1987; Tedder 1987; Lawrence 1990; Sandhoff 1991; Sa'adu 1993; Cone 1993a; Cuende 1994; Wilborn 1994a]. Different DNA sequences from subtype B HHV-6 strains have also been amplified from clinical specimens [Kondo 1990; Kondo 1991; Collandre 1991; Pruksananonda 1992; Cone 1993a].

Restriction enzyme digestion and HHV-6 strain variation

Variation between A and B strain HHV-6 have been demonstrated by different patterns of digestion by restriction enzymes which recognise and cut specific short sequences of nucleotide bases [Kikuta 1989a; Jarrett 1989; Pellet 1990; Ablashi 1991a; Aubin 1991; Aubin 1992a; Aubin 1993; Gompels 1993]. It has been possible to use these differences to differentiate between the two subtypes in clinical samples. For example, an 830 bp sequence within the large tegument protein (LTP) gene amplified from the pZVH 14 fragment of HHV-6 genome [Collandre 1991] contains a Hind III restriction enzyme digestion site at position 2945 of the LTP gene in "B" but not "A" strains (figure 1). This is the result of a substitution of a thymidine nucleotide base for a cytosine at this position [Aubin 1991; Aubin 1992a; Aubin 1993].This strain difference has been used in large clinical studies to discriminate between the two subtypes of HHV-6 [Dewhurst 1992; Dewhurst 1993].
Further to the discovery of these genomic strain variations sequence specific oligonucleotide probes have been devised to hybridise with the specific subtypes [Drobyski 1993a; Aubin 1994; Gautheret 1996]. However, in one comparative study hybridisation was found to be less sensitive and specific than subtype specific primers and PCR amplification [Aubin 1994].

Improvements in the technique for PCR

Many modifications to the basic method have led to great improvements in sensitivity and specificity of this technique [Cone 1992]. In particular, addition of a second round of amplification using "nested " primers which anneal to sequences within the fragment originally amplified will especially increase sensitivity. Use of various procedures such as a "hot start" can also improve the specificity of the reaction [D'Aquila 1991; Hebert 1993]. One of the greatest problems with PCR is the risk of contamination associated with such massive amplification of genome, this has been addressed by strict adherence to procedures which ensure separation of all pre-amplified and post-amplified products [Cone 1990]. Liberal use of negative control samples at all stages of the process is important to detect contamination. Co-amplification of host "house keeping" genes is often undertaken to ensure that a negative result is due to absence of viral template and not to inhibition of the PCR by substances within the clinical sample.

HHV-6 DNA PCR has been continuously developed and methods to quantify HHV-6 viral DNA in clinical samples now exist [Secchiero 1995b]. Multiplex PCR systems for detection of more than one herpesvirus in a single clinical sample have also been developed [Tenorio 1993; McElhinney 1995]. Using this highly versatile technique it has been possible to detect the presence of HHV-6 DNA in many tissues of the body during different clinical circumstances.

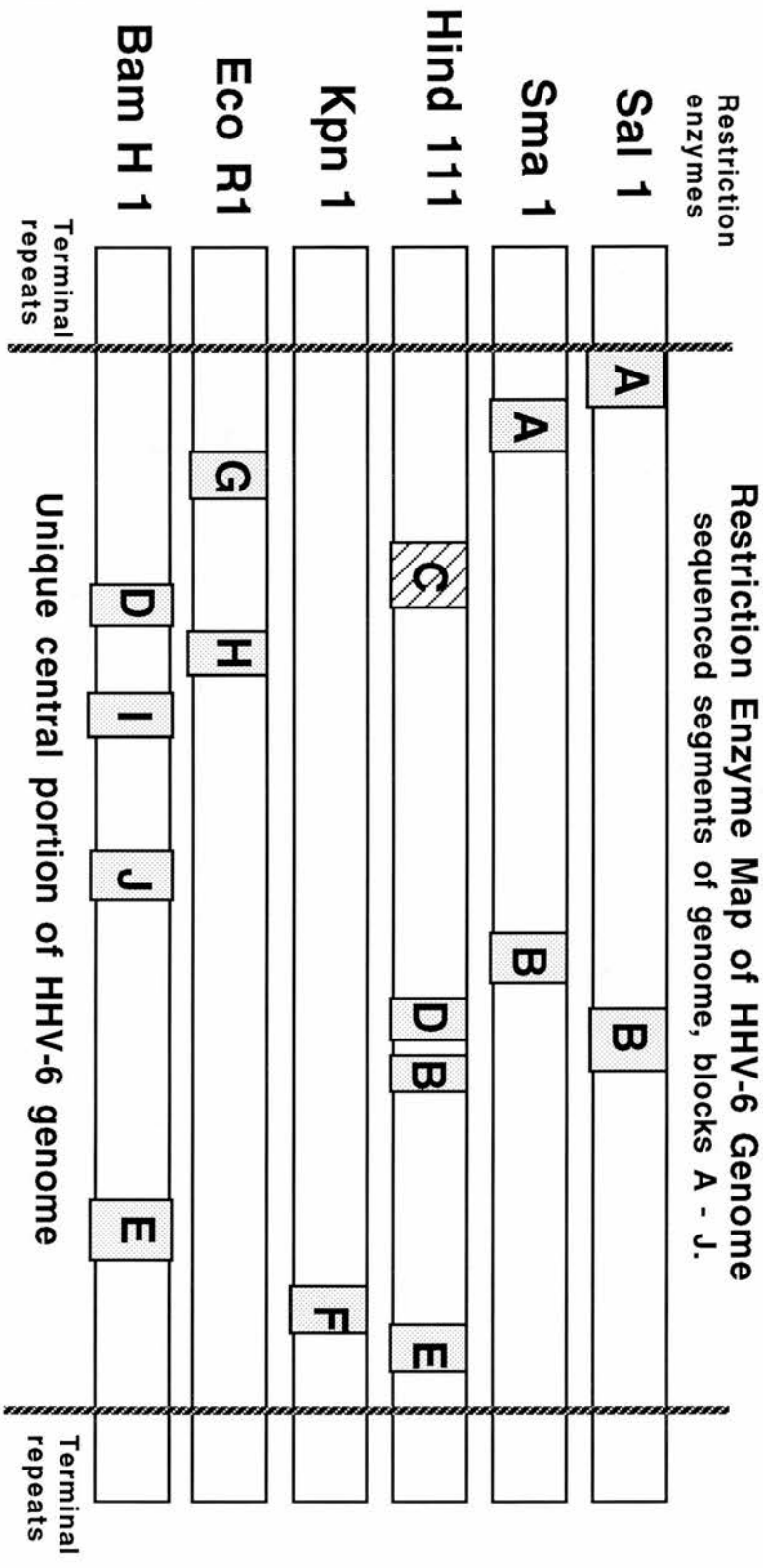
Legend to figure 1: Simplified restriction enzyme map of the HHV-6 genome, with parts of the HHV-6 genome sequenced at the time of commencement of this study.

6 long boxes each representing the digest map obtained with a different restriction enzyme. The names of the restriction enzymes are indicated on the left hand side of the diagram (Sal1, Sma1, Hind III, Kpn1, Eco R1, BamH1).

Segments of the genome which had been sequenced at the time are represented as fine dotted boxes (A,B, D,E,F,G,H,I,J). The segment of genome selected for amplification and restriction enzyme digestion in this study is represented by a lightly hatched box (C).

A [Efstathiou 1992]
B [Lawrence 1990; Littler 1990]
C Region chosen for amplification in this study
[Collandre 1991; Aubin 1993]
D [Josephs 1986 ; Josephs 1991a; Collandre 1991; Aubin 1993]
E [Thomson 1992]
F [Martin 1991a]
G [Neipel 1992]
H [Teo 1991]
I [Chou 1992]
J [Gompels 1993]

Figure 1: Simplified restriction enzyme map of the HHV-6 genome sequenced at the time of commencement of this study, see over.


Restriction Enzyme Map of HHV-6 Genome
sequenced segments of genome, blocks A - J.
Restriction enzymes
Sal 1
Sma 1
Hind 111
Kpn 1
Eco R1
Bam H 1
Terminal repeats
Unique central portion of HHV-6 genome
A
B
A
B
C
D
B
E
F
G
H
D
I
J
E
Terminal repeats

Retrospective Serological Study

Aim of this study

As described, this retrospective study was designed to examine whether children presenting with malignant disease had mounted an immune response to HHV-6, and if so whether it reflected the same pattern as that of normal children. Sera from a cross-sectional group of new oncology patients were compared with a group of control patients. The response of these children to four other herpes viruses (Herpes Simplex virus (HSV), Varicella Zoster virus (VZV), Cytomegalovirus (CMV), and Epstein Barr virus (EBV)) was also examined. Sera from a further group of patients were followed longitudinally to assess the effect of chemotherapy on the HHV-6 antibody response.

Patients and Samples

The approval of the local paediatric ethical committee was obtained for this study. Samples from paediatric oncology patients seen at the Royal Hospital for Sick Children are routinely sent for viral serology at presentation before administration of blood products, and at regular intervals during treatment. These samples are tested at the Regional Virus Laboratory (RVL), City Hospital, Edinburgh, and any surplus serum is stored long term at -20°C.

Cohort of new children presenting with malignancy & controls

Computer search was used to identify serum samples taken from oncology patients at presentation, from January 1987 to December 1991. In some cases samples had not been taken at presentation or were exhausted (table 1). Sixty six sera were identified (31 from acute leukaemics (AL) and 35 from patients with solid tumours (ST)). These samples were age and season matched to 66 stored sera from patients who presented to the hospital in the same year with minor illnesses (e.g. upper respiratory tract infection, pyrexia, or viral infection). The age range of patients was 1 month to 13 years with a bimodal distribution with peaks at 3 and 10 years of age, representing the typical age distribution of children presenting with malignancy (figure 1). These sera were tested for anti-HHV-6 IgG by IFA and ELISA, and for anti-HHV-6 IgM by IFA.

All the sera from presenting patients and controls, were also tested for anti-VZV IgG and anti-CMV IgG by commercial ELISA kits following the manufacturers instructions (SIGMA).

Previous results were available from some patients for EBV and HSV antibody responses. Anti-EBV IgG was tested by IFA on infected EB3 cells and antibodies to HSV were titrated by

the complement fixation test (RVL standard methods, unpublished).

Cohort of patients followed sequentially whilst on chemotherapy

Sequential sera from 45 patients (26 acute leukaemics and 19 with solid tumours) on continuing chemotherapy who presented between January 1989 and December 1991 were examined for anti-HHV-6 IgG by ELISA. Three to thirty three samples were tested per patient depending on the length of therapy. These samples were obtained approximately monthly and there were more from the leukaemics who underwent longer courses of therapy. In 13 of these patients where the sample taken at presentation was exhausted (9 acute leukaemics and 4 with solid tumours), samples were available from within the first month of treatment, usually but not always, before administration of blood products. The age range of the patients was the same as for the presenting group with a similar bimodal distribution (data not shown). Sequential sera from seven cases were selected and tested for anti-HHV-6 IgM by IFA. (Two asymptomatic seroconversions to CMV, four cases of primary varicella or shingles, and one case who remained IgG negative to HHV-6 throughout treatment.)

table 1: Patients presenting with malignancy from whom samples were available to test for HHV-6 serology.

Percentages in brackets are calculated from the number of children presenting each year.

Year	Patients presenting with malignancy	Patients from whom samples sent to virology	Patients with stored serum remaining
1987	38	25 (66%)	21 (55%)
1988	24	17 (71%)	10 (42%)
1989	30	23 (77%)	14 (47%)
1990	29	14 (48%)	11 (38%)
1991	19	14 (74%)	10 (53%)
total	140	93 (66%)	66 (47%)

table 1:Patients presenting with malignancy from whom samples were available to test for HHV-6 serology.

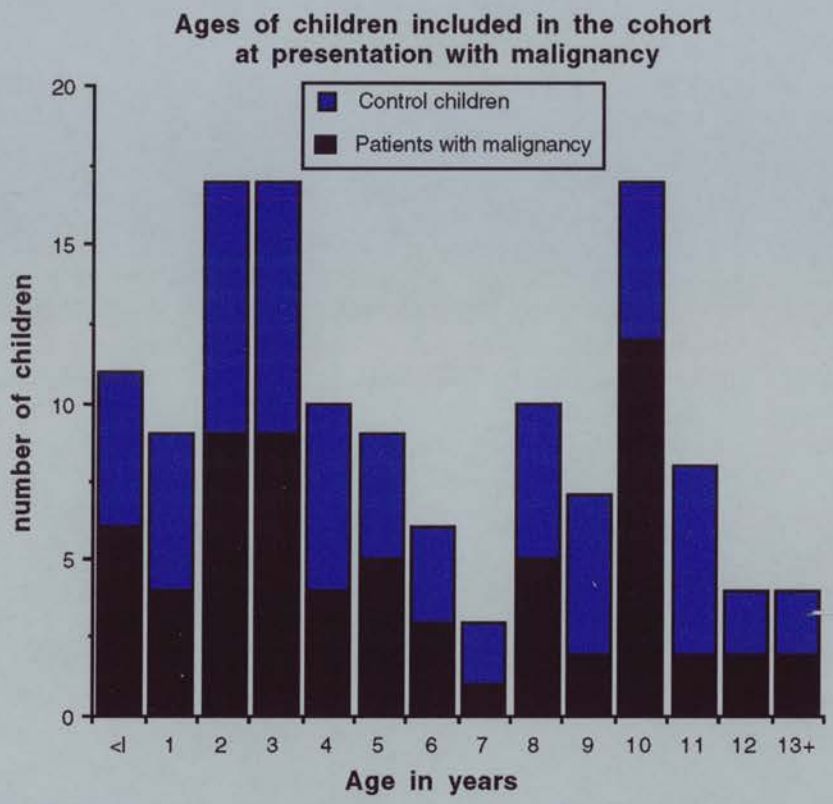


Figure 1: Age distribution of cases and controls in the presenting cohort.

Methods

Culture of HHV-6 in JJhan Cells

Cells of the lymphoblastoid cell line JJhan were grown in culture medium (RPMI with Hepes buffer, foetal calf serum (10%),glutamine (2 mM), and penicillin (100 iu /ml) and streptomycin (100 µg /ml)). The cells were activated with phytohaemaglutinin (5 µg / ml) and then infected with the AJ strain (sub type A) of HHV-6 from an infected aliquot of cells (a gift from Prof R Tedder) [Tedder 1987]. At the time of infection the maximal ratio of uninfected to infected cells was 10:1. After seven days, infection could be shown under light microscopy by the presence of HHV-6 induced cellular changes, numerous large ballooned cells and syncitia having formed (figure1a,b,c, from chapter 1). Successful infection of the culture was confirmed with mouse anti-HHV-6 monoclonal antibodies to an HHV-6 early antigen (H 64 A5), Late antigen (H61 G11), and envelope antigen (H68 H3) (kindly donated by Dr PA Coyle, Regional Virus Laboratory, Belfast) (figure1c from chapter 1 demonstrated the monoclonal H 64 A5 on infected cells). The cultures were then harvested for the IFA and ELISA tests.

Several batches of HHV-6 infected cells were cultured and it was apparent that some cultures were more successful than others, with more cytopathic effect (CPE) and and a stronger immunofluorescent response with the mouse anti-HHV-6 monoclonal antibodies. Electron microscopy of some of the different batches also demonstrated very scanty virus in the less successful. Slides made from the different batches were compared for the immunofluorescent response with the anti-HHV-6 monoclonal antibodies and only those which had abundant presentation of viral antigens were used for the tests (table 2).

HHV6 CULTURES TABLE	HV6	JJHAN	HV6	JJHAN	HV6	HV6	HV6	HV6	JJHAN	HV6	JJHAN
BATCH NO	1	1	2A	2A	2B	2C	3A	3B	3B	3C	3C
ANTIGEN MADE	26.5.92	26.5.92	3.8.92	3.8.92	7.8.92	10.8.92	-	19.9.92	22.9.92	2.10.92	2.10.92
PROTEIN CONT(mg/ml)	0.85	0.83	-	-	-	-	-	0.81	0.75	3.2	0.85
TOTAL CELL COUNT x 10(8)	0.2	1	1	1.78	0.7		-	2	1.8	1.3	0.2
VOL OF ANTIGEN IN GLY BUFFER(ml)	3	3	-	-	-			10	10	4.5	2
NO. ALIQUOTS(500ul)	6	5	-	-	-			19	18	9	4
NO DAYS HV6 IN CULTURE	8	8	10	10	14	7	7	8		13	
RATIO INFECTED:UNINFECTED CELLS	1:10		1:10		1:10	1:2.5	1:7	1:7		1:3	
PASS NO	1(1991)		1(1992)		1(1992)	2(1992)	1(1991)	2(1992)		3(1992)	
EM GRID	ND		N0		ND	ND	ND	ND	ND	ND	
EM SECTION	ND		ND		YES	ND	ND	YES	YES	YES	
					d/c, v/l/v			d/c	n/c	d/c	
BEST ESTIMATE OF VOL OF ANTIGEN											
OPTIMAL DILUTION	1/160										
TOTAL VOLUME OF ANTIGEN(ml)	480										
TOTAL NO OF PLATES(10ML/PLATE)	48										
TOTAL NO OF WELLS(N X 96)	4608										
NO DUPLICATE SERA TO TEST	2304										
MONOCLONALS, IFA ON CELLS											
HA5 EA(1/400)	+++				+ / -	+++	+++	-	+++		
HH3 LA(1/400)	+	-			-	+++	+	-	++		
HG11 ENV(1/400)	++	-			-	+++	+ / -	-	++		
ELISA PERFORMED	YES	-	ND		ND	ND	ND	YES	YES		
SLIDES MADE	YES		ND	ND	ND	YES	YES	YES	YES	YES	
NO OF SLIDES		YES			96	40 (11.9)	53	4	25	ND	
comparison of IF slides (cells)	poor				good	too thick	clumped		clumped		
Fluorescence for IgG	good				v poor	good	moderate		good @		
fluoresence for IgM											
COMMENTS	*		*		* *		* * *		* * * *		
* TITRATION PLATE											
** AUTO FLUORECENCE											
*** CLUMPED CELL DEBRIS											
**** SUPERNATE SAVED											
d/c disrupted cells											
v/l/v very little virus											
n/c normal cells											
@ less infected cells/slide											

table 2: Assessment of batches of HHV-6 infected cells produced for IFA and ELISA.

Indirect Immunofluorescent Antibody (IFA) Test for HHV-6

For this test between 10^4-10^5 JJhan cells from an HHV-6 infected culture were spotted onto each well of a 12 well slide. After drying in air, slides were fixed in acetone for five minutes at room temperature, and then stored at -20°C until required. On each slide well the ratio of infected to uninfected cells was usually less than 50%.

Sera were tested for anti-HHV-6 IgG at a dilution of 1 in 10 in phosphate buffered saline (PBS) pH 7.2, with 0.2% bovine serum albumin (BSA). A positive control serum and diluent only control were always run on each slide along with ten clinical samples. Slides were incubated in a moist chamber at 37°C for one hour, washed 3x with PBS and dried. Fluorescein isothiocyanate(FITC)-conjugated, anti-human IgG (Scottish Antibody Production Unit -SAPU) was applied to slides at a dilution of 1 in 80 and incubated for 30 mins at 37°C. Slides were then washed 2x in PBS and 1x in phosphate buffer (PB) pH 8.4, dried and mounted in glycerol / PBS (50:50) for examination under ultraviolet light. Large bright dots of fluorescence, mostly intracytoplasmic but also intranuclear, particularly apparent in ballooned cells and syncitia, were observed with anti-HHV-6 IgG positive sera (figures 2 ,3). The representative photograph of HHV-6 fluorescent cells was kindly donated by Dr KN Ward, University College, London.

Sera tested for anti-HHV-6 IgM were diluted 1 in 10 with IgG blocking agent (Incstar), incubated for 15 minutes at room temperature and after pulse centrifugation, the supernatant was applied to slides for 3 hours at 37°C. The slides were washed 3x with PBS, dried and FITC-conjugated, anti-Human IgM (SAPU) diluted 1 in 32 was applied for 1hour at 37°c. Slides were then washed and mounted, as above, for examination under ultraviolet light. The pattern of fluorescence with IgM positive sera was of finer cytoplasmic and surface brightness, often most marked in the ballooned cells.

figure2 : Image of the immunofluorecent pattern for IgG on HHV-6 infected JJhan cells with an IgG positive human serum.

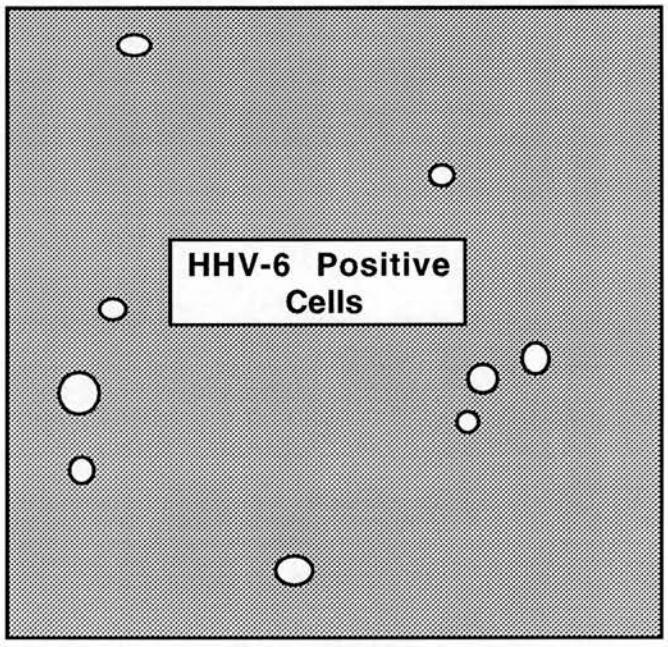
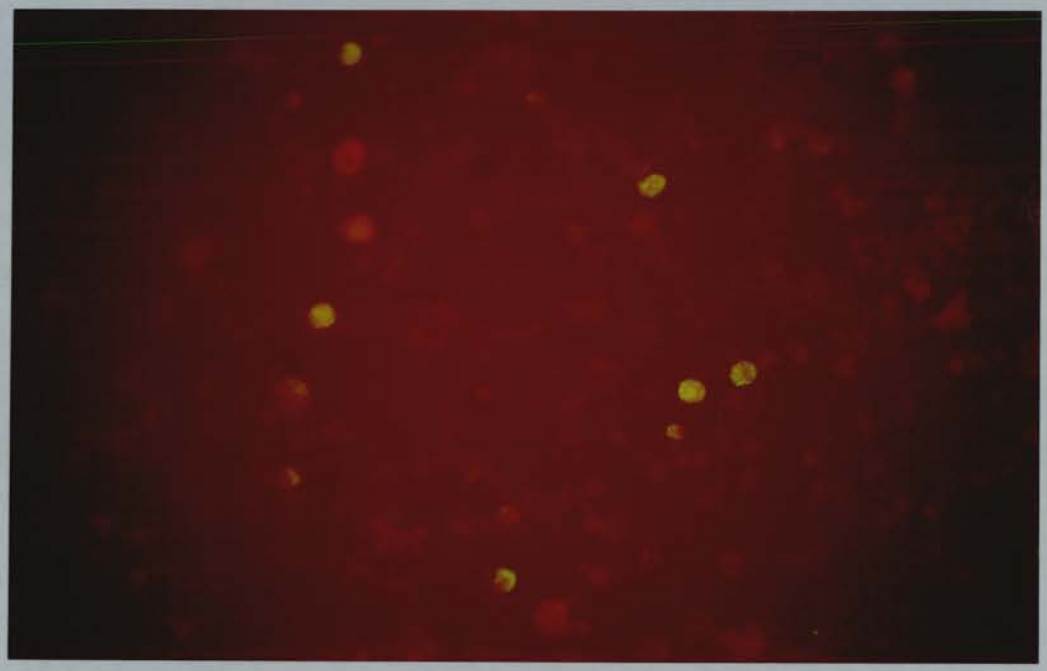


figure3 : Immunofluorecent pattern for IgG on HHV-6 infected JJhan cells with an IgG positive human serum (400 x magnification).



Cross-over sero-absorption experiment to ascertain specificity of IgM to HHV-6 and CMV

A cross over sero-absorption experiment was carried out with samples which were found to be IgM positive for HHV-6 and CMV to ascertain whether antibody was cross reactive or produced to both viruses. Nine samples from the two patients who seroconverted to CMV, and two positive and one negative well characterised control samples were included in the experiment. To absorb out HHV-6 antibodies from the serum 10µl of serum was incubated overnight at 37^{o}c with 10 µl of HHV-6 infected cell suspension. The cell suspension contained approximately 2×10^{5} cells per reaction tube. The next morning the suspension was centrifuged and 10 µl of the supernatant was then incubated with IgG removing solution (Incstar) for 15 mins, giving a final dilution of serum of 1/8. The method for IgM by IFA was then followed as above, and serum was spotted onto slides with HHV-6 infected lymphoblasts and CMV infected fibroblasts, also prepared in house. The same procedure was followed for CMV absorption of sera except that the cell suspension contained approximately 1.2×10^{5} CMV infected cells per reaction tube.

Enzyme Linked Immunosorbent Assay for IgG to HHV-6

The method of Chou [Chou 1990] was adapted and used to detect anti-HHV-6 IgG with an alkaline phosphatase detection system [Cubie 1993]. Antigen was prepared by the same method from infected and uninfected cells. Cells ($3x10^7$) were centrifuged at 1,200g, suspended in 4ml of chilled glycine buffer (pH 9.5), and sonicated on ice for 30 seconds (see page 93 for buffer recipes). The sonicate was centrifuged at 5,000g for 20 minutes and the supernatant stored at -70°C.

Antigen was diluted in glycine coating buffer (pH 9.5) and a series of different dilutions were used in the ELISA to ascertain the optimal working dilution. This was found to be a 1 in 160 dilution of the lysate with a protein content of 5 µg/ml (kindly measured by Dr Jean Kirk Biochemistry, RHSC, using the Lawrie method). Alternating 8 well rows of a ninety-six well microtitre plate (Nunc) were coated with either 100 µl of infected or uninfected antigen at this dilution and incubated overnight at 4°C. The plate was washed 3x with PBST washing buffer (Phosphate buffered saline pH 7.2, with Tween (0.05%), and BSA (0.1%)). Remaining binding sites were blocked with 200 µl of PBST (with 5% BSA) at 37°C for 1 hour and the plate was then washed 3x. Coated and blocked plates could be stored at 4°C for at least one week before use. An automated ELISA plate washer was used for all washes.

100 µl of sera diluted 1 in 100 in PBST (with 1% BSA) was applied to pairs of wells and incubated for two hours at 37°C. After three washes, 100 µl of diluted (1 in 1000) anti-human IgG conjugated with alkaline phosphatase (Sigma) was added to each well for one hour at 37°C. After five washes, 100 µl of the substrate para-nitrophenyl phosphate (1mg/ml in freshly made diethanolamine buffer, pH 9.8), was added to each well and incubated at room temperature, in the dark, for 30 minutes. The reaction was stopped with 50 µl of 3 M sodium hydroxide and the absorbance read at 405 nm.

At the outset a series of experiments were carried out to obtain the optimal dilutions of ELISA antigen, patient serum, and antihuman IgG-alkaline phosphatase conjugate concentrations (figures 4,5,6,7,8). The ELISA antigen concentration chosen was 1/160 which corresponded with an appropriate protein content (see above) and was also most economical of antigen. The patient serum concentration selected was 1/100 as this showed adequate discrimination between positive and negative samples. The concentration of antihuman IgG-alkaline phosphatase selected was 1 / 1000 for simplicity of ditution, and because it gave the most discrimination between positive and negative sera (figures 7,8).

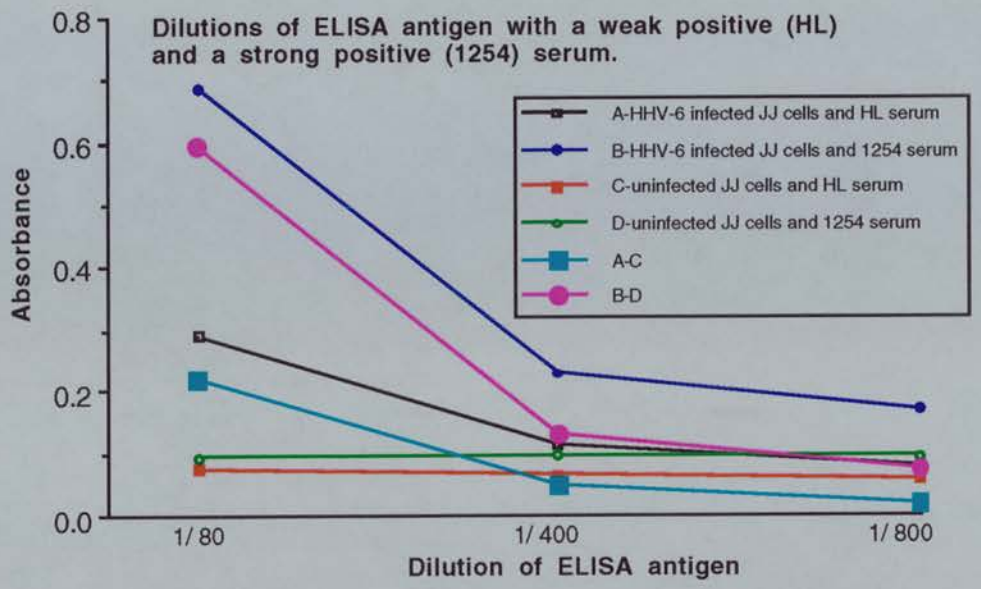


Figure 4 : Varying dilutions of ELISA antigen with serum dilutions of 1 /100 and antihuman IgG-alkaline phosphatase conjugate of 1/800. The sera tested were a known strongly positive serum (1254) and a known low positive serum (HL).

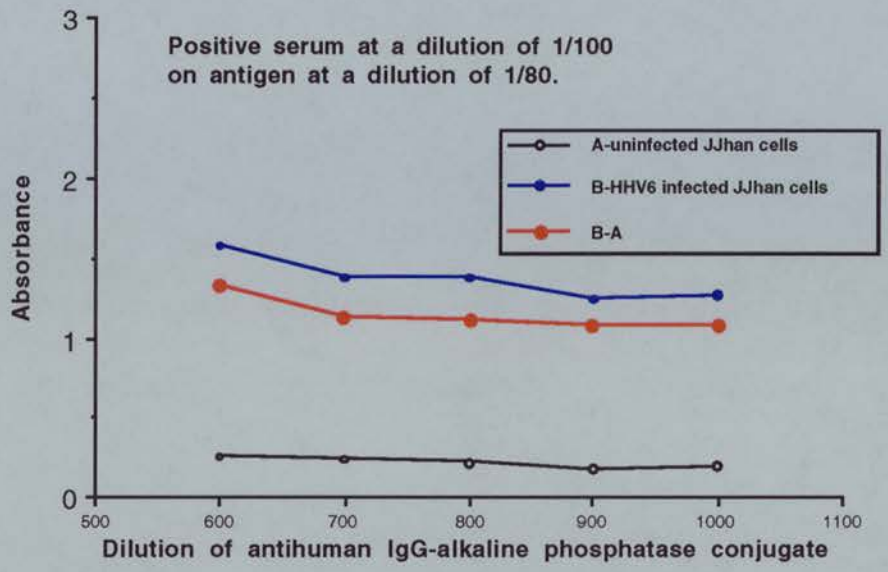


Figure 5: Varying dilutions of antihuman IgG-alkaline phosphatase conjugate with serum dilution of 1 /100 and antigen of 1/80

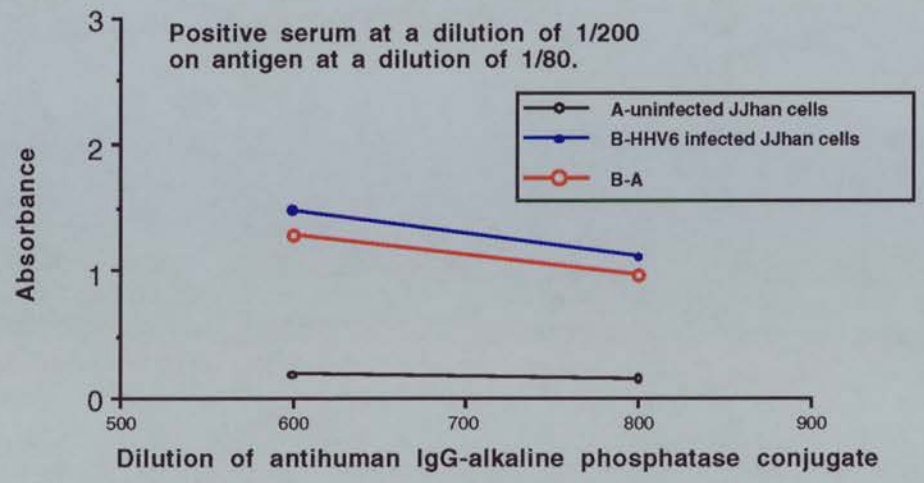


Figure 6: Varying dilutions of antihuman IgG-alkaline phosphatase conjugate with serum dilution of 1 /200 and antigen of 1/80

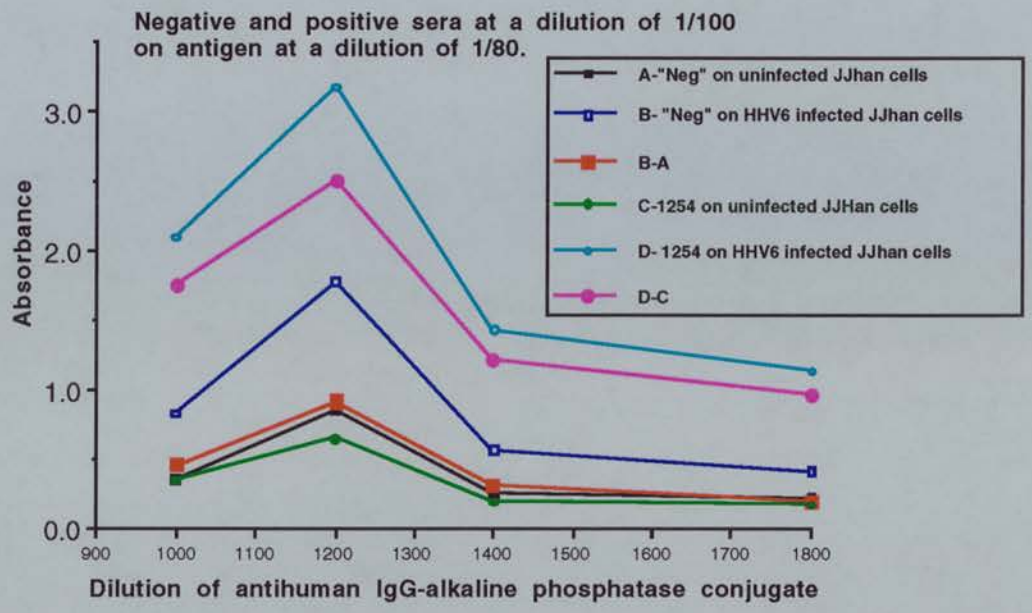


Figure 7: Varying dilutions of antihuman IgG-alkaline phosphatase conjugate with serum dilution of 1 /100 and antigen of 1/80. The sera tested were a known strongly positive serum (1254) and a known negative control serum.

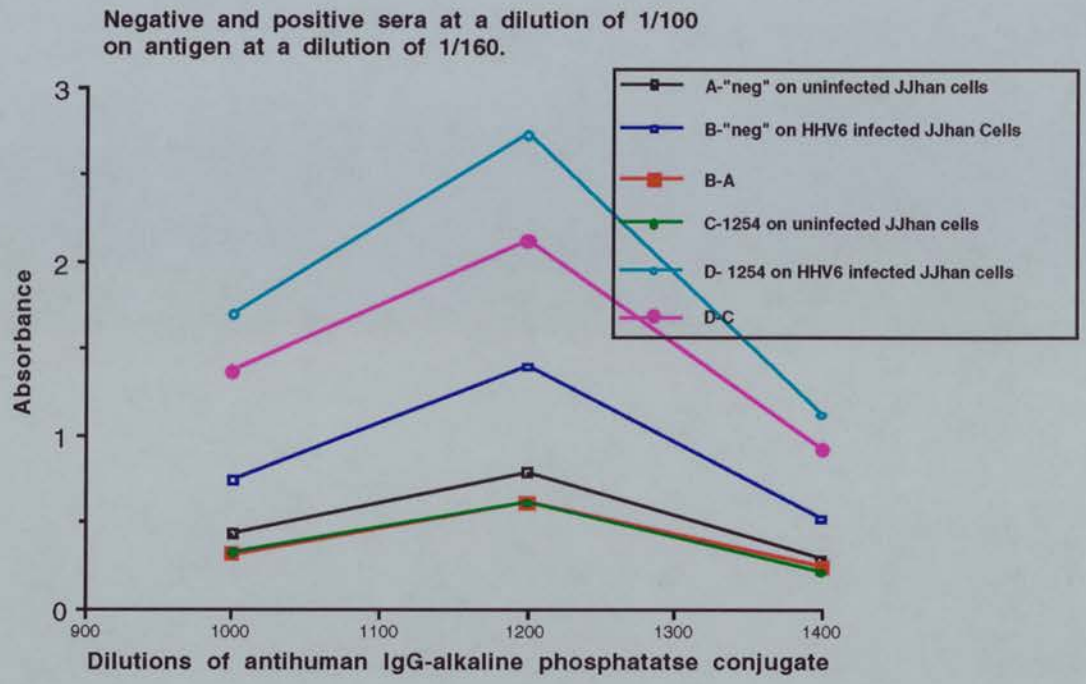


Figure 8: Varying dilutions of antihuman IgG-alkaline phosphatase conjugate with serum dilution of 1 /100 and antigen of 1/160. The sera tested were a known strongly positive serum (1254) and a known negative control serum.

The ELISA Index

As the antigen was a crude cell lysate, it was necessary to test each serum sample on both infected and uninfected antigen. Some sera had a stronger background reaction with the uninfected antigen than others and this was compensated for by using the difference between the two absorbances to calculate the ELISA Index:

$$\text{ELISA Index} = \frac{\text{Absorbance Infected cells - Absorbance Uninfected cells}}{\text{Negative Cut Off}}$$

The negative cut off for the test was the mean difference in absorbance of the three negative controls plus 3 standard deviations, but if this figure was less than 0.1 then 0.1 was used. The ELISA index was considered to be positive if greater than 1.1, negative if less than 0.9 and equivocal between 0.9 and 1.1.

The following control sera were included on each microtitre plate: a strong positive; a low positive; and three negatives. Positive and negative control sera were initially tested by IFA. Sera from infants presenting with minor illnesses (upper respiratory tract infection, pyrexia, or viral infection) were screened to obtain negative control samples. A well characterised strong positive control sample (1254) was obtained from Dr M Ogilvie (Medical Microbiology, University of Edinburgh), a low positive control sample was provided by HL. The plate was blanked on a well incubated with diluent only.

Statistical Methods

The results from the ELISA of the presenting cohort of patients were converted to positive, negative or equivocal for comparison with the IFA results. The data was analysed by the Chi-squared test or Fisher's exact test, for groups of data with very small numbers. A repeated measures model which allows correlation between the repeated values from the same patient, was applied to the sequential data from patients on treatment so the effects of time, age at presentation and diagnosis on the trends in the ELISA values could be analysed [Jennrich 1986]. The statistical package used was SAS (PROC MIXED). Dr Helen Brown, Department of Medical Statistics, University of Edinburgh, undertook the statistical analysis using the repeated measures model.

Recipes for Buffers

Glycine Coating Buffer pH 9.8
0.1M NaCl
0.1 M glycine
Na OH (5M) to achieve pH 9.8.

PBS
To make 10 x stock solution
Add 80g NaCl
2g KCl
14.4g Na_2HPo_4
2.4g KH2Po4 to 800ml of distilled H_2O.
Adjust the pH to 7.2 with HCl. Make up to 1L with H_2O.
Dispense and sterilise.

PBST
Dilute 10 x PBS (pH 7.2) to 1 x PBS
Add Tween 20 to final concentration of 0.05%
Add BSA to a final concentration of 0.1%
Add 0.2g NaN_3

PBST + 1% BSA - for serum and conjugate dilution
Add 1g of BSA to 100 ml PBST. Dispense as 20ml aliquots and store at -20°c.

PBST + 5% BSA - for blocking
Add 5g of BSA to 100 ml PBST. Dispense as 10ml aliquots and store at -20°c.

Diethanolamine Buffer pH 9.8

Diethanolamine (store in hot room)	970µl
Distilled water	8ml
1M HCl	approx 1.1.5 ml to correct pH.

Make up immediately prior to use.

Retrospective Serological Study Results

Comparison of IFA and ELISA for Anti-HHV-6 IgG

There was no significant difference in the results obtained by these two tests in the presenting cohort of patients, although there were more equivocal and negative sera by IFA (table 3). The high degree of concordance between the tests is demonstrated graphically (figure9). In particular all of the sera which tested negative by ELISA also did so by IFA. Haemolysis of these small paediatric samples did not significantly alter the pattern of results in either test (figures 10 & 11.) The ELISA performed consistently as the repeated values obtained from the controls on different runs demonstrates (figure 12, table 4). For all ELISA runs the control values fell within +/- 2 Standard Deviations (SD) of the mean control value and for the majority of runs within +/- 1 SD of the mean control value. The variation in values obtained appears wider for the negative controls, but this is a reflection of the sigmoidal nature of the ELISA curve with a drop off of accuracy in readings at the top and bottom end of the curve [Wreghitt 1990].

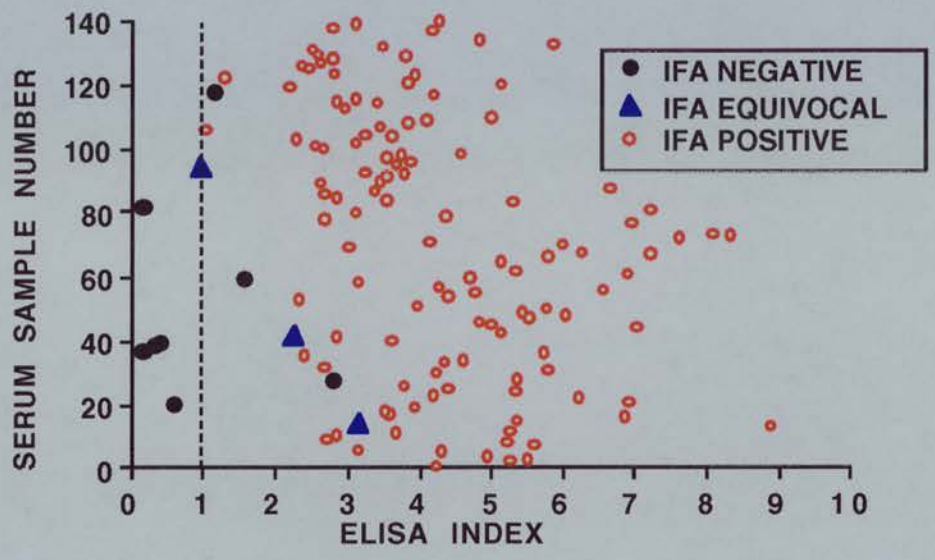


figure 9: Concordance of results obtained by ELISA and IFA

Table 3: Seroprevalence of HHV-6 IgG in patients and controls by ELISA and IFA
(including all patients and controls and those under one year).

	Positive	Negative	Equivocal	Not Suitable	Total
ELISA Patients (ALL)	64 (97%)	2 (3%)	-	-	66
IFA Patients (ALL)	61 (92.5%)	4 (6%)	-	1 (1.5%)	66
ELISA Controls (ALL)	61 (92.5%)	3 (4.5%)	2 (3%)	-	66
IFA Controls (ALL)	58 (88%)	4 (6%)	3 (4.5%)	1 (1.5%)	66
ELISA Patients & Controls (<1 yr)	6 (60%)	4 (40%)	-	-	10
IFA Patients & Controls (<1 yr)	5 (50%)	5 (50%)	-	-	10

table 3: Seroprevalence of HHV-6 IgG in patients & controls by ELISA & IFA (including all patients and controls and those under one year).

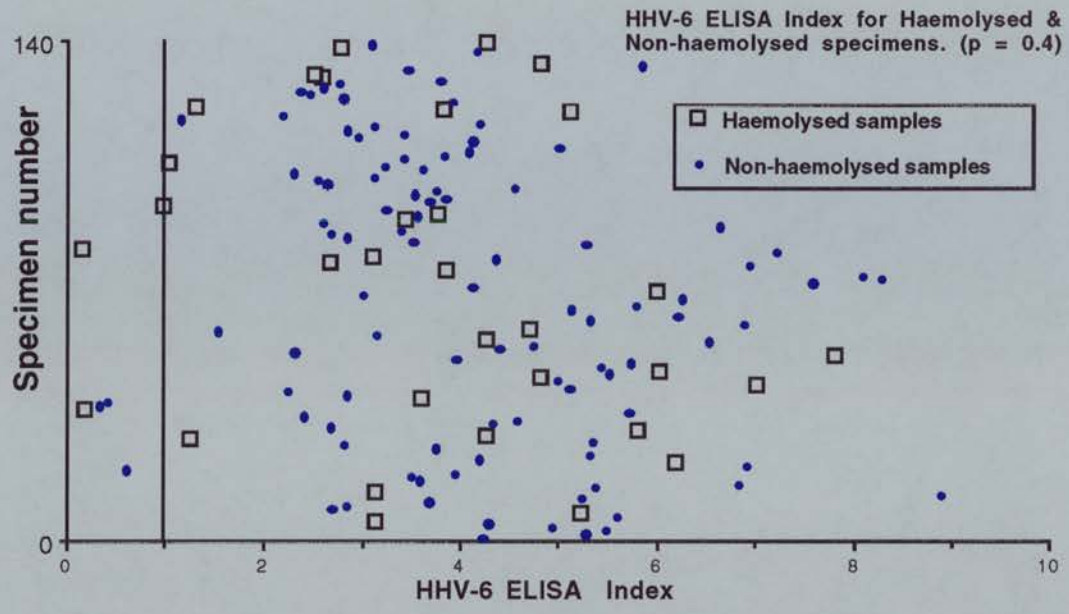


figure 10: Effect of sample haemolysis on HHV-6 antibody result by ELISA

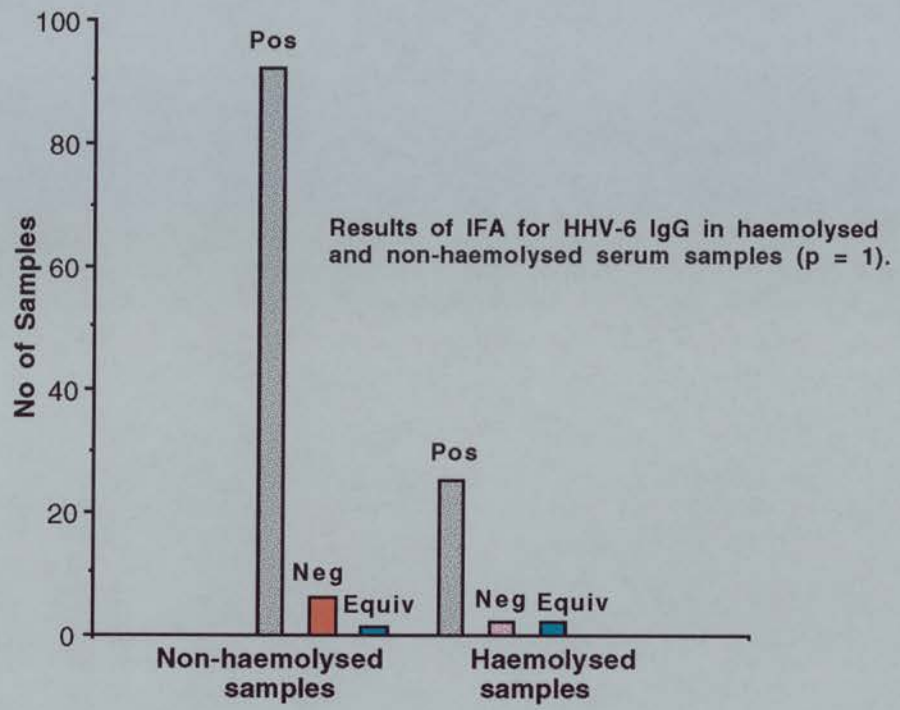


figure 11: Effect of sample haemolysis on HHV-6 antibody result by IFA

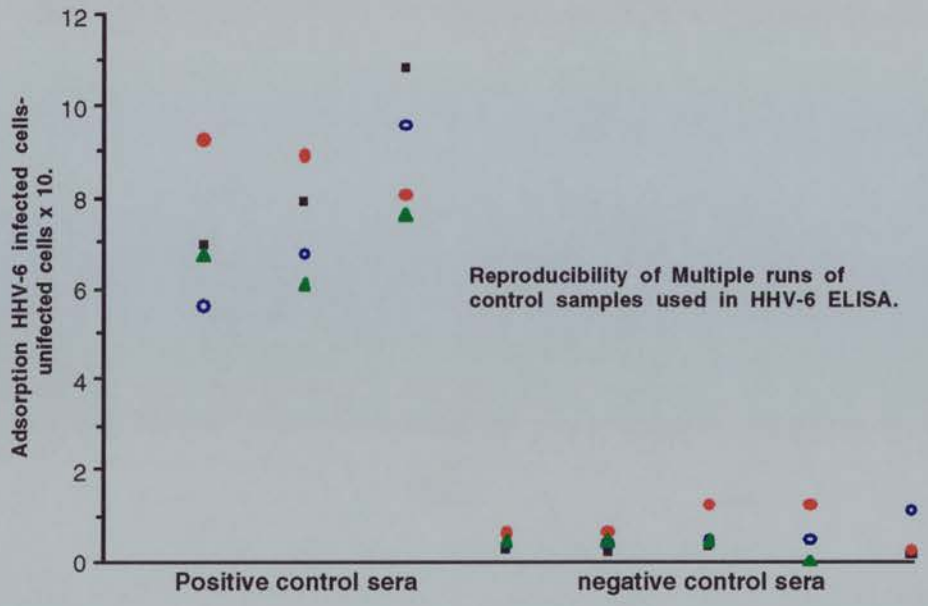


figure 12: Reproducibility of ELISA with positive and negative control sera. Each set of symbols represents a different ELISA run (i.e. red closed circles, blue open circles, black closed squares, green closed triangles). The same 7 samples (3 positive control sera & 4 negative control sera) were tested on each run and gave reproducible results (see table 4).

Table 4: Comparison of ELISA readings (OD 450) for HHV-6 positive & negative controls samples between plates for presenting cohort of patients.

Control sample number	Pos / Neg	Mean ELISA reading	1 x SD	2 x SD	Number of runs where the mean lay within +/- 2 x SD	Number of runs where the mean lay within +/- 1 x SD	Coefficient of variance (%)
7	pos	0.725	0.185	0.37	3 / 3	2 / 3	26
13	pos	0.784	0.108	0.216	3 / 3	3 / 3	14
1254	pos	0.9	0.148	0.296	4 / 4	3 / 4	16
20	neg	0.04	0.018	0.036	3 / 3	2 / 3	45
37	neg	0.04	0.023	0.046	3 / 3	1 / 3	57
38	neg	0.065	0.048	0.096	3 / 3	2 / 3	74
39	neg	0.07	0.046	0.092	3 / 3	2 / 3	66
82	neg	0.061	0.066	0.132	2 / 2	2 / 2	107

table 4: Comparison of ELISA readings for HHV-6 positive & negative controls samples between plates for presenting cohort of patients.

Serological response to HHV-6

Presenting Samples from Oncology Patients and Controls

IgG antibodies to HHV-6 at presentation

Around 90% of patients and controls were anti-HHV-6 IgG positive by both tests and there was no significant difference in seropositivity for anti-HHV-6 IgG between patients or controls (table3). Solid tumour patients were as likely as leukaemics to have HHV-6 antibody. The proportion of seropositive cases in children under one year was lower (50-60%), but there was no significant difference between the patients or controls in this small group . By the more sensitive ELISA, only five children had no antibody to HHV-6. Two were patients, a six month old with neuroblastoma and a nine year old with acute leukaemia (see below). The HHV-6 antibody negative control patients were infants aged, four, six, and seven months.

IgM Antibodies to HHV-6 at presentation

IgM antibodies to HHV-6 were found by IFA in two control patients. An eight year old with a febrile illness and a thirteen month old with an upper respiratory tract infection. One leukaemic patient was also positive for IgM at presentation (see below). All of these patients also had IgG antibodies to HHV-6.

Antibodies to Five Herpesviruses at Presentation

There was no significant difference in antibody response to VZV or CMV between patients or controls. Two thirds of patients and controls had IgG to VZV and one third of patients and controls had IgG to CMV (table 5). The acquisition of antibodies to HHV-6, CMV and VZV according to age for the whole group, including patients and controls, is demonstrated in figures 13,14 & 15. The majority of children are seropositive for HHV-6 by one year of age, where seropositivity to VZV increases progressively during childhood. In this group of Scottish children seropositivity to CMV shows a much slower increase during childhood. Evidence of past EBV infection was found in 33 (62%) of the 53 patients' samples available for testing while HSV antibodies were detected in only 9 (15%) of 61 patients tested by complement fixation. There was no significant difference in response between patients with solid tumours or leukaemia.

In the patient group complete results of serological response to the five herpes viruses were available for 79 %, and demonstrated an IgG response to an increasing number of herpesviruses with increasing age. Where the child had antibody to one herpesvirus this was usually HHV-6 (7/66). Those with IgG to only HHV-6 had a mean age of 3.7 years compared to 6.6 years for those with IgG to two or more herpesviruses, including HHV-6 (table 6).

Table 5: Seroprevalence for HHV-6, CMV, VZV, EBV, and HSV IgG in presenting patients and controls.

Pos- positive, Neg- negative, Equiv- Equivocal, NA- not available,
*- equivocal results added to positive results.

	CMV			VZV			EBV		HSV	
	Pos	Neg	Equiv	Pos	Neg	Equiv	Pos	Neg	Pos	Neg
HHV-6 Positive Patients *	18	39	7	41	22	1	32	19	9	50
HHV-6 Positive Controls	21	38	4	40	20	3	NA	NA	NA	NA
HHV-6 Negative Patients	-	2	-	2	-	-	1	1	-	2
HHV-6 Negative Controls	1	2	-	-	2	1	NA	NA	NA	NA

table5: Seroprevalence for HHV-6, CMV, VZV, EBV, and HSV IgG in presenting patients and controls.

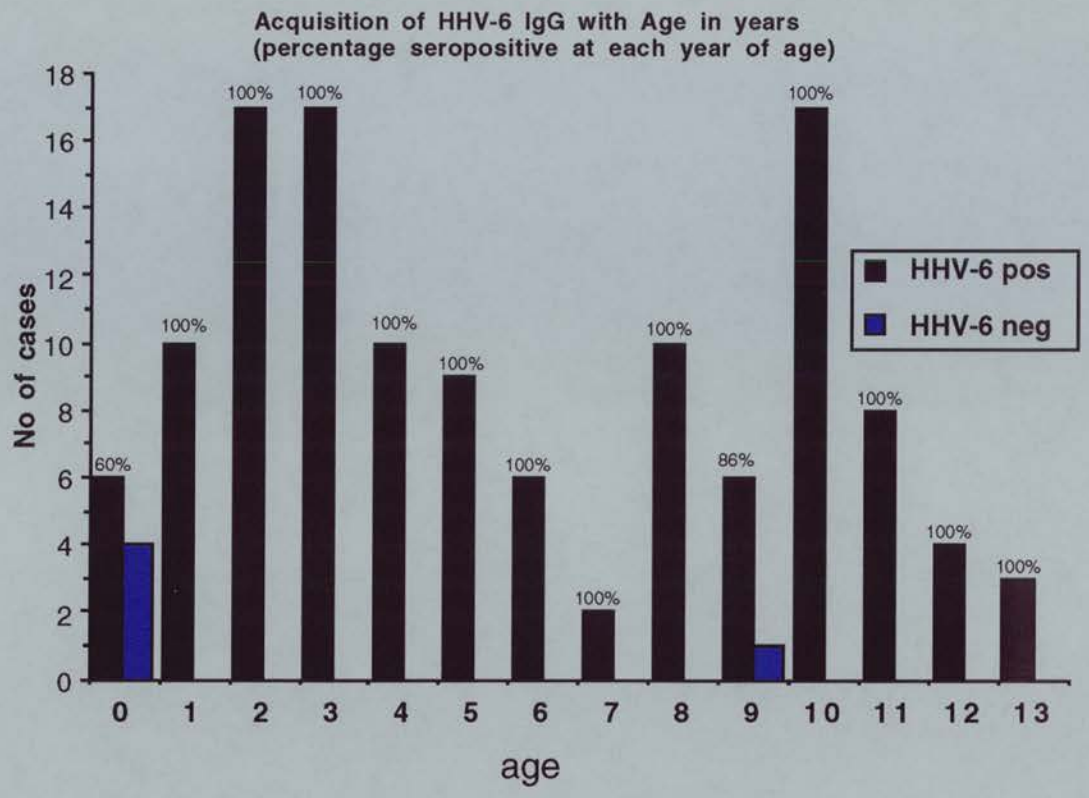


Figure 13: Seropositivity to HHV-6 in the presenting cohort of patients and controls, according to age.

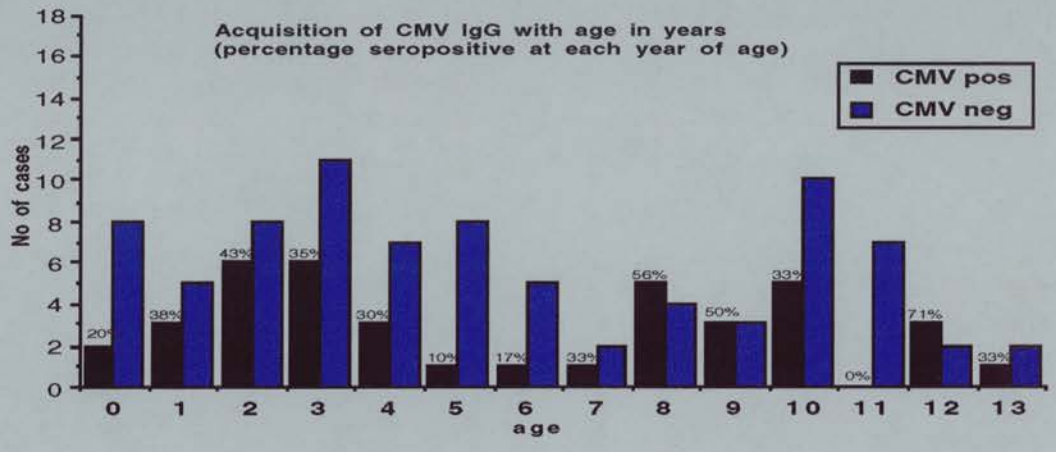


Figure14: Seropositivity to CMV in the presenting cohort cohort of patients and controls, according to age.

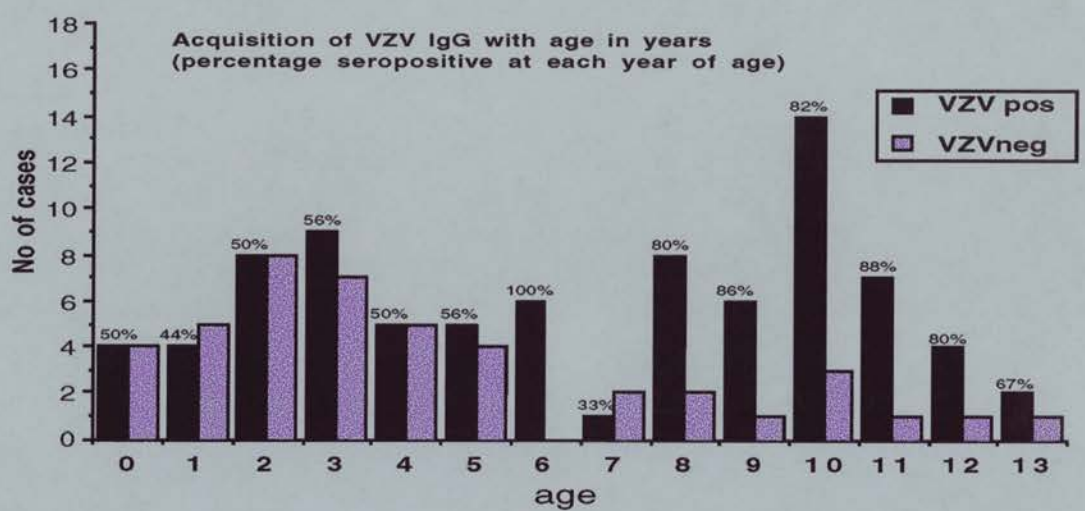


Figure15: Seropositivity toVZV in the presenting cohort of patients and controls, according to age.

Table 6: Herpesvirus group serology and patient age at presentation with malignancy.

	Age in Years		Total (%)
HHV-6 Positive Patients	**Mean**	**(Range)**	
Antibody to one Herpesvirus (HHV-6)	3.7	(0.8-10)	7 (10.62)
Antibody to two Herpesviruses (HHV-6+1)	6.3	(1-13)	12 (18.12)
Antibody to three Herpesviruses (HHV-6+2)	6.2	(1-12)	17 (25.75)
Antibody to four Herpesviruses (HHV-6+3)	7.1	(1-11)	10 (15.15)
Antibody to five Herpesviruses (HHV-6+4)	6.8	(2-12)	4 (6.06)
Incomplete data	3.9	(0.2-13)	14 (21.2)
HHV-6 Negative Patients			
Antibody to one Herpesvirus	0.5		1 (1.5)
Antibody to two Herpesvirus	9		1 (1.5)
Total	5.6	(0.2-13)	66 (100)

table 6: Herpesvirus group serology and patient age at presentation with malignancy.

Sequential Sera from Patients on Chemotherapy

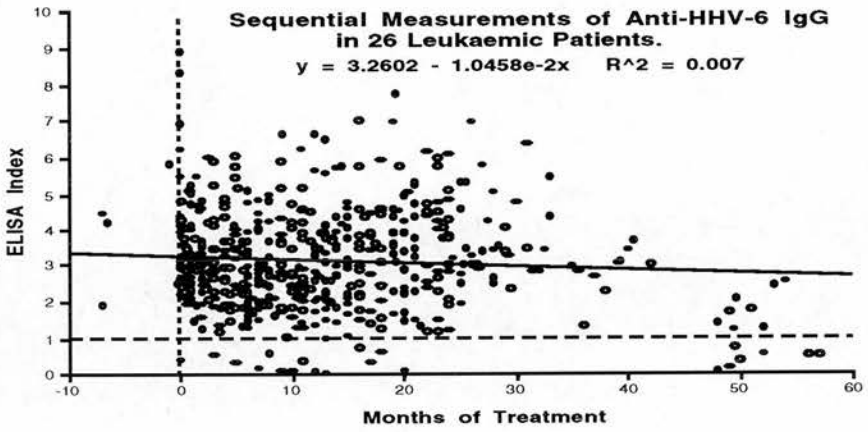
Anti-HHV-6 IgG in sequential sera

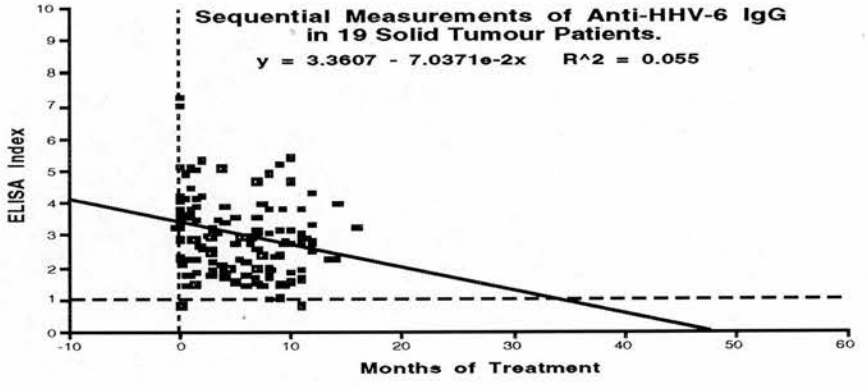
676 sequential specimens from 45 patients were examined for anti-HHV-6 IgG by ELISA, and the test performed consistently (table 7). Five hundred and forty-four samples were obtained from the 26 leukaemia patients (9-33 samples per child) and 132 samples were tested from the 19 patients with solid tumours (3-15 per child). The majority of samples were positive for anti-HHV-6 IgG (figure 16). One nine year old leukaemic child had no antibody to HHV-6 in the first sample tested, and 23 further samples from this child were tested, 3 had an ELISA index of just over one, however each time this subsequently became negative and he remained negative two years later when his leukaemia relapsed (figure 17). All sera from this child were negative for anti-HHV-6 IgM. This group of children had a similar serological pattern of response to the five herpes viruses tested as the other patients and controls (table 8).
A repeated measures model was fitted to all the data from these patients to analyse the effects of time, age at presentation, and diagnosis (AL - acute leukaemia or ST - solid tumour) on any trends in the ELISA Index values obtained. There was no overall significant difference between AL or ST patients with both groups showing a decline in ELISA Index over time. The slope of this decline was not significantly different between the two groups of patients when compared over a one year time period. Most of the solid tumour patients were only treated for one year or less, whereas many leukaemic patients were treated for at least twice that time (figure 16). The relationship between age and ELISA Index was different between the two groups of patients. In the ST patients there was little difference in ELISA Index values with age, but older AL patients tended to have lower ELISA Index values ($p = 0.039$).

Table 7: Comparison of ELISA readings (OD 450) for HHV-6 positive & negative controls samples between plates for the sequential cohort of patients.

Control sample number	Pos / Neg	Mean ELISA reading	1 x SD	2 x SD	Number of runs where the mean lay within +/- 2 x SD	Number of runs where the mean lay within +/- 1 x SD	Coefficient of variance (%)
1254	pos	0.74	0.109	0.218	8 / 8	6 / 8	15
1	pos	0.295	0.052	0.104	8 / 8	7 / 8	18
37	neg	-0.025	0.054	0.108	3 / 3	2 / 3	-214
38	neg	0.19	0.02	0.04	5 / 8	2 / 8	101
39	neg	0.061	0.015	0.03	8 / 8	7 / 8	25
82	neg	0.016	0.011	0.022	5 / 5	3 / 5	69

table 7: Comparison of ELISA readings for HHV-6 positive & negative controls samples between plates for the sequential cohort of patients.





Figures 16 a + b: Sequential sera tested for HHV-6 IgG, from leukaemia patients above (16a), and solid tumour patients below (16b). The scattergrams represent the range of HHV-6 IgG concentrations obtained from all the patients over time, see text for statistical interpretation.

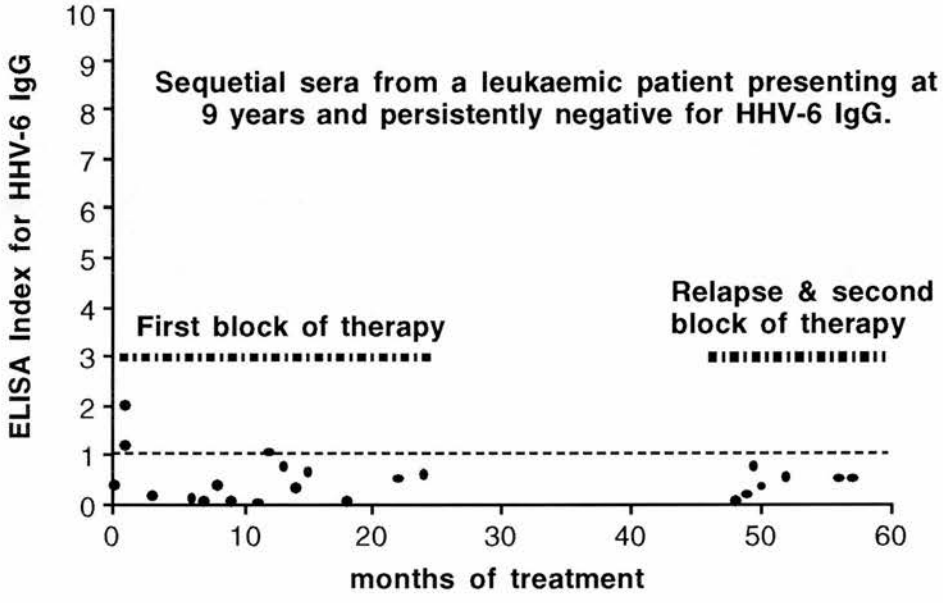


Figure17: Sequential sera tested for HHV-6 IgG from a leukaemic patient who was seronegative at presentation and continued to be so subsequently.

Table 8: Presenting herpesvirus serology in patients followed with sequential samples for HHV-6.
Pos- positive, Neg- negative, Equiv- equivocal.

	HHV-6			CMV			VZV			EBV		HSV	
	Pos	Neg	Equiv	Pos	Neg	Equiv	Pos	Neg	Equiv	Pos	Neg	Pos	Neg
Acute Leukaemic Patients	24	1	-	5	18	-	17	8	-	12	11	7	17
Solid Tumour Patients	19	-	-	4	12	3	9	8	2	10	5	1	16

table 8:Presenting herpesvirus serology in patients followed with sequential samples for HHV-6.

Anti-HHV-6 IgM in sequential sera

With the resources available it was not possible to test all sequential sera for anti-HHV-6 IgM, so sequential sera from six patients with changes in activity against other herpesviruses were selected to be examined for anti-HHV-6 IgM. These included two children who sero-converted to CMV asymptomatically during treatment. One produced a four fold rise in anti-CMV IgG titre and was strongly positive for anti-CMV IgM at the time of seroconversion, and again three months later, but in no other subsequent samples. None of the 29 samples from this child had anti-HHV-6 IgM (figure 18). The other patient had a four fold rise in anti-CMV IgG titre two months after starting treatment and soon after was strongly positive for anti-CMV IgM, three further samples were also positive for anti-CMV IgM. Anti-HHV-6 IgM was present prior to the rise in CMV antibody, but insufficient sample remained to test for anti-CMV IgM. Over the 24 months of therapy ten samples were positive for anti-HHV-6 IgM, three of these were also anti-CMV IgM positive (figure19). None of the 4 clinical cases of varicella -zoster infection which occurred during therapy were associated with production of IgM to HHV-6.

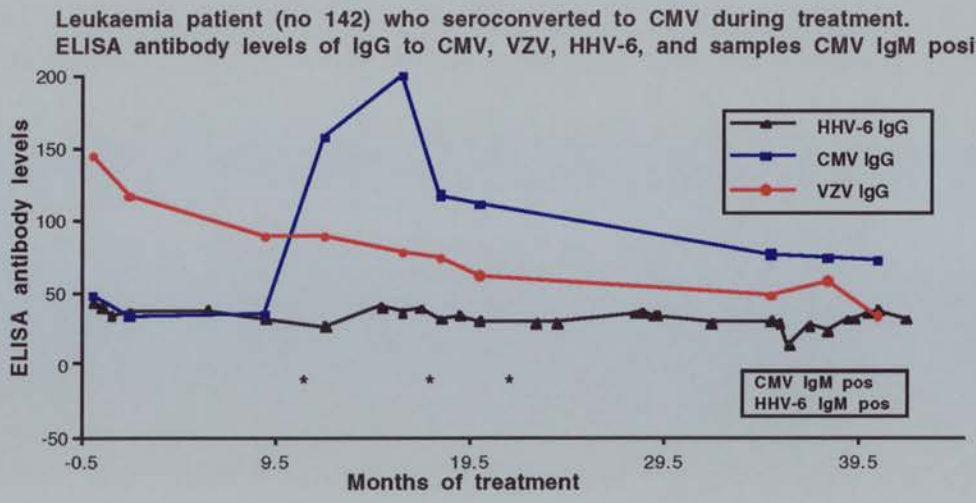


Figure 18: A leukemia patient who seroconverted to CMV and produced anti-CMV IgM but did not produced any change in antibodies to HHV-6.

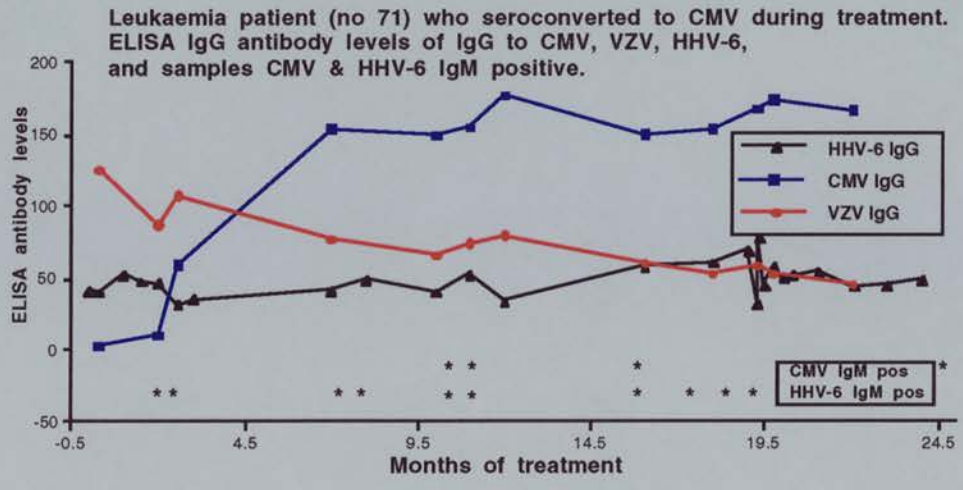


Figure19: A leukemia patient who seroconverted to CMV and produced anti-CMV IgM but also IgM antibodies to HHV-6.

Cross over IgM sero-absorption experiment for HHV-6 & CMV antibodies

On nine selected samples from the two patients who seroconverted to CMV a cross over sero-absorption experiment to test whether the IgM produced was specific or cross reactive for HHV-6 or CMV was carried out. Using the method described, after over night incubation of test sera with infected cells It was not possible to completely absorb out all the IgM antibodies. For patient 142, it appeared that only IgM to CMV was produced. For patient 71, the results of the experiment were not completely consistent, however for some of the samples it did appear that antibodies could have been cross reactive to HHV-6 and CMV (table 9).

Table 9: Cross absorption study to ascertain whether IgM antibody produced from two patients and three controls was specific for HHV-6 or CMV or cross reactive antibodies.

Serum	Unabsorbed	HHV-6 absorbed	HHV-6 absorbed	Unabsorbed	CMV absorbed	CMV absorbed	Comment	Interpretation
Test slide	HHV-6 slide	HHV-6 slide	CMV slide	CMV slide	HHV-6 slide	CMV slide		IgM antibody to:
samples								
142 a	neg	neg	neg	neg	neg	neg	always neg	none
142 b	neg	neg	pos	pos	neg	pos	Strongly positive CMV IgM, not absorbed	CMV
							out with HHV-6 or CMV infected cells	
142 c	neg	neg	pos	neg	neg	pos	Weakly positive CMV IgM, not absorbed	CMV
							out with HHV-6 or CMV infected cells	
71 a	w pos	w pos	pos	neg	w pos	pos	Positive on both slides with either	CMV & HHV-6
							absortion on CMV or HHV-6	
71 b	w pos	pos	pos	neg	w pos	pos	"	CMV & HHV-6
71 c	pos	pos	w pos	pos	pos	neg	Positive on both HHV-6 slides but not on	? both, ? only HHV-6
							CMV absorbed CMV slide	
71 d	pos	pos	w pos	pos	w pos	w pos	"	? both, ? only HHV-6
71 e	neg	pos	pos	pos	w pos	neg	Positive on both HHV-6 slides but not on	? only HHV-6, ? and CMV
							CMV absorbed	
71 f	neg	pos	pos	pos	w pos	neg	"	? only HHV-6, ? and CMV
Controls								
HHV-6 & CMV Neg	neg	neg	neg	neg	neg	neg	always neg	none
HHV-6 & CMV pos	pos	pos	neg	pos	pos	pos	Positive on both HHV-6 slides & CMV	HHV-6 & CMV
HHV-6 pos	pos	pos	neg	neg	neg	neg	Positive only on HHV-6 infected cells	HHV-6
							whether absorbed or not	

table 9: Cross absorption study to ascertain whether IgM antibody produced was specific for HHV-6 or CMV or cross reactive.

Interpretation of the findings of the Retrospective Serological Study of HHV-6 antibodies in paediatric oncology patients

Technical details of IFA & ELISA

More than 90% of the presenting patients and controls in this study had IgG to HHV-6 by ELISA and slightly less by IFA (table 3). The interpretation of the fluorescent antibody test, being visual, is more subjective and the differentiation of low positives from negatives can be difficult. Although the ELISA used a crude cell lysate as antigen, lysates of both infected and uninfected cells were used in parallel and the difference in absorbance was used to calculate an HHV-6 specific ELISA index. The ELISA test results demonstrated a relatively clear separation between positive and negative sera with very few equivocal results and all the samples which were negative by ELISA were also negative by IFA (figure 9). The ELISA was also robust and performed consistently for each batch of samples tested as demonstrated in tables 4 & 7. However, the ELISA could only be considered to be a semi-quantitative measure of HHV-6 antibody trends, and was not set up as a specifically quantifiable test.
For this study it was not possible to titrate out sera for the IFA to obtain information on the concentration of antibody present in the samples, nor was it possible to examine the avidity of the antibody which could have given usefull information on the age of the antibody response and the possibility of cross reactivity of antibody response [Ward 1993a; Ward 1993b]
Although all the presenting sera were tested for anti-HHV-6 IgM by IFA , this was not possible using this time consuming test for all the sequential sera. Ideally the ELISA protocol would have been adapted for anti-HHV-6 IgM detection, but time was not available.

Cross reactive antibody responses

The absorption experiment carried out in this study, could not be repeated because of scarcity of samples, but the inconsistency of the results also seem to point to the same findings as others. Although in one child no HHV-6 IgM was produced with CMV seroconversion, in the other cross reactive IgM probably was produced.
As described in "The Host and HHV-6" antigenic cross-reactivity with other herpesviruses, especially CMV may affect the apparent response to HHV-6. This is not usually a problem for primary HHV-6 infection, perhaps because HHV-6 may be this first herpesvirus infection [Okuno 1989; Asano 1990a]. Nor is it usually seen in the IgG responses of healthy donors [Salahuddin 1986; Downing 1987; Tedder 1987; Briggs 1988; Lopez 1988; Saxinger 1988; Buchbinder 1989; Fox 1990b]. However in both immunocompetent and immunosuppressed patients having primary CMV infection cross reactive antibodies probably are produced, certainly to IgG and possible also to IgM [Irving 1988; Larcher 1988; Linde 1988; Morris 1988; Chou 1990; Enders 1990; Fox 1990b; Irving 1990a; Irving

1990b; Sutherland 1991; Ward 1991; Adler 1993]. Divergent results have been obtained using selective absorption of sera with cells infected with CMV or HHV-6, some groups not finding antibody cross reactivity [Irving 1990a; Ward 1991] and others demonstrating depletion of cross reactive antibodies [Sutherland 1991; Adler 1993]. These differences may be due in part to the complexities of absorption studies, but also to the possibility that in different circumstances different responses or combinations of antibody responses may be occurring. Most studies have examined the possiblility of cross reactive IgG responses and there is very little information on IgM responses, as were studied in this experiment. In retrospect, since this was a study of IgM antibody, a clearer result might have been obtained if the IgG antibody had been removed prior to the cross-absorption. Unfortunately insufficient sera remained to repeat the experiment. Elucidation of this relationship will require not only further serological information on cross reactive epitopes, but also quantitative assessment of HHV-6 and CMV viraemia during the infection.

Interpretation of patient results

Presenting Samples from Oncology Patients and Controls

The percentage of anti-HHV-6 IgG seropositivity in previous studies has varied according to the tests used, the dilution of serum tested, and the age range of the subjects. In paediatric populations studied beyond the age of one year, the anti-HHV-6 IgG seropositivity is up to 100%, with the highest seropositive rate obtained by ELISA and neutralising antibody tests (see table 2, "The Host and HHV-6"). The results obtained in this study are therefore in agreement with other studies.

This study confirms that most paediatric oncology patients have encountered HHV-6 at presentation and appear to have a similar serological response to the virus as age matched controls. The findings are in agreement with a study which examined sera from 50 patients with acute lymphoblastic leukaemia (age range 1-52 years, mean age 17.6 years), in which there was no significant difference in titres for anti-HHV-6 IgG between cases and controls, by IFA or ELISA [Levine 1992b]. Also with a recent serological study of 121 German children with leukaemia, with age and gender matched controls, where again there was no difference in seroprevalence or titres of antibody to HHV-6 [Schlehofer 1996]. In contrast, earlier IFA test studies of adult patients with leukaemia and lymphoma had suggested the seroprevalence and antibody titres to be greater in patients than controls [Ablashi 1988b; Clark 1990].

Further investigation of the serological response of paediatric oncology patients could include examination of the pattern of avidity of the IgG response to HHV-6 as well as the response to different HHV-6 antigens. In a study by Iyengar most healthy control sera tested positive for a late HHV-6 antigen and only one third for an early HHV-6 antigen. Seropositivity

for the early antigen was increased in patients with lymphoproliferative disease, which could indicate increased activity of the virus and possibly an altered immune response of the host [Iyengar 1991]. This is similar to the altered patterns of serological responses to EBV seen in immunocompromised patients. In a study of patients with various myelodysplastic syndromes, elevation of titres of antibody to EBV, HHV-6 and CMV was associated with expression of viral antigens in the bone marrow, implying active viral replication [Krueger 1994a].

Five presenting sera had neither IgG nor IgM to HHV-6 by ELISA or IFA, three were from control patients and one from a solid tumour patient all under the age of one year. According to their age the children under one year of age had most likely not yet had a primary HHV-6 infection. The fifth negative serum was from a nine year old leukaemic patient who was seronegative at presentation but tested IgG positive in three samples out of twenty-three, and who was negative at relapse two years later. No samples from this child were IgM positive making it unlikely that he underwent primary seroconversion. It is much more likely that these unsustained rises in IgG antibody were related to blood products received during treatment. Althought at presentation this child did have IgG to a single herpesviruses (VZV), it might be speculated that his lack of responses to the other herpesviruses could have been related either to an immunological problem or lack of exposure . It could also be speculated that such an immune response problem may in some way be related to his risk for leukaemia. Of the two control patients with IgM to HHV-6, the infant was probably having a primary infection, and the older child may have been having a primary infection or a reactivation. The leukaemic patient positive for IgM at presentation was further investigated in the cross over absorbance study and may have been having a cross reactive response to CMV or reactivation of HHV-6, but without direct viral detection this cannot be elucidated further.
While the serological response to all five herpesviruses was examined for 52 (79%) of the patients, data was only available for CMV and VZV for the control patients. However, in this group of children aged from less than one to thirteen years the overall seropositivity for patients and controls was similar for VZV (65% and 60% respectively) and CMV (27% and 33% respectively). This pattern is similar to that reported by others for children in the UK [Kangro 1994]. In the patient group the acquisition of herpesvirus infection increased with time, and not surprisingly it appeared that when a child had antibodies to only one herpesvirus this was usually HHV-6. Further examination of control and patient responses to the other herpesviruses including EBV would have been a useful addition to the study as differential responses to EBV have been shown in paediatric oncology patients [Schlehofer 1996].

Sequential Sera from Patients on Chemotherapy

Anti-HHV-6 IgG in sequential sera

Examination of the sequential sera from patients on chemotherapy for anti-HHV-6 IgG demonstrated no significant difference between solid tumour (ST) and acute leukaemic (AL) patients when compared over the same time period. Although the trend in anti-HHV-6 IgG was downward with time, there was little evidence of large fluctuations in IgG levels which might be expected if reactivation of HHV-6 was occurring. In a longitudinal study of 37 patients with Hodgkin's disease (age range 5-72 years) IFA titres increased significantly in patients who relapsed and decreased significantly over time in those who did not [Levine 1992c]. It was not possible to examine any relationship between changes in ELISA indices and relapse of disease in the current study. The finding that older children had lower ELISA Indexes for HHV-6 reflects that of other workers who have shown that the highest geometric mean titres for HHV-6 IgG are found in young children under five years [Brown 1988a; Yoshikawa 1990].

In addition to changing titres of IgG, IgM can also be produced in reactivation or reinfection with HHV-6. It was not possible to test all the sera for anti-HHV-6 IgM and so sera from only 5 patients who had demonstrated laboratory and / or clinical evidence of herpesvirus activity were selected. In this small group VZV primary and reactivated infection did not appear to cause reactivation of HHV-6 with IgM or increased IgG production. However this situation was different for the two children with primary CMV infection. One made no IgM to HHV-6 at all and the ELISA index for anti-HHV-6 IgG remained unchanged over time. The other produced anti-HHV-6 IgM on several occasions, but again the ELISA index for anti-HHV-6 IgG remained steady with no significant change in relation to the rise of anti-CMV IgG (figures 18 & 19) (see above - "Cross reactive antibody responses").

Summary

In summary, this serological study has demonstrated that paediatric oncology patients , whether solid tumour or leukaemic patients, have a similar response to HHV-6 as age matched controls and that almost all have encountered the virus at the time of presentation with disease. The seroprevalence for the two herpesviruses, VZV and CMV, is also similar between controls and patients. Acquisition of a response to an increasing number of herpesviruses increased with age in the patients and where there was only a response to one virus it was usually HHV-6. Sequential samples from patients during immunosuppressive treatment showed a gradual decline in ELISA index for IgG to HHV-6 with time but did not give any helpful information as to whether reactivation of HHV-6 was occurring. IgM to HHV-6 was produced by one child with a primary CMV seroconversion but again this did not confirm reactivation and the possibility of antibody cross reactivity could not be excluded. Assessment of serological response in the immunocompromised will always give an incomplete picture of the host response. To ascertain further whether this virus is pathogenic in immunosuppressed children the next phase of the study examined for evidence of viral activity in immunosuppressed paediatric patients.

Prospective PCR study of HHV-6 in paediatric oncology patients

Aim of this study

HHV-6 can be a pathogenic virus for immunosuppressed patients including bone marrow transplant patients, liver transplant patients and others, but whether it is pathogenic for paediatric oncology patients on chemotherapy is not known. The majority of such children have IgG for HHV-6 at presentation and levels of antibody wain with treatment, but detection of IgG or IgM antibodies to HHV-6 is not a reliable indicator of current virus activity in these patients.

The aim of this study was to develop a nested polymerase chain reaction (PCR) for HHV-6 which could be used to examine the saliva of paediatric oncology patients and controls for HHV-6 DNA, giving direct evidence of HHV-6 excretion. Saliva was chosen for examination as a source for HHV-6 because it has frequently been shown by PCR to contain HHV-6 DNA in adults, but to date has only been examined in a few children [Cone 1993a]. Saliva is also an acceptable secretion to obtain voluntarily from healthy children. The study aimed to compare findings in the saliva of normal healthy children with sequential samples from paediatric oncology patients. In addition, serum samples were collected from patients at routine visits for chemotherapy and during febrile episodes, these were also examined by PCR for HHV-6 DNA for evidence of any active viraemia.

Patients and Samples

Local ethical committee approval and consent of parents and children were obtained for this study. All new patients old enough and willing who attended the oncology service of the Royal Hospital for Sick Children between August 1992 and September 1993 were invited to take part. The first patient saliva sample was obtained before starting chemotherapy and thereafter samples were obtained when the patient attended for treatment or was febrile and unwell. Healthy siblings and local volunteer children with no clinical oral disease acted as normal controls. Only children over 3 years of age were able to give saliva samples .
Saliva samples were obtained after a fifteen second gargle with 5 mls of normal saline and processed as soon as possible. After rota mixing the saliva was centrifuged at 1000 rpm for 8 mins and the supernatant separated from the cell pellet. Viral transport medium was added to the pellets and foetal calf serum (1.5%) and ampicillin to the supernates. The samples were stored at -70^{o}C and were tested together in batches.

Serum from each patient, taken at presentation, was tested for anti-HHV-6 IgG by IFA . Serum from a group of these children obtained at routine visits for chemotherapy and during febrile episodes and stored at -70^{o}c, was also tested for HHV-6 DNA by PCR.

Methods

Preparation of Saliva and Serum for PCR - Extraction of DNA

Saliva, supernate or pellet were thawed, and 200µl diluted in an equivalent volume of double strength lysis buffer (0.1M TRIS, 0.02M EDTA, 1% Tween 20) with proteinase K (100µg/ml). The samples were incubated on a heating block for 90 mins at 55°c and 10 mins at 95°c, this enabled lysis of cellular material and digestion of protein with final inactivation of proteinase K (PK) at 95°c.

DNA was then extracted from the sample using a phenol / chloroform method and precipitated over night at -20°c in pure ethanol [Sambrook 1989]. An equal volume of commercially prepared Phenol:Chloroform:Isoamyl alcohol (P:C:I) (25:24:1) (Sigma) was added to the lysis buffer containing the PK digested sample and mixed well to form an emulsion. This was spun at 12,000g for 1 min at room temperature, and the aqueous phase (top) carefully transferred to a fresh tube with an equal volume of P:C:I, leaving the white proteinacious layer. The process was then repeated but after spinning the second aqueous phase was added to an equal volume of chloroform and then spun again. To the third aqueous phase sodium acetate was added to a final concentration of 0.3M, and mixed well, the salt being important to assist the precipitation of the DNA from solution. Two volumes of ice-cold ethanol (100%), were added to the solution and the mixture stored over night at -20°c.

DNA was recovered by centrifugation at room temperature at 12,000g for 10 mins. Sometimes a whitish pellet was visible on the shoulder of the tube at this stage, the supernate was removed very carefully and the tube half filled with ice cold ethanol (70%) and centrifugation repeated at 12,000g for 2 mins. After the supernate was removed the tubes were left open in an isolation hood until the last traces of ethanol had evaporated. The pellet, which was sometimes invisible, was dissolved in 100µl of warm (37°c), sterile, distilled water. The extracted DNA was either stored at -20°c until required or 10µl of this solution was used directly for the first round PCR.

The same procedure of digestion with proteinase K and DNA extraction was also followed for serum samples except that 100µl of serum was added to 100µl of sterile water and the diluted serum added to the lysis buffer.

Sequence of HHV-6 genome chosen for amplification

The original 9000bp probe for HHV-6 developed from a fragment named pZVH 14 from a Bam H1 digest of the GS strain has been shown not to react with any other herpesviruses [Josephs 1986]. This fragment contains the open reading frame (ORF) for the large tegument protein gene (LTP gene) [Josephs 1991a]. An 830 bp sequence from this part of the genome was later amplified [Collandre 1991] and strain variations were demonstrated by restriction enzyme digestion in this area [Aubin 1991; Aubin 1992; Aubin 1993]. A Hind III digestion site at position 2945 of the LTP gene, present in "B" but not "A" subtype strains of HHV-6 has been used in paediatric studies to discriminate between the two types of HHV-6 [Dewhurst 1992; Dewhurst 1993].

To increase sensitivity and specificity a nested PCR was designed to amplify a 287bp outer segment and 163bp inner segment across the HindIII restriction enzyme site (position 2817-3103) (figure 1). Details of the primers chosen including: position on the LTP gene; base pair composition; length; optical density; molecular weight; melting temperature; and amplimer lengths are given on table 1. Primers were made by Oswel, Department of Chemistry, King's Buildings, University of Edinburgh.

Legend to figure 1: Sequence of HHV-6 genome chosen for amplification

diagramatic representation of sequence chosen for amplification:

	TR	terminal repeats
	LTP gene	Large tegument protein gene
Products of nested PCR for HHV-6	First round	287 base pairs
	Second round	163 base pairs
Products of Hind 111 digestion	Type "A" HHV-6	163 base pairs
	Type "B" HHV-6	66 & 97 base pairs

Figure 1: Sequence of HHV-6 genome chosen for amplification

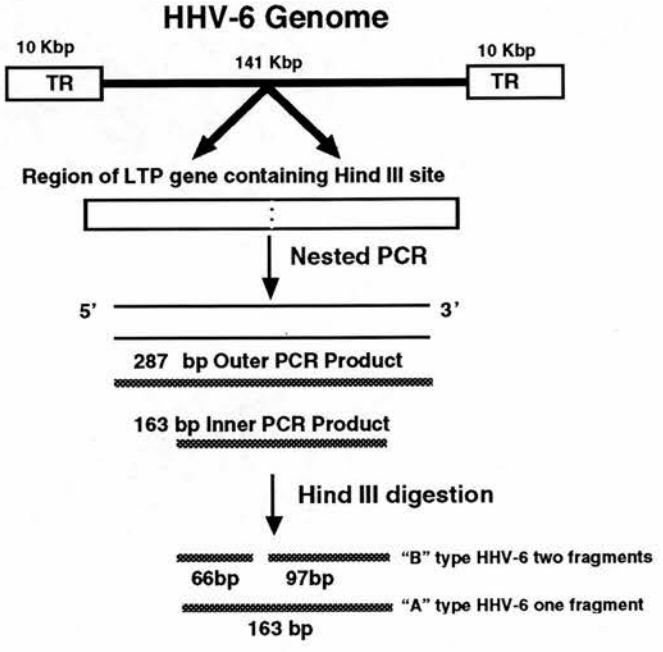


table 1: Details of Primers for HHV-6 and Human Betaglobin PCR.

bp - base pairs; % C+G - percentage of cytosine and guanosine bases;
Tm - melting temperature; OD / ml - Optical density / ml; Mol Wt - molecular weight.

HHV-6 primers										
Primer	Position on LTP gene 5' end	3' end	Length bp	% C+G	Tm	OD/ml	MolWt	Amplimer	Sequence	
Round 1										
1 outer	2817	2840	24	54	74	12	7920		agt cat cac gat cgg cgt gct atc	
2 outer	3103	3081	23	48	62	19	7590	287bp	tat cta gcg caa tcg cta tgt cg	
Round 2										
3 inner	2879	2902	24	54	74	17	7920		tcg act ctc acc cta ctg aac gag	
4 inner	3041	3018	24	46	70	18	7920	163bp	tga cta gag agc gac aaa ttg gag	
Human Betaglobin (HB) primers										
Primer	Position on HB gene 5' end	3' end	Length bp	% C+G	Tm	OD/ml	MolWt	Amplimer	Sequence	
1	54	73	20	50	60	20.6	5934		caa ctt cat cca cgt tca cc	
2	195	176	20	55	62	21.9	6186	268bp	gaa gag cca agg aca ggt ac	

table 1: Details of Primers for HHV-6 and Human Betaglobin PCR

Source of β globin primers, Bauer H et al, JAMA 1991. 265. 472-77.

Development and optimisation of the PCR

A series of experiments were undertaken to optimise the nested PCR for the detection of low copy numbers of HHV-6 genome (table 2). A hot start (80^{o}C) was used for the first round PCR to minimise nonspecific priming and increase sensitivity. In the first and second rounds of amplification 30 cycles were used. The first denaturation was for 5 minutes at 94^{o}C and all subsequent denaturations for 1 minute, annealing was for 1 minute at 60^{o}C, and extension for 1 minute at 60^{o}C, except for the last cycle where extension was for 8 minutes. The first round reaction volume was 100µl and the second round 50µl, and the same reaction mix was used in each round. Two units of Taq polymerase (Perkin Elmer) with the manufacturer's buffer, 1µM of primers, 0.2mM of all four d-Nucleoside triphosphates (dNTP's), and 1.5mM of magnesium chloride were used per reaction. 10µl of extracted sample were added to the first round PCR and 2µl of the subsequent product were then added to the second round. All samples were amplified twice.

Sensitivity and Specificity of the Nested PCR for HHV-6

During the development of the HHV-6 nested PCR, sensitivity was assessed by using a dilution series of measured HHV-6 DNA. Initially dilution series of extracted DNA from the supernatant of cultured HHV-6 were used to obtain a rough estimate of the sensitivity of the PCR and ultimately this was confirmed by using a pure dilution series of HHV-6 amplimers.

Dilution series of extracted HHV-6 DNA

The sensitivity of the nested PCR was assessed making a dilution series of freshly extracted DNA from the supernatant of cultures of AJ strain HHV-6 (figure 2a). The virus culture supernatant samples were ultracentrifuged to concentrate viral particles and subjected to PK digestion and DNA extraction as described above. It was assumed therefore, that the majority of DNA extracted would be of viral origin and the DNA content of the samples was measured by spectrophotometry. The first sample V1, contained 12.75 µg DNA / ml and the second sample V3, contained 16 µg DNA /ml. Tenfold dilutions of these samples were made in sterile water down to 0.1 fg per sample of DNA and then the dilution series were amplified according to the initial nested protocol. Figure 2a demonstrates that for supernatant V1, HHV-6 could be amplified down to an input of 1.28 pg of DNA and for supernatant V3, HHV-6 could be amplified down to an input of160 fg of DNA. One HHV-6 genome is equivalent to 0.172 femtogram (fg) of DNA (assuming the mass of a nucleotide pair is 660 daltons, and one dalton, is equal to 1.67 x 10^{-24} grams, [Sambrook 1989]). Thus, in the two dilution series described V1 and V3, the lowest concentrations amplified contained 7440 and 930 HHV-6 genomes respectively. With use of an initial hot start and increased primer

concentrations in both rounds of the PCR (see table 2) it was possible to improve the sensitivity to give a positive band on agarose gel down to an input of 16 fg of DNA, equivalent to 93 HHV-6 genomes. This is demonstrated in figure 2b which shows the result of an experiment where the use of wax or mineral oil to cover the PCR reaction was compared. In this experiment clearer bands were obtained with mineral oil as well as amplification to a lower concentration, oil was therefore used for the patient samples. A PCR sensitive to the level of around 100 HHV-6 copies per reaction was considered acceptable for this study.

Dilution series of HHV-6 amplimers

In addition a dilution series of measured DNA from first round HHV-6 amplimers was subjected to the full nested PCR to assess the PCR sensitivity . A positive band on agarose gel was seen consistently at 10-100ag of input DNA and sometimes at 1ag of input DNA (figure 2c). One attogram of DNA is equivalent to 3 HHV-6 amplimers (assuming the mass of a nucleotide pair is 660 daltons, and one dalton, is equal to 1.67×10^{-24} grams, [Sambrook 1989]).

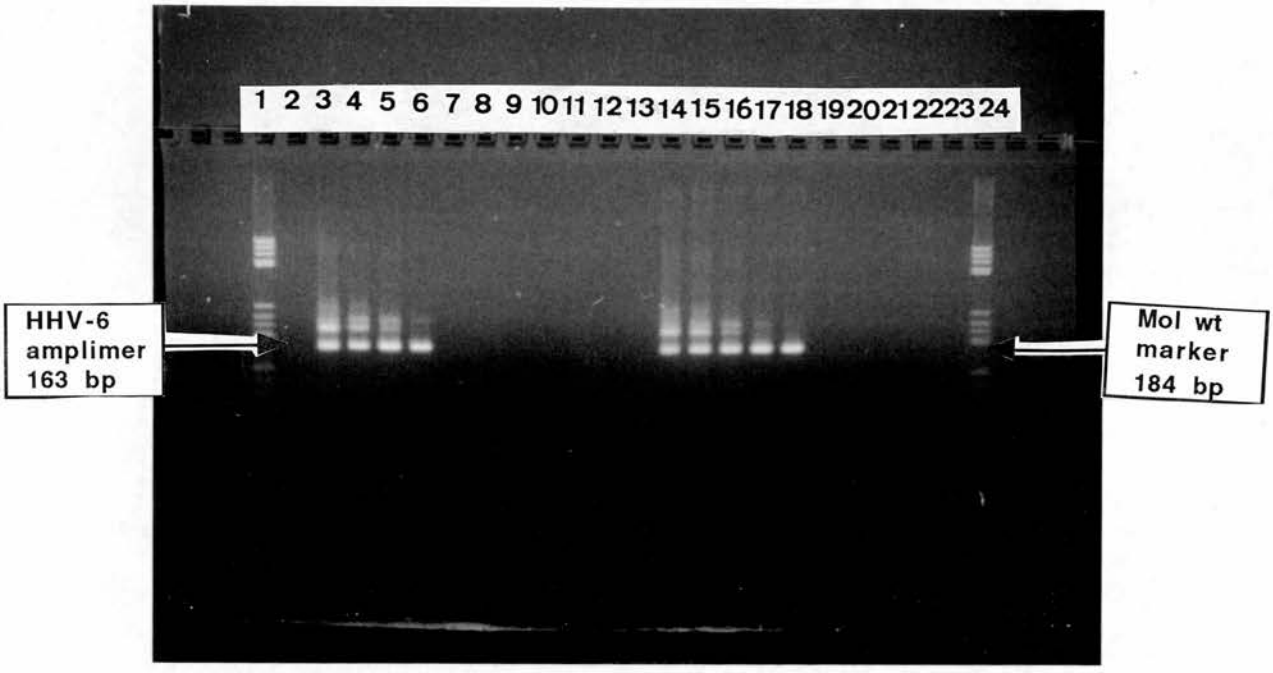
The specificity of the primer set for HHV-6 was demonstrated in an experiment where DNA was extracted from all seven herpesviruses, known at the time, and subjected to the nested PCR. Herpes simplex types 1 and 2, varicella zoster and cytomegalovirus were all obtained from laboratory isolates confirmed by fluorescent monoclonal antibodies. Epstein-Barr virus was cultured from the EB3 cell line. HHV-6 type "A" (AJ strain) was cultured from JJhan cells. Extracted HHV-7 DNA (MK strain) was a kind gift from Dr Duncan Clark, Royal Free Hospital. The input DNA was not measured for any of the laboratory isolates, but the HHV-7 DNA was at a concentration of 200 ng / µl and 400ng were added to the first round reaction . Three dilutions of HHV-6 amplimers equivalent to 3000, 300 and 30 amplimers were also amplified. The products of the nested PCR were run on agarose gel and a positive band was only seen in two lanes. One corresponding to the extract of cultured HHV-6 (lane H) and the other to a dilution equivalent to 3000 HHV-6 amplimers (lane L) ((figure 3).

table 2: Optimisation experiments for HHV-6 nested PCR (HHV-6 DNA extracted from the AJ strain of the virus was used these experiments)

Feature to be tested	Experiments		Outcome
Annealing Temps for the:			
Outer sets of primers	**Temperature (oc)**	**Results of EB gel**	
Primer Pair 1&2	52,55,58,60,62	Inc temp= Inc intensity of product	chose annealing temp of 60oc for round 1 PCR
Primer Pair 1&2	60,62,65,68,70,72	Inc temp = dec intensity of product	
Inner sets of primers	**Temperature (oc)**	**Results of EB gel**	
Primer Pair 3&4	60,62,65,68	dec temp= Inc intensity of product	chose annealing temp of 60oc for round 2 PCR
Primer Pair 3&4	52,55,58,60	Inc temp= Inc intensity of product	
Primer Concentrations	**0.1 µM**	**0.5 µM**	**5.0 µM**
Round 1 PCR	No product	Product	Product + Primer-dimers
Round 2 PCR			The best combination of primers was
(from round 1 with 0.1µM of primers)	Product	Product	Product + lots of extra bands 0.1-0.5 µM for both rounds.
	With higher round one primer concentrations there was more 1st round product after the 2nd round PCR.		
Number of Cycles of PCR	**25,30,35, cycles.**		
Round 1 PCR	There were more nonspecific products with 25 cycles, but no increase in products with increased number of cycles.		Chose 30 cycles for each round of the PCR
Round 2 PCR	No of cycles in round 2 were not tested.		
Hot Start	A dilution series of HHV-6 DNA was amplified after incubation of sample, primers, buffer & MgCl at 80oc for 10 mins prior to addition of dNTP's, buffer & Taq polymerase		Hot start decreased the number of non-specific bands seen after PCR but did not increase the sensitivity of the PCR.
DNA Polymerase	A pair of dilution series of HHV-6 DNA were amplified with Taq polymerase or "Dynazyme" as the DNA polymerase		Taq polymerase amplified down to 160fg DNA Dynazyme only amplified down to16pg DNA therefore Taq polymerase was used for the PCR.
Oil versus Wax	A pair of dilution series of HHV-6 DNA were amplified with a hot start and either paraffin oil or wax between layers		The PCR with wax was positive down to 160fg The PCR with oil was positive down to 16fg therefore oil was used for the PCR,. this was also easier to handle in the Nested PCR
Beta-globin Primers	addition of Beta-globin primers to the PCR mix decreased the sensitivity of the PCR		The concentration of HHV-6 primers used was therefore increased to 1µM for each round of the PCR.

table 2: Optimisation experiments for HHV-6 nested PCR

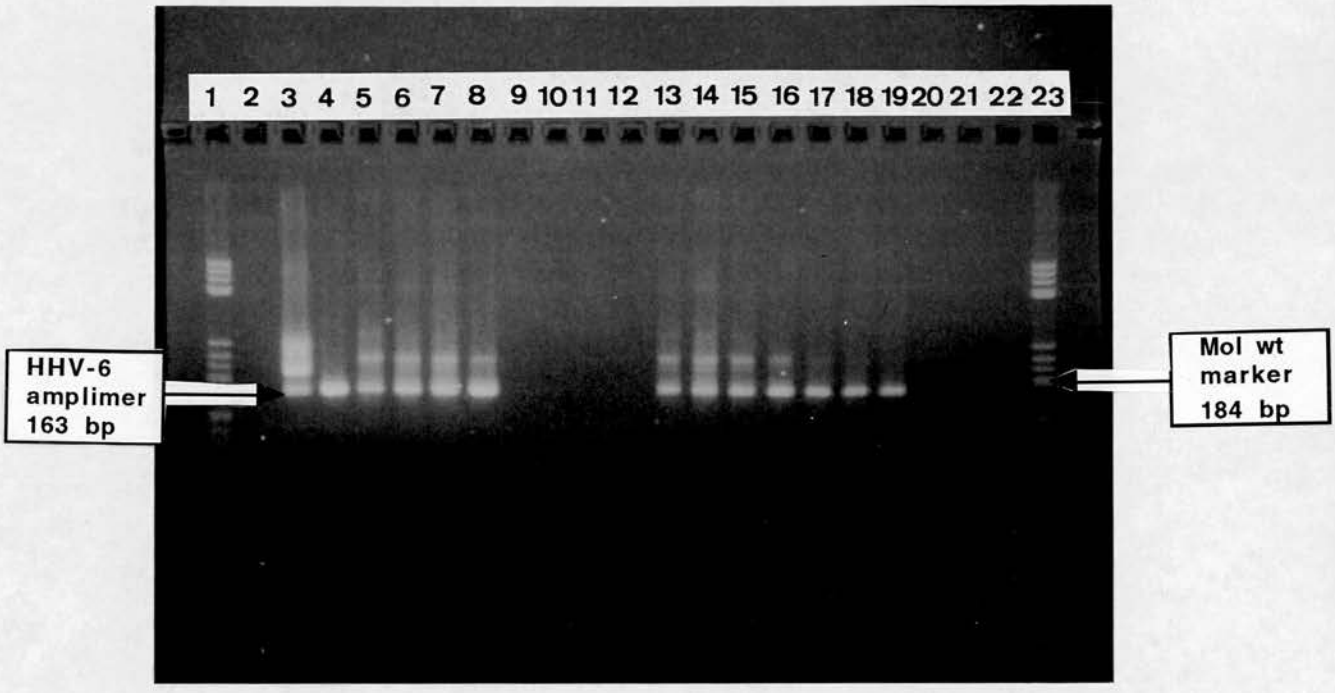
figure 2a : Sensitivity of the nested PCR for HHV-6, dilution series of DNA extracted from the supernate of cultured AJ strain HHV-6.



Legend to figure 2a : Sensitivity of the nested PCR for cultured HHV-6.
Dilution series of DNA extracted from supernate of two different cultures AJ strain HHV-6, V1 and V3. Lanes positive for HHV-6 amplimers indicated in bold type.

		Extract of V1		Extract of V3
Lane	1	DNA ladder	13	empty
	2	empty	**14**	**1.6 ng of DNA**
	3	**1.28 ng of DNA**	**15**	**160 pg of DNA**
	4	**128 pg of DNA**	**16**	**16 pg of DNA**
	5	**12.8 pg of DNA**	**17**	**1.6 pg of DNA**
	6	**1.28 pg of DNA**	**18**	**160 fg of DNA**
	7	128 fg of DNA	19	16 fg of DNA
	8	12.8 fg of DNA	20	1.6 fg of DNA
	9	1.28 fg of DNA	21	0.16 fg of DNA
	10	0.128 fg of DNA	22	water
	11	water	23	empty
	12	empty	24	DNA ladder

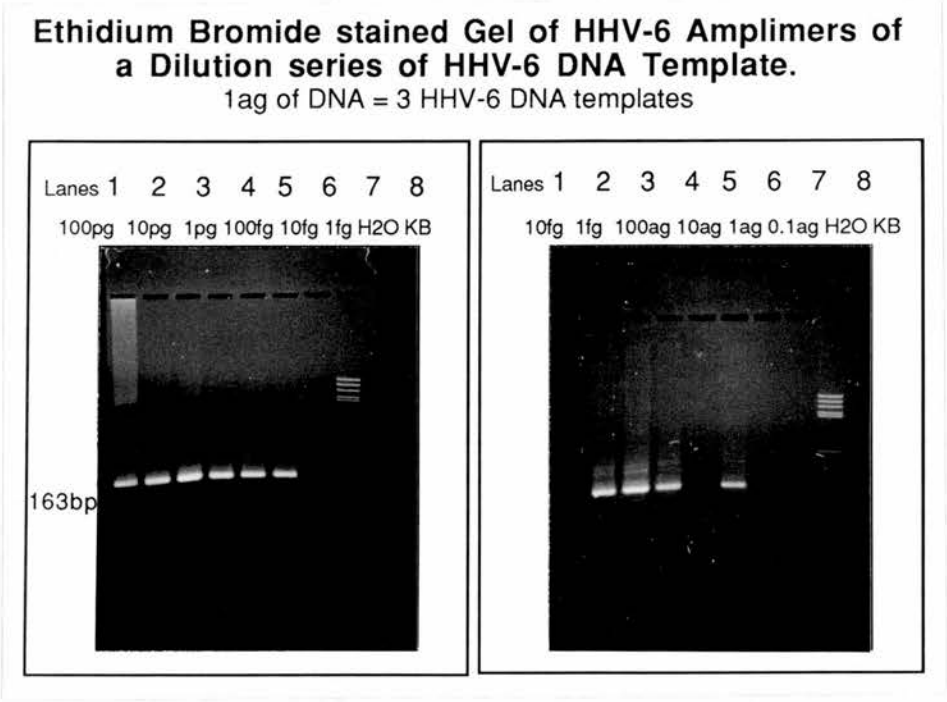
figure 2b : Sensitivity of the nested PCR for HHV-6, dilution series of DNA extracted from the supernate of cultured AJ strain HHV-6, comparison of wax or oil to cover the PCR reaction.



Legend to figure 2b : Sensitivity of the nested PCR for cultured HHV-6. Dilution series of DNA extracted from the supernate of culture V3 of AJ strain HHV-6. Left hand lanes covered with wax and Right hand with oil. Lanes positive for HHV-6 amplimers indicated in bold type.

		Wax Overlay		OIL Overlay
Lane	1	DNA ladder	12	empty
	2	empty	**13**	**16 ng of DNA**
	3	**16 ng of DNA**	**14**	**1.6 ng of DNA**
	4	**1.6 ng of DNA**	**15**	**16 pg of DNA**
	5	**160 pg of DNA**	**16**	**160 pg of DNA**
	6	**16 pg of DNA**	**17**	**16 pg of DNA**
	7	**1.6 pg of DNA**	**18**	**1.6 pg of DNA**
	8	**160 fg of DNA**	**19**	**160 fg of DNA**
	9	16 fg of DNA	**20**	**16 fg of DNA**
	10	1.6 fg of DNA	21	1.6 fg of DNA
	11	water	22	water
			23	DNA ladder

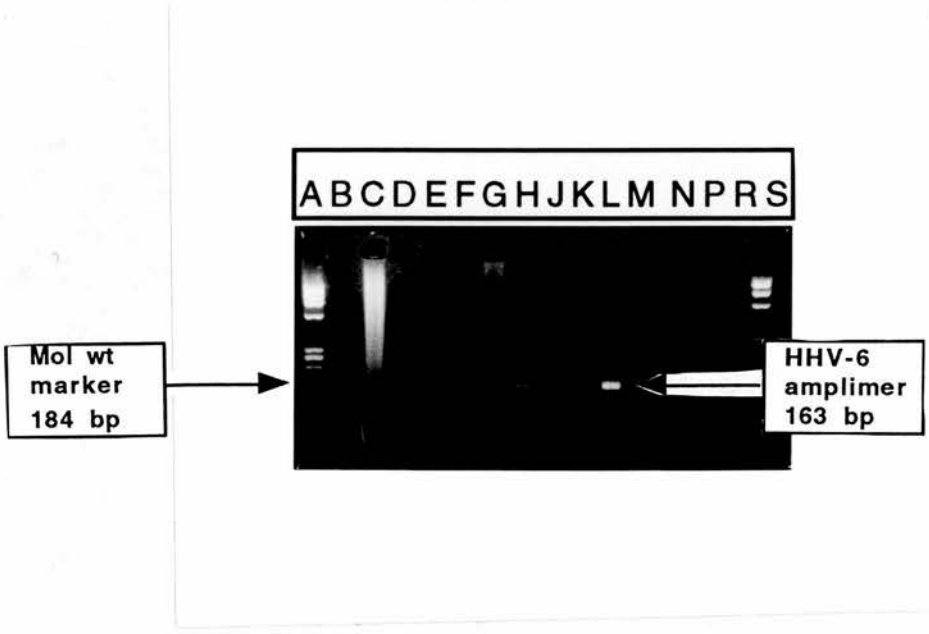
figure 2c : Sensitivity of the nested PCR for HHV-6, dilution series of HHV-6 templates.



Legend to figure 2c : Sensitivity of the nested PCR for HHV-6 amplimers. Dilution series of amplified HHV-6 templates on a left and right hand ethidium bromide stained gel. 1 ag of HHV-6 DNA is equivalent to 3 HHV-6 DNA templates (see text for calculation). Lanes positive for HHV-6 amplimers indicated in bold type.

Left Gel			**Right Gel**		
Lane	**1**	**100pg of HHV-6 DNA**	Lane	**1**	**10fg of HHV-6 DNA**
	2	**10pg**		**2**	**1fg**
	3	**1pg**		**3**	**100ag**
	4	**100fg**		4	10ag
	5	**10fg**		**5**	**1ag**
	6	1fg		6	0.1ag
	7	water		7	water
	8	DNA KB ladder		8	DNA KB ladder

figure 3: Specificity of the nested PCR for HHV-6



Legend to figure 3: Specificity of the nested PCR for HHV-6

Only two positive bands seen at the level equivalent to 163 bp: lane H from extracted HHV-6 from culture supernatant; lane L from stored, aliquoted, HHV-6 amplimers. Lanes positive for HHV-6 amplimers indicated in bold type.

Lanes	A	DNA ladder (Phi x 174 RF DNA / Hae 111, fragments - 1353, 1078, 872, 603, 310, 271 / 281, 234, 194, 118, 72), Gibco / BRL.
	B	empty
	C	HSV 1
	D	HSV 2
	E	VZV
	F	EBV
	G	CMV
	H	**HHV-6 (extracted supernatant from cultured AJ strain)**
	J	HHV-7
	K	Water
	L	**dilutions of HHV-6 amplimers ~ 3000**
	M	dilutions of HHV-6 amplimers ~ 300
	N	dilutions of HHV-6 amplimers ~ 30
	P	Water
	R	empty
	S	DNA mass ladder (fragments - 2000, 1200, 800, 400, 200, 100)

PCR Reaction Mix Components

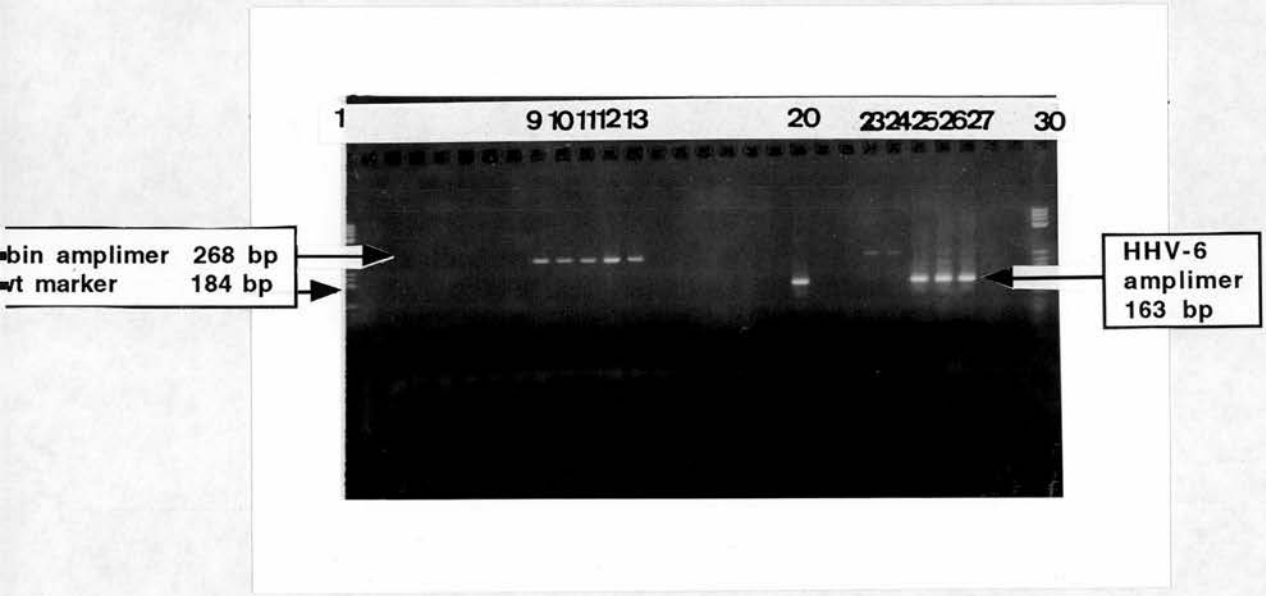
All reaction mixes were prepared in a designated clean pre-PCR laboratory where gloves, aprons and lab coats were worn at all times. Filter tips were used for all pipetting to avoid contamination from pipette hubs. Reaction mixes for the first and second round PCR's were prepared in a still air hood which had been illuminated with UV light for at least 30 mins, and wiped down with 70% ethanol before and after use.

A "hot start" method was used for the first round PCR only. The first round reaction mixes were made up as "top" and "bottom" batches with a total volume of 100µl per reaction. In the "bottom" of the eppendorf tube was placed PCR buffer (Perkin Elmer), magnesium chloride (1.5mM)(supplied as part of buffer), first round primers for HHV-6, and Betaglobin primers, this was overlayed with 75µl of paraffin oil. The sample of extracted DNA was added to this in a different class II extraction hood. The tubes were then taken to the thermal cycler (Hybaid) and heated up to 80^{o}c. As the samples were heating up, Taq polymerase (Perkin Elmer) was added to the "top" solution which contained PCR buffer, and dNTP's. This was then layered on top of the hot oil at the thermal cycler and the cycles of amplification allowed to commence. For the second round of PCR all the elements, except target, were added to the tubes in the still air hood (PCR buffer, magnesium chloride (1.5mM) (supplied as part of buffer), second round primers for HHV-6, dNTP's and Taq polymerase) to make a total volume of 48µl per reaction. Two µl of first round product was added to the tube in another room and the second round of amplification allowed to proceed.

Betaglobin Primers

Although the conditions for this nested PCR were optimised for HHV-6 nested primers alone it was decided to include primers for genomic DNA (beta-globin) in the first round PCR (table 1). The aim was to identify the presence of host DNA in the samples and ascertain whether this bore any relation to the presence of HHV-6 DNA. This also acted as an internal control for inhibition of the PCR. Figure 4 demonstrates the amplimers generated by the betaglobin PCR on one of the first runs of saliva samples tested, where most pellet samples amplified betaglobin. As a result of this betaglobin primers were added to the first round PCR mix, but the clear results demonstrated in this run were rarely repeated when all the test samples were run.

figure 4: Betaglobin primers added to the first round of the HHV-6 nested PCR.



Legend to figure 4: Betaglobin primers in the HHV-6 nested PCR.
This agarose gel demonstrates a series patient samples (supernatant -S, pellet -P) with HHV-6 and betaglobin PCR. All but one of the pellet samples amplified betaglobin, indicated with an asterisk* in round one and ** in round two. One supernatant and three pellets amplified HHV-6, indicated in bold type.

		First round PCR		Second round PCR
Lane	1	DNA KB ladder	16	empty
	2	empty	17	patient sample S
	3	patient sample S	18	patient sample S
	4	patient sample S	19	patient sample S
	5	patient sample S	**20**	**patient sample S**
	6	patient sample S	21	patient sample S
	7	patient sample S	22	patient sample S
	8	patient sample S	23	patient sample P**
	9	patient sample P*	24	patient sample P**
	10	patient sample P*	**25**	**patient sample P**
	11	patient sample P*	**26**	**patient sample P**(w)**
	12	patient sample P*	**27**	**patient sample P**(w)**
	13	patient sample P*	28	patient sample P
	14	patient sample P	29	empty
	15	empty	30	DNA KB ladder

PCR Negative Samples

Any samples repeatedly negative to HHV-6 nested amplification or beta-globin amplification were spiked with 600 HHV-6 (AJ strain) first round amplimers and the PCR repeated for evidence of inhibition of the PCR.

Samples included in each PCR amplification run

Samples were amplified in batches of 21 with three water controls, one uninfected lymphoblastoid cell line negative control, and three positive controls containing 30, 300, and 3,000 HHV-6 amplified genome segments of type "A" from the AJ strain of the virus. The products of the first and second round amplification were run concurrently on an ethidium bromide stained gel (figure 5).

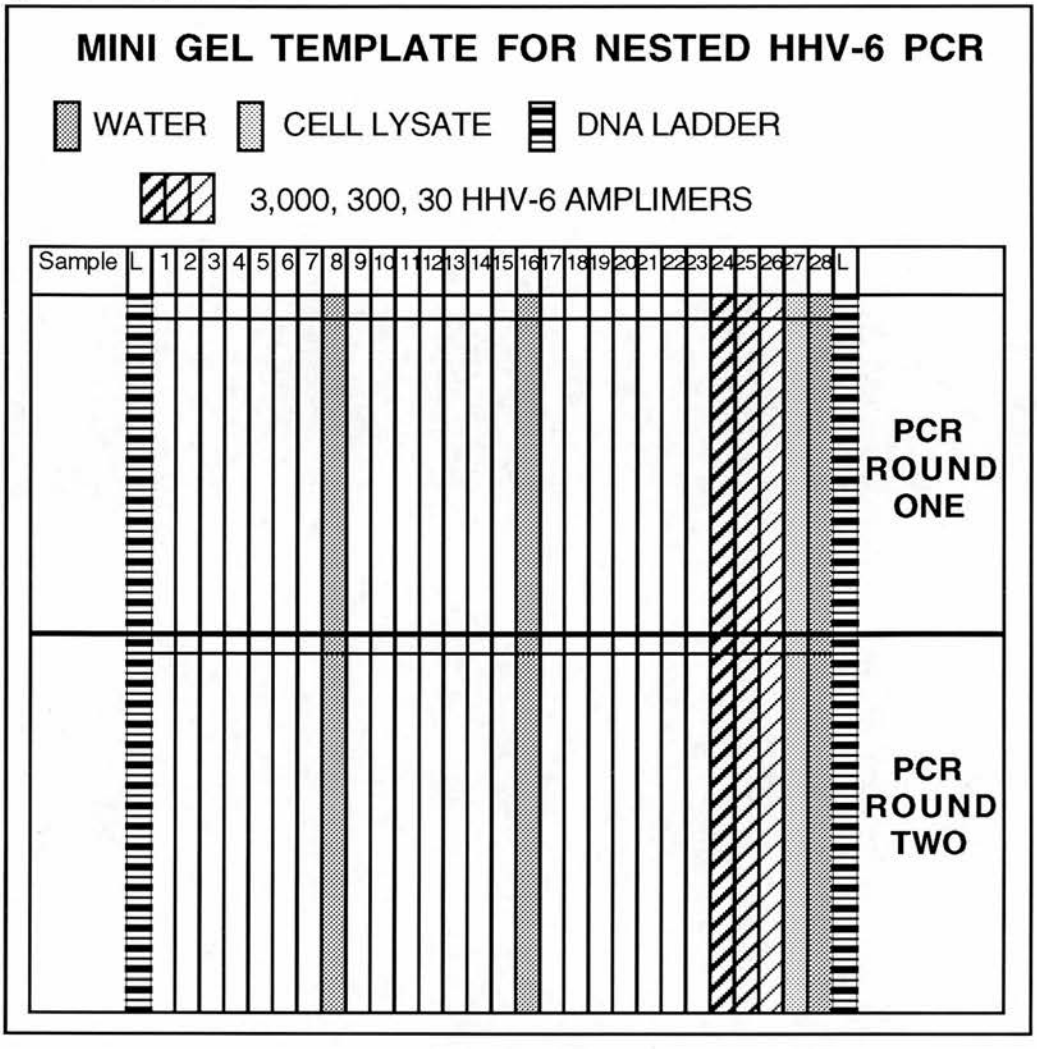


figure 5: Template for PCR Gel Runs used for each batch of PCR amplified samples.

Detection of Amplified HHV-6 - Ethidium Bromide stained Gels

Agarose mini-gels (60 ml) were prepared with 3% agarose (2% Nuseive and 1% Seakem) and 0.5 x TBE buffer. After dissolving the gel and cooling to about 60°c, 2µl of ethidium bromide was added to the gel which was then poured into the mould, edged with autoclave tape and the comb added. The gel was cooled to room temperature and then to 4°c. The electrophoresis tank was filled with 0.5 x TBE buffer of the same batch and the tape removed from the gel before it was submerged in buffer to 2-3 mm. The comb was carefully removed from the gel.

Two µl of loading buffer stained with bromophenol blue (0.25% in 40% sucrose) was added to 14 µl of sample and, 12µl of the mixture was then carefully added to each well.

The DNA ladder used to compare with the length of amplified fragments visualised was "DNA molecular weight marker noV" (Boehringer Mannheim). This is a mixture of fragments from cleavage of plasmid pBR322 DNA and contains 22 fragments from 587 to 8 bp in length (587/ 504/ 458/ 434/ 267/ 234/ 213/ 192/ 184/ 124/ 123/ 104/ 89/ 80/ 64/ 57/ 51/ 21/ 18/ 11/ 8). The ladder was diluted 1/15 in sterile water, to approximately 3 µg of DNA per well, and run in at least two wells on each gel.

Electrophoresis of the gel was for about one hour over 14.8 cm; at 100 volts and 50 mAmps, ie 6.75 volts / cm. When the dye had reached to within 1 cm of the end of the gel the current was discontinued, the gel removed from the buffer, wrapped in "Saran" film and examined under UV light on the transilluminator and either photographed or transcribed to the template diagram (figure 5). Ethidium bromide stained gels were disposed of with autoclaved rubbish and used buffer washed down the drain with copious running water.

HHV-6 PCR Positive samples and Hind III digestion

Any sample which amplified HHV-6 DNA and gave a band at 163bp after the second round of PCR was subjected to Hind III digestion to ascertain whether it was sub-type A or B HHV-6. For this restriction enzyme (RE) digestion, 5µl of amplified product, 3µl of sterile water, and 1µl of 10 X RE buffer B (the appropriate buffer for this enzyme,Boehringer Mannheim) were added to a microcentrifuge tube. At -20°c the RE, Hind III, was diluted to 10 units per µl in buffer B and 2 units added immediately to each reaction. The reaction tubes were incubated for 1 hour in a water bath at 37°c and after this 2µl of 100mM EDTA was added to each tube to stop the reaction. As the products of this digestion gave small fragments (97 and 66 bp) a 3% "Metaphor " agarose gel (Flowgen) with 1 x TBE, stained with ethidium bromide was used for better definition of fragments. The samples were tested in batches of up to 12 with an HHV-6 type A positive control (which does not digest with Hind III) and the "DNA molecular weight marker noV" (Boehringer Mannheim) on each gel.

Statistical Methods

Nonparametric tests were used to compare the small groups of patient samples including the Chi-squared test and the Mann-Whitney-U test.

Recipes for Buffers

1M TRIS stock solution

121.1g of TRIS dilute in 800ml distilled water
Adjust pH to 7.5 with HCl (5M)
Make to 1l, aliquot and autoclave.

0.5 M EDTA stock solution

18.61g EDTA dilute in 80ml distilled water
Adjust pH to 8.0 with NAOH pellets (approx 2g)
(NB EDTA not go into solution without NaOH)
Make to 100ml, aliquot and autoclave.

TE Lysis buffer

To make 100ml
5ml stock solution 1M TRIS (final conc 50mM TRIS)
2ml stock solution 0.5M EDTA (final conc 10mM EDTA)
0.5ml Tween 20 (final conc 0.5%)
92.5 ml distilled water
Add proteinase K to a final conc of 100g/ml for protein digestion.

TBE electrophoresis buffer, 5 x stock solution

TRIS base	54g (89mM)
Boric acid	27.5g (89mM)

0.5M EDTA stock (pH 8.0) 20ml (2mM)
Make to 1L with distilled water, dispense as 50ml aliquots and autoclave.
(On long term storage TBE can precipitate, if so it should not be used).

Prospective Saliva PCR Study

Results of PCR experiments

Patients and Controls

A total of 115 saliva samples, including pellets and supernates, were examined for HHV-6 DNA. Seventy seven saliva samples were obtained from 12 patients (2-13 per child), age range 3-13 years. Thirty eight samples were obtained from 10 siblings (two from one sibling at different times), and 27 healthy volunteer children, age range 4-16 years. Fifty one serum samples were obtained from the 12 patients (1 - 9 per child), it was only possible to test an initial 25 of these by PCR.

Performance of the HHV-6 PCR

No amplified HHV-6 DNA was ever found after PCR in any of the water or uninfected lymphoblastoid cell controls. The lymphoblastoid cell control was only intermittently positive for the betaglobin amplimer, which may have been related to lack of genomic DNA in some samples , but may also have been related to the fact that the betaglobin primers were used in a system which was not optimised specifically for them. All three concentrations of HHV-6 positive control were amplified on 65% of runs, however on 35% of runs only 3,000 and 300 HHV-6 amplimers were amplified. It appeared that the addition of the betaglobin primers to the reaction mix could have affected the sensitivity of the HHV-6 nested PCR.
There were 36 completely PCR negative samples (both HHV-6 and betaglobin), and after spiking these with 600 HHV-6 amplimers and reamplifiying them, only one saliva pellet and two supernates were found to be inhibitory for the PCR.

Concordance of Results between two PCR runs

Over all for the HHV-6 PCR the positive concordance between the replicate PCR runs was similar for both supernate (74%) and pellet (76%). The betaglobin PCR positive concordance rate was much higher for supernate specimens (88%) than pellet specimens (67%), probably because the majority of supernate specimens were, as expected, betaglobin PCR negative. A similar pattern was seen when the results were divided into patient and control groups (table 3).
These results demonstrate that some PCR runs were more sensitive than others. As there was no obvious problem with contamination at any time, it was decided for interpretation of the patient data, to consider a sample which had been PCR positive on at least one of the runs as a positive result.

table 3: Concordance of PCR results between runs for the different patient and control groups

"same" - same result between two runs, "different" - different result between two runs.

	betaglobin		betaglobin		betaglobin		HHV6		HHV6		HHV6	
Result	Same		different		same		different		same		different	
	supernate		supernate		pellet		pellet		supernate		pellet	
	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)
patient first samp n = 12	11	(92)	1	(8)	7	(58)	5	(42)	12	(100)	0	(0)
All patient samples n = 77	73	(95)	4	(5)	52	(68)	25	(32)	59	(77)	18	(23)
Siblings n = 11	7	(64)	4	(36)	5	(45)	6	(55)	6	(55)	5	(45)
Volunteers n = 27	21	(78)	6	(22)	20	(74)	7	(26)	20	(74)	7	(26)
All controls n = 38	28	(74)	10	(26)	25	(66)	13	(34)	26	(68)	12	(32)
All samples	101	(88)	14	(12)	77	(67)	38	(33)	85	(74)	30	(26)

	HHV6		HHV6	
	same		different	
	pellet		pellet	
	No	(%)	No	(%)
patient first samp n = 12	9	(75)	3	(25)
All patient samples n = 77	58	(75)	19	(25)
Siblings n = 11	7	(64)	4	(36)
Volunteers n = 27	22	(81)	5	(19)
All controls n = 38	29	(76)	9	(24)
All samples	87	(76)	28	(24)

Prospective Saliva PCR Study
PCR Results for all samples

HHV-6 PCR

When the total number of samples obtained were considered and the results obtained for the pellet and supernate were summed then 28/38 (74%) of healthy control saliva samples and 45/77 (58%) patient saliva samples were PCR positive for HHV-6. When the supernate and pellet results were considered separately then 23/38 (61%) of healthy control supernate samples and 30/77 (40%) patient supernate samples were PCR positive for HHV-6 and 21/38 (55%) of healthy control pellet samples and 31/77 (40%) patient pellet samples were PCR positive for HHV-6 (table 4). The overall findings are represented diagrammatically on bar charts (figure 6 -8). All the patients were seropositive for HHV-6 IgG by IFA.

Comparison of all the results obtained for the patient and control groups demonstrate that HHV-6 DNA could be amplified from fewer patient supernate specimens than controls (Chi squared test $p = 0.04$), but there was no significant difference for amplification of HHV-6 from pellets or when the results from pellets and supernates were summed. In the above comparison a single specimen obtained from each control patient (except one sibling who gave 2) was compared with a series of samples obtained from patients. When the results from only the first patient sample obtained (n=12) were compared with those of the control group (n=38), then the same pattern of results was demonstrated, but there was no significant difference between patients or controls for supernatants, pellets or the combined results (table 4).

Photographs of representative agarose gels of amplified HHV-6 DNA from salivary specimens from patients and controls with a diagrammatic key are shown on figures 15 - 17.

table 4: Results of HHV-6 PCR for Patients and Healthy Controls

			HHV6		HHV6	
Cases	**No**	**Type of Samples**	**Ever**	**Positive**	**Never**	**Positive**
First Patient sample	12	Supernate	2	(17%)	10	(83%)
		Pellet	7	(58)	5	(42)
		Both	7	(58)	5	(42)
All Patient samples	77	Supernate	30	(40)	47	(60)
		Pellet	31	(40)	46	(60)
		Both	45	(58)	32	(42)
All Sibs samples	11	Supernate	5	(45)	6	(55)
		Pellet	6	(55)	5	(45)
		Both	8	(73)	3	(27)
All Volunteer samples	27	Supernate	18	(67)	9	(33)
		Pellet	15	(56)	12	(44)
		Both	20	(74)	7	(26)
All Control samples	38	Supernate	23	(61)	15	(39)
		Pellet	20	(53)	18	(47)
		Both	26	(68)	12	(32)

table 4: Results of HHV-6 PCR for Patients and Healthy Controls

figure 6: HHV-6 DNA in saliva supernatant samples from patients & controls

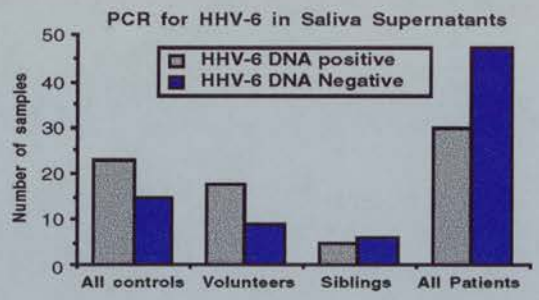


figure 7: HHV-6 DNA in saliva pellet sample from patients & controls

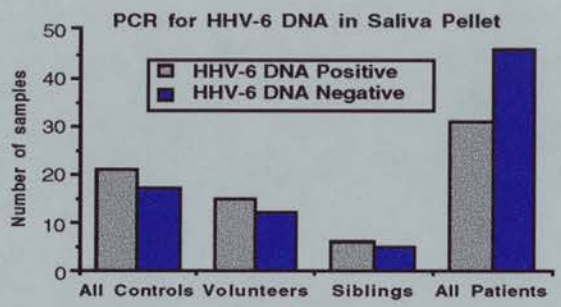
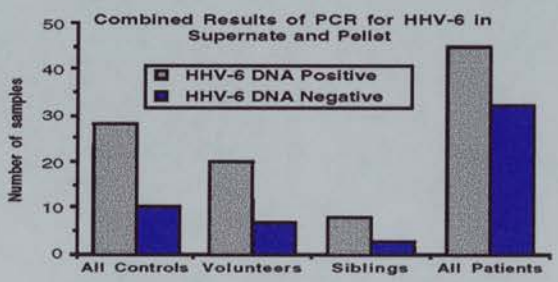


figure 8: Combined result of HHV-6 DNA in saliva pellet and supernatant



Comparison of saliva results from Well and Febrile Patients

When the PCR results from the patients who were well were compared with those who were unwell and febrile at the time of collection, fewer supernate samples from unwell children amplified HHV-6 DNA (chi-squared test p=0.06), but there was no significant difference in HHV-6 DNA in the pellet samples or when the results were summed for supernate and pellet (figures 9-11, and table 5).

Comparison of saliva results according to Patients' White Blood Count

Only one third of supernate samples from children with a total white blood count (WBC) less than 1×10^{9}/l and absolute neutrophil count (ANC) less than 1×10^{9}/l amplified HHV-6 DNA whereas in samples from children with counts greater than 1×10^{9}/l nearer half the samples amplified HHV-6. However, these differences did not reach significance by the chi-square test. Whether the absolute lymphocyte count was greater than or less than 1×10^{9}/l did not affect the salivary HHV-6 DNA PCR result. (figures 12-14, and table 6).

Figure 9: HHV-6 DNA in saliva supernatant from well & febrile patients

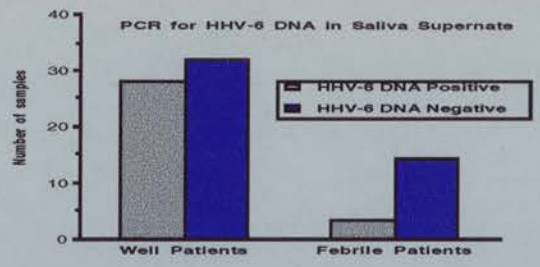


Figure 10: HHV-6 DNA in saliva pellet from well & febrile patients

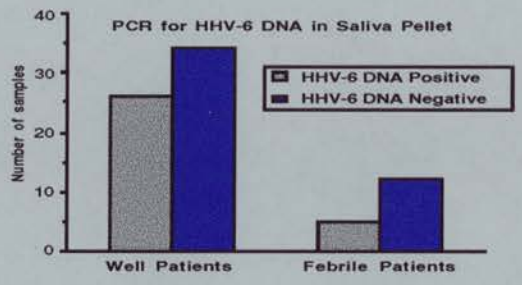


Figure 11: Combined result of HHV-6 DNA in saliva pellet and supernatant

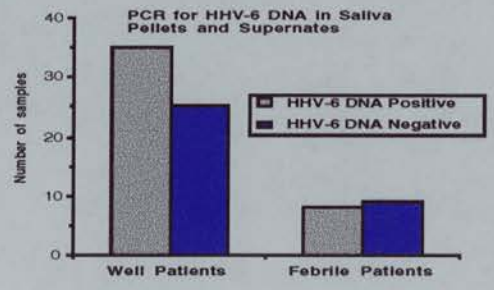


table 5: HHV-6 DNA PCR results in well and febrile patients

			HHV6		HHV6	
Cases	**No**	**Type of Samples**	**Ever**	**Positive**	**Never**	**Positive**
All Patient samples	77	Supernate	30	(40%)	47	(60%)
		Pellet	31	(40)	46	(60)
		Both	45	(58)	32	(42)
Well patients	60	Supernate	28	(47)	32	(53)
		Pellet	26	(43)	34	(57)
		Both	35	(58)	25	(42)
Febrile patients	17	Supernate	3	(18)	14	(83)
		Pellet	5	(29)	12	(71)
		Both	8	(47)	9	(53)

table 5: HHV-6 DNA PCR results in well and febrile patients

figure 12: HHV-6 DNA in saliva supernatants according to WBC

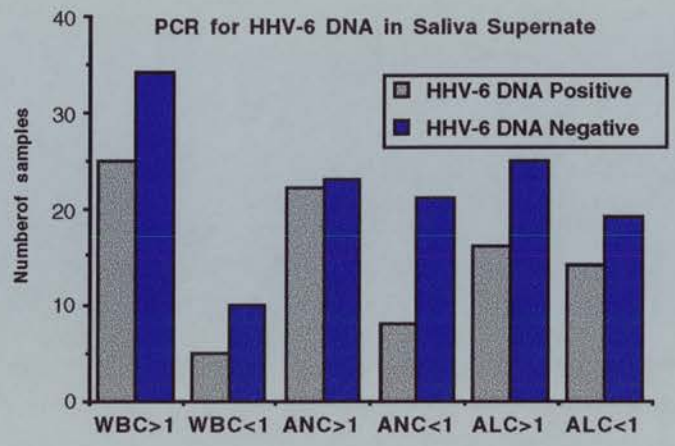


figure 13: HHV-6 DNA in saliva pellets according to WBC

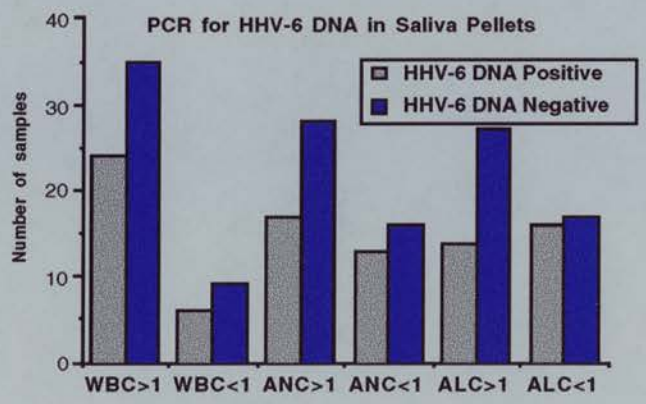
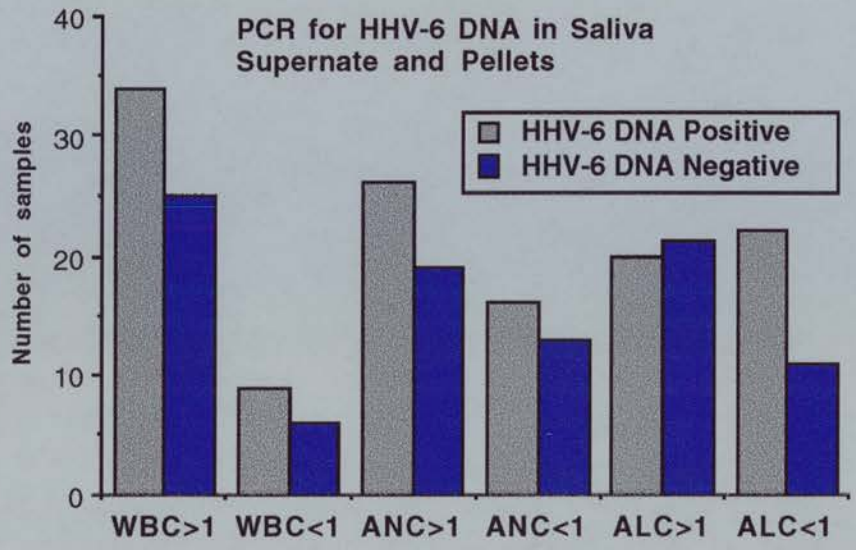


Figure 14: Combined result of HHV-6 DNA in saliva pellet and supernatant

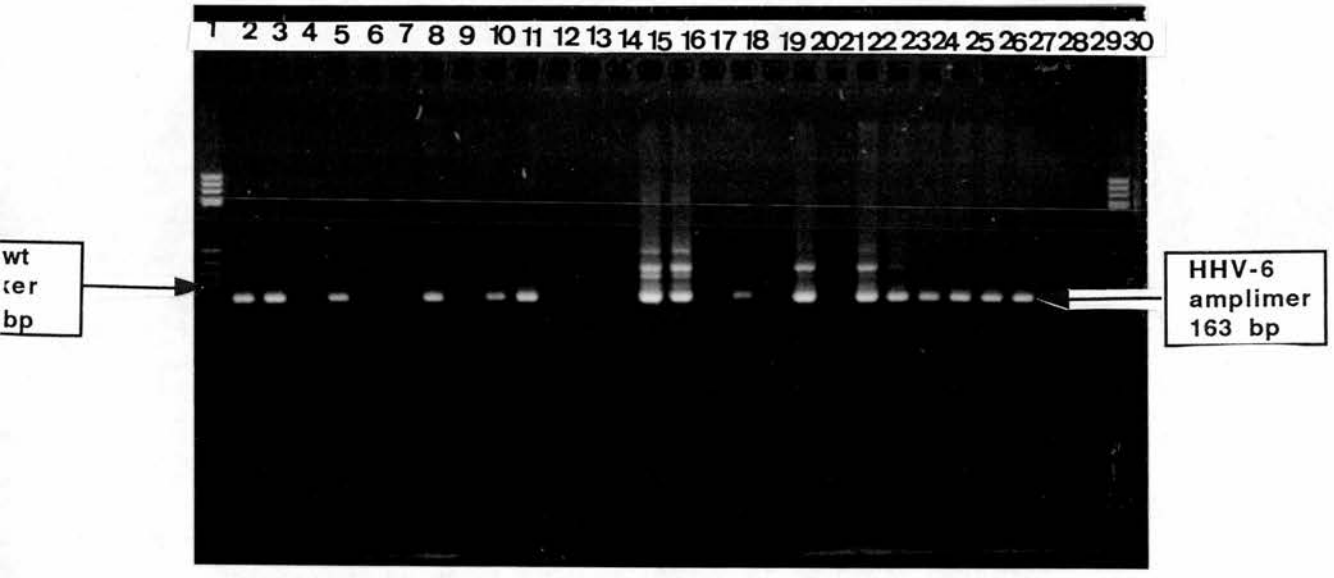


Legend to graphs 12 -14:

WBC> 1	Total white blood count greater than 1 x 10^{9}/l
WBC < 1	Total white blood count less than 1 x 10^{9}/l
ANC > 1	Absolute neutrophil count greater than 1 x 10^{9}/l
ANC < 1	Absolute neutrophil count less than 1 x 10^{9}/l
ALC > 1	Absolute lymphocyte count greater than 1 x 10^{9}/l
ALC < 1	Absolute lymphocyte count less than 1 x 10^{9}/l

Photographs of HHV-6 nested PCR Gel runs

figure 15: Series of patient saliva samples amplified for HHV-6 DNA

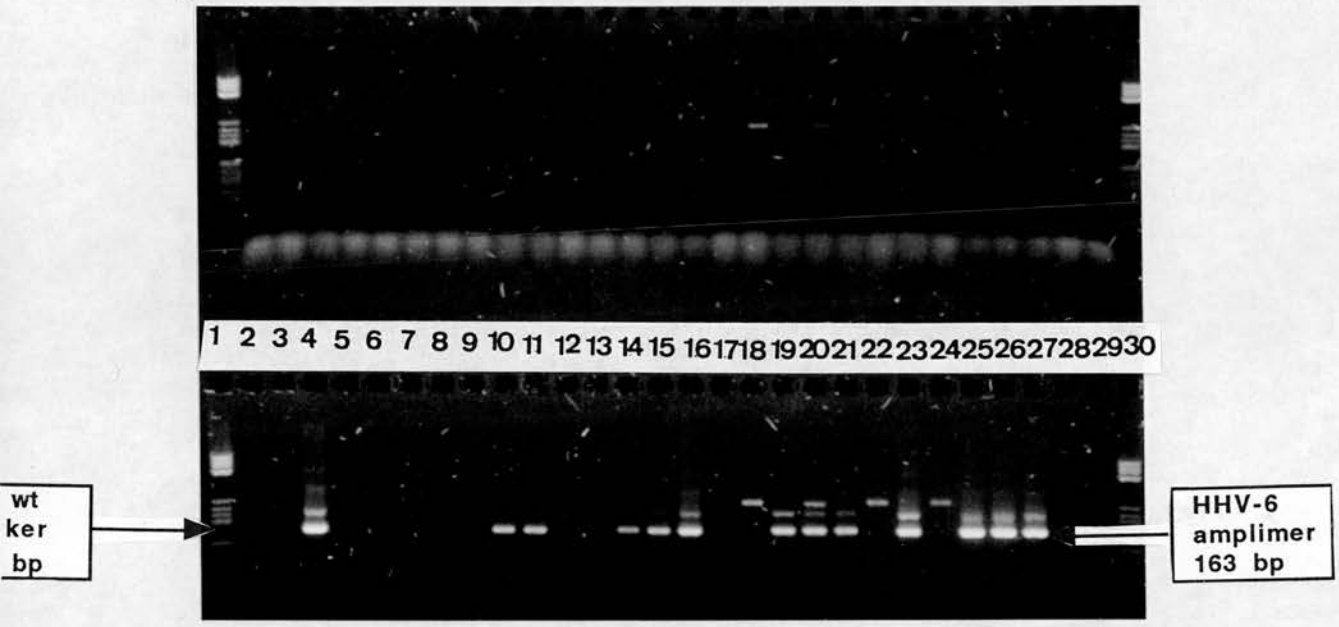


Legend to figure 15: A series of patient samples amplied for HHV-6 DNA. All the batches of samples were tested according to the template shown previously (figure 4). Only the second round PCR products are shown, samples which amplfied HHV-6 are indicated in bold (S - supernatant, P - pellet). Samples which were Betaglobin PCR positive on the first round indicated by an asterisk *. The lymphoblastoid cell line did not amplifiy betaglobin.

Lane	1	DNA KB ladder	**16**	**patient sample P***
	2	**patient sample S**	17	water
	3	**patient sample S**	**18**	**patient sample S**
	4	patient sample S	19	patient sample S
	5	**patient sample P**	**20**	**patient sample S**
	6	patient sample S	21	patient sample S*
	7	patient sample S	**22**	**patient sample P***
	8	**patient sample S**	**23**	**patient sample P**
	9	water	**24**	**patient sample P**
	10	**patient sample S**	**25**	**3000 HHV-6 amplimers**
	11	**patient sampleS**	**26**	**300 HHV-6 amplimers**
	12	patient sample P	**27**	**30 HHV-6 amplimers**
	13	patient sample P*	28	lymphoblastoid cell DNA
	14	patient sample P*	29	water
	15	**patient sample P***	30	DNA KB ladder

Photographs of HHV-6 nested PCR Gel runs

figure 16: Series 1 of control saliva samples amplified for HHV-6 DNA



Legend to figure 16: A series of control samples amplied for HHV-6 DNA. All the batches of samples were tested according to the template shown previously (figure 4). The first and second round PCR products are shown, upper part of gel - first round, lower part -second round. Samples which amplfied HHV-6 are indicated in bold (S - supernatant, P - pellet). Samples which were Betaglobin PCR positive on the first round only are indicated by an asterisk *, and those positive on the second round ** . The lymphoblastoid cell line did not amplifiy betaglobin.

Lane	1	DNA KB ladder	**16**	**Control sample P**
	2	Control sample S	17	water
	3	Control sample S	18	Control sample P*, **
	4	**Control sample S**	**19**	**Control sample P**
	5	Control sample S**(+/-)	**20**	**Control sample P*,****
	6	Control sample S	**21**	**Control sample P**
	7	Control sample S	22	Control sample P*, **
	8	Control sample S**	**23**	**Control sample P**
	9	water	24	Control sample P*, **
	10	**Control sample S**	**25**	**3000 HHV-6 amplimers**
	11	**Control sample S**	**26**	**300 HHV-6 amplimers**
	12	Control sample S	**27**	**30 HHV-6 amplimers**
	13	Control sample P	28	lymphoblastoid cell DNA
	14	**Control sample P**	29	water
	15	**Control sample P**	30	DNA KB ladder

Photographs of HHV-6 nested PCR Gel runs

figure 17: Series 2 of control saliva samples amplified for HHV-6 DNA

Legend to figure 17: A series of control samples amplied for HHV-6 DNA. All the batches of samples were tested according to the template shown previously (figure 4). The first and second round PCR products are shown, upper part of gel - first round, lower part -second round. Samples which amplfied HHV-6 are indicated in bold (S - supernatant, P - pellet). Samples which were Betaglobin PCR positive on the first round only are indicated by an asterisk *, and those positive on the second round ** . The lymphoblastoid cell line did amplifiy betaglobin.

Lane	1	DNA KB ladder	**16**	**Control sample S**
	2	Control sample P	17	water
	3	**Control sample P**	**18**	**Control sample S**
	4	Control sample P	**19**	**Control sample S**
	5	Control sample P	**20**	**Control sampleS**
	6	**Control sample P**	21	Control sample S
	7	**Control sample P**	22	Control sample S
	8	Control sample P	**23**	**Control sample S**
	9	water	**24**	**Control sample S**
	10	Control sample P	**25**	**3000 HHV-6 amplimers**
	11	Control sample P	**26**	**300 HHV-6 amplimers**
	12	Control sample P	27	30 HHV-6 amplimers
	13	**Control sample S**	28	lymphoblastoid cell DNA**
	14	**Control sampleS**	29	water
	15	Control sampleS	30	DNA KB ladder

table 6: HHV-6 PCR results according to patient white blood count

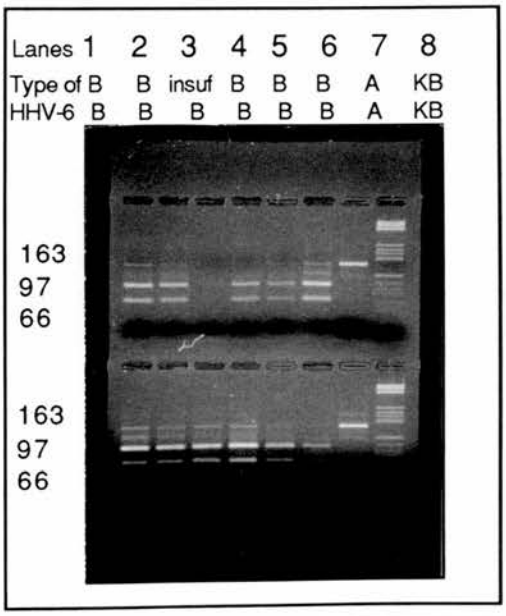
WBC - total white blood cell count; ANC - absolute neutrophil count; ALC - absolute lymphocyte count.

			HHV6		HHV6	
Cases	**No**	**Type of Samples**	**Ever**	**Positive**	**Never**	**Positive**
All Patient samples	77	Supernate	30	(40%)	47	(60%)
		Pellet	31	(40)	46	(60)
		Both	45	(58)	32	(42)
WBC>1	59	Supernate	28	(47)	32	(53)
		Pellet	26	(43)	34	(57)
		Both	35	(58)	25	(42)
WBC<1	15	Supernate	25	(42)	34	(58)
		Pellet	24	(41)	35	(59)
		Both	34	(58)	25	(42)
ANC>1	45	Supernate	22	(49)	23	(51)
		Pellet	17	(38)	28	(62)
		Both	26	(58)	19	(42)
ANC<1	29	Supernate	8	(28)	21	(72)
		Pellet	13	(45)	16	(55)
		Both	16	(55)	13	(45)
ALC>1	41	Supernate	16	(39)	25	(61)
		Pellet	14	(34)	27	(67)
		Both	20	(49)	21	(51)
ALC<1	33	Supernate	14	(42)	19	(58)
		Pellet	16	(48)	17	(52)
		Both	22	(67)	11	(33)

Results of Hind III Restriction Enzyme Digestion

All the HHV-6 PCR positive saliva samples (53 supernatant extracts and 52 pellet extracts) tested from patients and controls digested with Hind III to give two fragments and were therefore "B" type HHV-6 (figure 18).

figure 18: A representative gel of the products of the Hind III restriction enzyme digestion.



legend to figure 18: Agarose gel of Hind III digested products of PCR for HHV-6 DNA. Upper and Lower rows lanes 1-6 patient samples, lane 7 positive control A type HHV-6. All the patient samples digested to give two products of 97 and 66 bp, except top row lane 3 where there was insufficient sample in this run. The control A type HHV-6 did not digest giving only one product of 163 bp. All the patient samples evaluable were therefore type B HHV-6.

Betaglobin PCR

When the total number of samples obtained were considered and the results obtained for the pellet and supernate were summed then 26/38 (68%) of control saliva samples and 38/77 (49%) patient saliva samples were first round PCR positive for betaglobin. When the supernate and pellet results were considered separately then 11/38 (29%) of control supernate samples and 4/77 (5%) patient supernate samples were PCR positive for betaglobin and 18/38 (47%) of control pellet samples and 34/77 (44%) patient pellet samples were PCR positive for Betaglobin (table 7) (see figures 15,16,17 above). Comparison of all the results obtained for the patient and control groups demonstrate that betaglobin DNA could be amplified from significantly fewer patient supernate specimens than controls ($p < 0.001$), but there was no significant difference for amplification of betaglobin from pellets. When the results from pellets and supernates were summed this difference remained significant ($p = 0.03$). There was no significant difference in betaglobin amplification within patient groups compared. Saliva samples from which Betaglobin gene sequence could be amplified, whether pellet or supernatant, were not any more likely to amplify HHV-6 than those which did not.

table 7: Betaglobin DNA PCR for Patients and Controls

			Betaglobin		Betaglobin	
Cases	No	Type of Samples	Ever	Positive	Never	Positive
First Patient sample	12	Supernate	1	(8)	11	(92)
		Pellet	6	(50)	6	(50)
		Both	6	(50)	6	(50)
All Patient samples	77	Supernate	4	(5)	73	(95)
		Pellet	34	(44)	43	(56)
		Both	38	(49)	39	(51)
All Sibs samples	11	Supernate	4	(36)	7	(64)
		Pellet	7	(64)	4	(36)
		Both	10	(91)	1	(9)
All Volunteer samples	27	Supernate	7	(26)	20	(74)
		Pellet	11	(41)	16	(59)
		Both	16	(59)	11	(41)
All Control samples	38	Supernate	11	(29)	27	(71)
		Pellet	18	(47)	20	(53)
		Both	26	(68)	12	(32)

table 7: Betaglobin DNA PCR for Patients and Controls

Sequential Salivary HHV-6 PCR Results from Individual Patients

A chronological series of samples was obtained from 12 patients, 7 with leukaemia and 5 with solid tumours, and all these patients were seropositive for HHV-6. The samples were obtained at regular intervals when the children were either well or febrile and unwell from the time of diagnosis until the end of the collection period. The timing of sampling from these children and results of the nested HHV-6 PCR are shown in figure 19.

Since the number of samples obtained per child varied from 2 to 13, to enable differences in the sequential data to be considered between patients, for each child the ratios of the summed, pellet & supernatant, results were compared (table 8 and figure 20). Comparison of these ratios revealed two distinct groups of patients, those with mainly HHV-6 positive saliva (group B) and those with mainly HHV-6 negative saliva (group A) (CHi-sq $p<0.0001$). There was no difference between these two groups for, age, betaglobin PCR, total white blood count, absolute lymphocyte count, or diagnosis (solid tumour / leukaemia). However, in the mainly HHV-6 negative, group A, there were more febrile unwell children (10/30 group A versus 7/47 group B, CHi-sq $p=0.05$) and they had lower absolute neutrophil counts (M- W -U test $p=0.02$).

The results from unsummed supernates and pellets from the children were also expressed as ratios and the results were very similar. For the supernates one child from group B had a ratio of less than 0.5, for the pellets three children from group B had ratios of less than 0.5, but they were all greater than the ratios from the patients in group A and none of the group A children had higher ratios (data not shown).

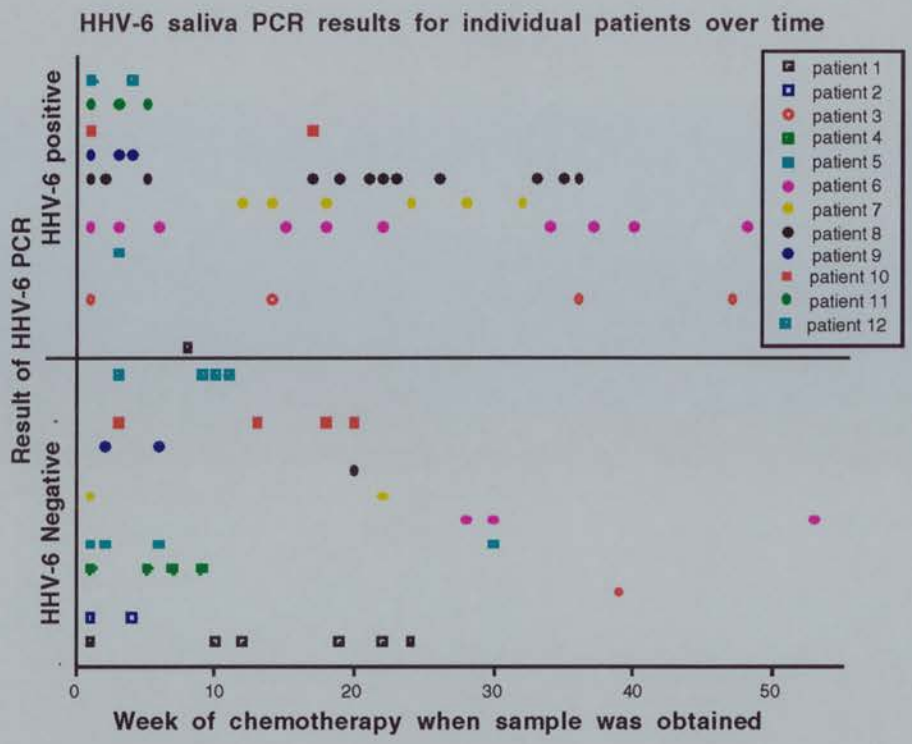


figure 19: sequential series of HHV-6 saliva PCR results from 12 patients

HHV-6 saliva PCR results are presented for 12 patients as they were collected chronologically over the weeks of chemotherapy. Samples from each patient are represented by a different coloured symbol. Those patients who had mainly HHV-6 positive samples are represented by circles and those with mainly HHV-6 negative samples are represented by squares. In a week when a patient had an HHV-6 positive sample it appears above the line and if it was an HHV-6 negative sample below the line.

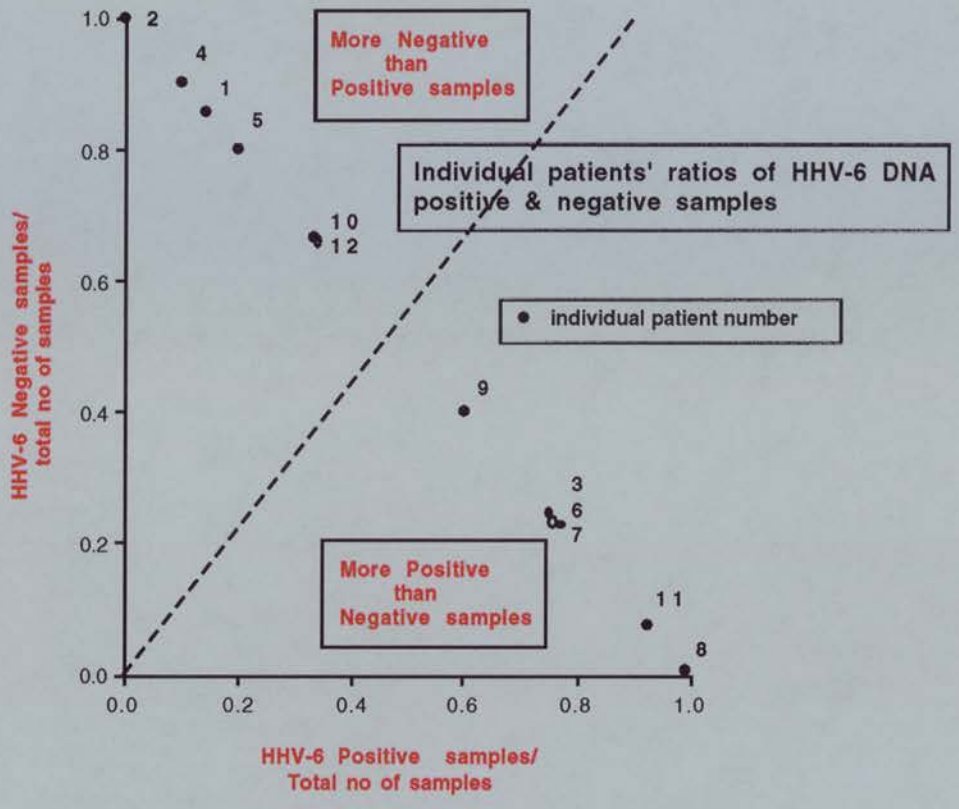


figure 20: Ratios of HHV-6 positive / negative samples for each patient

Group A patients - mainly HHV-6 positive saliva (patients 3,6,7,8,9,11)

Group B patients - mainly HHV-6 negative saliva (patients 1,2,4,5,10,12)

table 8: Details of HHV-6 PCR results over time from individual patients.

Group A - rarely HHV-6 PCR positive, Group B - frequently HHV-6 PCR positive

Patient group & no	Diagnosis ST/AL	Age yrs	Weeks followed	HHV-6 PCR positive	HHV-6 PCR negative	HHV-6 pos / total samples	Febrile Pos	Febrile Neg	Well Pos	Well Neg
A1	ST	3	9	-	4	0	-	-	-	4
A2	Al	4	4	-	2	0	-	2	-	-
A4	AL	8	24	1	6	0.14	1	2	-	4
A5	ST	13	30	1	4	0.2	-	-	1	4
A10	AL	6	20	2	4	0.33	1	4	1	-
A12	AL	5	11	2	4	0.33	-	-	2	4
B3	ST	7	47	4	1	0.8	-	-	4	1
B6	ST	5	59	10	3	0.77	1	-	10	2
B7	ST	10	32	6	2	0.75	2	-	4	2
B8	AL	8	36	12	1	0.92	3	-	9	1
B9	AL	12	6	3	2	0.6	-	1	3	1
B11	AL	4	5	3	-	1	-	-	3	-

table 8: Ratios of positive to negative saliva samples for HHV-6 DNA from patients over time

PCR for HHV-6 and Betaglobin in Patient Serum Samples

An initial 25 / 51 serum samples from ten of the patients were examined for HHV-6 and betaglobin DNA by PCR. These samples had been collected at routine visits as well as during admissions when the patients were febrile and unwell. None of the samples amplified HHV-6 DNA or betaglobin DNA. When spiked with 600 HHV-6 amplimers all but one amplified. There was insufficient time to amplify all 51 samples.

Anti-viral treatment and HHV-6 PCR results

During this sequential study, none of the 12 children were treated with ganciclovir, which is known to interrupt HHV-6 excretion [Russler 1989]. Three samples were obtained from children whilst on aciclovir therapy, which has a much weaker negative effect on HHV-6 excretion [Russler 1989]. Two saliva samples from children on oral aciclovir were PCR positive for HHV-6. One saliva sample from a child receiving intravenous aciclovir was HHV-6 PCR negative. Numbers are too small to assess whether aciclovir therapy had any effect on results obtained for the HHV-6 PCR.

Findings of the Prospective Study of HHV-6 DNA in the saliva of paediatric oncology patients

Performance of the nested HHV-6 PCR

The sensitivity of a PCR will determine the number of positive results obtained and the degree of positivity in any sample examined. In this study an indirect measure of the sensitivity of the system could be gained from the dilution series of cultured virus and of first round amplimers. However this does not necessarily relate directly to findings in saliva where different biochemical conditions could alter the sensitivity of the system.

Only one saliva pellet and two supernatants, one pair being from the same child, were inhibitory to the PCR and all the other repeatedly negative samples gave positive results when spiked with 600 first round HHV-6 amplimers. Some of the PCR runs appeared more sensitive than others, according to the dilutions of first round amplimer which could be amplified, but with an end point dilution of 30 amplimers in the most dilute sample it is possible that on occasions lack of amplimer in the dilution led to the negative result rather than decreased sensitivity of the system. Indeed, although PCR is an extremely sensitive technique it is also dependent on highly defined conditions and there are many variables which can affect the performance of any particular run.

The decision to include Betaglobin primers as an internal control in the first round of the PCR may also have affected the sensitivity of the HHV-6 PCR. Unfortunately the sensitivity experiments for the HHV-6 PCR were undertaken prior to this and they were not repeated with the Betaglobin primers in the mix. It is also the case that the PCR was optimised for the HHV-6 primers and not the Betaglobin primers and they also may not have been amplifying at maximal efficiency. In retrospect, it would have been preferable either to optimise the PCR for both sets of primers or to have carried out separate PCRs for each primer set .

All samples were amplified at least twice, and it was decided to consider a sample positive even if it amplified HHV-6 only once out of two tests. This was considered acceptable: 1) because of the possibility of variable performance of the PCR; 2) because of the likelihood that HHV-6 could been present very small numbers variable between replicates; and 3) because of the fortunate lack of any contamination of water or uninfected cell extract controls. Although the positive concordance between the two HHV-6 PCR runs was similar for both supernate (74%) and pellet (76%), in a larger more definitive study more stringent concordance between replicates would be required.

HHV-6 and HHV-7 are genetically closely related viruses [Frenkel 1990a; Frenkel 1991; Schirmer 1991; Berneman 1992], and controversy over salivary findings for HHV-6 DNA could be related to the specificity of the PCR used. Earlier studies did not necessarily test primers for specificity to HHV-6 alone allowing the possibility for amplification of both viruses.

The primers used in this study did not amplify HHV-7 DNA when tested. Therefore the results obtained in this pilot study would appear to be specific for HHV-6 in the saliva. Of note though, the sensitivity of the PCR in the specificity experiment was not as good as in most of the patient runs as only the 3000 HHV-6 amplimer dilution was amplified where in most of the patient runs 300 or even 30 amplimers were amplified. However this is balanced against the fact that the concentration of HHV-7 DNA added to the sample was quite high and therefore it might still be expected that any amplification would have been detected.

Interpretation of patient results

HHV-6 in Saliva

This preliminary examination of sequential saliva samples from paediatric oncology patients for HHV-6 DNA demonstrated that "B" type HHV-6 DNA can frequently be found in the saliva of healthy children (74%) and those with malignancy (58%). "B" type HHV-6 has been found almost exclusively in children with primary infection and increasingly frequently in immunosuppressed patients. Although there are only a small number of patients in this study the finding that they could be clearly separated into two groups, one where the saliva was frequently positive for HHV-6 DNA (81% of samples tested positive) and the other rarely positive (20% of samples tested positive) is of interest. The former group appeared to most closely resemble the healthy control children, not only in terms of HHV-6 positivity but also they were generally well with fewer febrile episodes and less neutropaenia.

That HHV-6 is more frequently present in the saliva of more healthy children appears at first surprising as latent herpes viruses are considered more often recurrent and more likely to cause problems in immunosuppressed patients. A number of factors may be acting to produce this seemingly paradoxical result in the "well" children. Immunosuppressed children on chemotherapy could secrete less HHV-6 when they are unwell if the turnover of cells in the salivary glands, a likely source of virus, is affected by the treatment. Chemotherapy also tends to deplete the lymphocyte population which is a reservoir for HHV-6. Although in this study, no relation between low blood lymphocyte counts and HHV-6 excretion was seen, it is possible that the local lymphocyte situation in the mouth mucosa could be different. An age effect might have meant that younger children more recently infected with HHV-6 would be more likely to be secreting the virus. However there was no significant difference in age between the groups. It could be that children who were less well were not so good at producing saliva specimens and the findings are simply an artifact of technique. However this did not appear to be the case when the specimens were collected. Further investigation to confirm or refute this preliminary paradoxical finding would require: larger numbers; better matching of patients and controls; sequential sampling of healthy controls as well as patients;

and a fully quantitative HHV-6 PCR.
Saliva samples were divided into pellet and supernatant to examine for cell free HHV-6 DNA in the supernatant. HHV-6 DNA was more often present in control sample supernates (23/38, 79%) than patient sample supernates (31/77, 40%). Strikingly, when the patients were divided into the frequently HHV-6 positive and rarely HHV-6 positive groups, again the frequently HHV-6 positive group most closely resembled the healthy children with a similar percentage of supernate samples HHV-6 DNA positive (29/47, 61%), very few supernate samples had HHV-6 DNA in the rarely HHV-6 positive group (2/30, 6%). That healthy children and the patients who are well are more likely to be secreting HHV-6 into the saliva again implies that in the others secretion is impaired for some reason. The factors which might cause this were discussed above, and a chemotherapy effect on salivary glands or mononuclear cells would seem a plausible explanation.
Pellet samples tested for HHV-6 DNA probably contained a mix of cellular material from the mouth, including monocytes and lymphocytes, and HHV-6 detected in these samples could have been latent or actively secreted. The percentage of samples positive for HHV-6 was similar between the whole patient group and the healthy controls (31/77 (40%) v 21 / 38 (55%)). There was also no difference where patients were unwell or well, or according to the blood white cell count. This would seem to imply that cell associated virus can be detected in a constant proportion of patients, but that there may be a decrease of secretion of virus in the less well children. Further examination of this would again require, larger numbers; better matching of patients and controls; sequential sampling of healthy controls as well as patients; and a quantitative HHV-6 PCR.

There are few published studies on secretion of HHV-6 in saliva of children. In a small study of primary infection with HHV-6 and HHV-7, 41 cases with fever +/- convulsions and 7 control patients were recruited [Clark 1997]. HHV-6 DNA was amplified from the saliva in 54% of cases and 40% of controls and HHV-7 DNA was found in saliva in 34% of cases and 20% of controls. These were all children who were seropositive and previously infected with HHV-6 or HHV-7. In this study the saliva was collected by rubbing the gums with a swab. The largest study of primary HHV-6 infection and subsequent persistence of the virus has been carried out in Rochester, USA, where over a number of years cases of primary HHV-6 infection have been identified and subsequently followed for evidence of persistence of the virus in the index case as well as other family members. Saliva, PBMC's and CSF specimens have all been examined [Hall 1998]. PCR analysis of 258 saliva samples, obtained from children 2-5 years after primary infection, demonstrated 52% to be HHV-6 positive. In family members, after PCR for HHV-6, 24% of 204 saliva samples collected from adults, and 52% of 235 samples collected from children were found to be positive. In sequential samples of saliva obtained from 199 children over a period of 6 months to 5 years after primary infection (median 2.2 years), HHV-6 DNA was detected in: 70% after 6 months - 1 year; 33% after 2

years; 67% after 3 years; and 50% after 4-6 years. Only 15 % of follow up adult specimens were repeatedly HHV-6 positive. These sequential findings from impressive numbers of children give similar percentage positivity to that observed in our small cross-sectional cohort of children.

In the early adult studies, HHV-6 was frequently found in saliva by PCR (63 - 90% of samples tested positive) [Gopal 1990; Jarrett 1990; Cone 1993a] and virus isolation (92 - 100% of samples tested positive) [Pietroboni 1988a; Harnett 1990; Levy 1990a] . However, in a small study where throat swabs from adults (n = 30) and children (n = 49) were examined for HHV-6 DNA , only 3% of adults and 12% of children had HHV-6 positive swabs [Kido 1990]. In another small Japanese study HHV-7 was isolated frequently from the saliva of immunocompetent adults (13 /16) and children over one year (7/10), and no HHV-6 was isolated [Hidaka 1993]. Cone et al demonstrated HHV-6 DNA in the saliva of 18 out of 20 healthy individuals, including one child, and the copy number ranged from zero - 200,000 HHV-6 genome equivalents per ml of saliva [Cone 1993a]. In 15 cases repeat samples were obtained after several months and similar results were obtained.Yamanishi has also described higher rates of HHV-6 DNA in throat swabs from children (age range 2-8 years, 78.6% positive) (International Herpesvirus workshop, Vancouver, 1994).

In a more recent study, where salivary HHV-6 and HHV-7 secretion as well as presence of the viruses in salivary gland biopsies were tested. DNA of each virus was found in 63% and 75% of the biopsies (n = 8), respectively [Di Luca 1995a]. HHV-7 was found more frequently in all the saliva samples tested (HHV-6 & HHV-7: healthy adults (n = 31), 3% & 55%; patients with recurrent aphthous ulcers (n = 12), 17% & 66%; adults with upper respiratory infections (n = 16), 0% & 56%). These results are very different from those obtained in the recent study mentioned above where 24% of adult samples were HHV-6 DNA positive [Hall 1998]. Another recent study of saliva from 44 healthy young adults (aged 19 -38 years, median 23) demonstrated 95% HHV-6 DNA positive [Aberle 1996].

Difference in the frequency of amplification of HHV-6 and HHV-7 from saliva may be related to technical problems, as at the time of the earlier studies HHV-7 had not yet been identified and the specificity of primers for HHV-6 alone had not been tested. However recent primers used are known to be specific for HHV-6, implying that frequent shedding of HHV-6 in saliva is a true finding [Di Luca 1995a; Aberle 1996; Hall 1998]. The volume of saliva tested may also be important if the virus is secreted in relatively low concentration, Di Luca et al extracted DNA from 150µl of saliva , where Cone et al used 400µl of saliva. Differences in isolation of virus may also be due to differences of technique, and direct comparison of throat swabs and saliva samples may not be appropriate.

Although there is some controversy about frequency of secretion of HHV-6 and HHV-7 in the saliva of adults, it would appear that it is less frequently shed than in children. As the large study of Hall et al has demonstrated, HHV-6 is frequently shed in the saliva of children, who have relatively recently been infected with the virus [Hall 1998]. It could be hypothesised that secretion of HHV-6 in saliva may wain with increased age or other factors.

Most studies where saliva has been examined for HHV-6 DNA in immunocompromised adults have only small numbers and conflicting findings. In a study were only 2 / 25 healthy controls were HHV-6 DNA positive in saliva, 2 / 8 patients with lymphoproliferative disease, 4 /25 patients with Sjogren's syndrome and 3 /18 patients with nasopharygeal carcinoma were also HHV-6 PCR positive [Saito 1991]. In this study the group with the highest positivity for HHV-6 were 4 / 8 patients with infectious mononucleosis who were also all EBV DNA positive.
In the small studies where HHV-6 has been cultured from saliva, no difference in isolation rate was found between HIV positive or negative individuals [Pietroboni 1988a; Harnett 1990 ; Levy 1990a]. By PCR however, one study demonstrated that patients with symptomatic HIV infection had a lower rate of HHV-6 positivity in saliva and PBMC than healthy adults [Gopal 1990]. Another study of 26 HIV positive patients demonstrated none to have HHV-6 in the saliva although this was found in only 3% of healthy controls, but significantly more HIV patients (81%) had HHV-7 than healthy controls (55%) [Di Luca 1995a]. Nearly half of these patients had AIDS and 77% were on AZT, implying that this was quite an immunosuppressed group. In a cohort with less severely affected HIV patients a higher frequency and higher copy numbers of HHV-6 DNA was demonstrated in the PBMC's of patients with high rather than low CD4 counts, but no significant correlation was seen with the copy number of HHV-6 in the saliva and stage of HIV disease or CD4 count [Fairfax 1994].
In a larger study HHV-6, HHV-7 and CMV were amplified from saliva and urine of 125 HIV positive adults and 29 controls [Gautheret-Dejean 1997]. All the viruses were found frequently in the saliva of patients and controls (HHV-6 - 43%, HHV-7 - 63%, and CMV - 61%) . All three viruses together were detected in 28% of samples and none in 18%.
HHV-7 and CMV detection did not differ between HIV seropositive and negative individuals, but the rate of HHV-6 detection was higher in saliva of seronegatives (HIV negative - HIV positive, 62% - 38%) ($p = 0.023$). The rate of DNA detection in saliva in the HIV positive was not affected by CD4 count for any of the three viruses. However, CMV was detected most frequently in the urine and its presence was associated with immunodeficiency and lower CD4 counts (HHV-6 - 2%, HHV-7 - 6.5%, and CMV - 37%).
Where quantitative PCR is used then more information can be gained as to the amount of virus present in different situations. A semi-quantitative PCR based on a known dilution series demonstrate a positive relationship of CD4 count to HHV-6 genome copies in PBMC's in patients with HIV, but no such relationship was found in the saliva [Fairfax 1994]. Healthy controls were not included in the study , so it was not possible to compare directly salivary HHV-6 concentrations between HIV positives and negatives. However, the median concentration of HHV-6 in saliva of the HIV patients was 20,000 genome equivalents / ml (range 0-125,000), this compares with a median value of 5,000 genome equivalents / ml (range 0 - 200,000) obtained in healthy adults in a previous study by the same group of researchers using the same methods [Cone 1993a].
Two of the above four studies in HIV infected adults demonstrated less frequent HHV-6 DNA

in the saliva of patients than controls [Gopal 1990; Gautheret-Dejean 1997]. This finding is of interest as it appears to concur with the findings of the present investigation where HHV-6 was found most frequently in the healthy children and least frequently in the most unwell patients, with the important proviso, that the immunodeficiencies of the different groups are not necessarily the same. Large scale saliva studies of HHV-6 in immunocompetent and immunosuppressed children have not been undertaken, the small number tested in this study being amongst the largest group. Small studies of HHV-6 transmission have looked at the DNA of salivary virus in mothers and infants for evidence of transmission with inconclusive results [Suga 1995; van Loon 1995]. There is no other published information about salivary secretion of this virus in immunosuppressed children to date.

HHV-6 in Serum

It was only possible to test 49% of serum samples collected from patients in this study for HHV-6 DNA and none of them amplified the virus. The implication being that none of these children had an active HHV-6 viraemia at the time of sampling. However, this number is too small to make any real assessment of whether any of these children who were know to be seropositive for HHV-6 were having any reactivation, or re-infection with HHV-6 during their treatment.

Since this study was undertaken a number of other studies have examined the presence of HHV-6 DNA in plasma or serum by qualitative and quantitative PCR. Prior to this, it was demonstrated during primary infection that the virus could be cultured from plasma in 26 % (25 /98) of cases where the sample was obtained within 4 days of onset of the fever but in no (0 /78) samples obtained after 5 days from onset of fever [Asano 1991a]. It also appeared that the children with more severe symptoms had more infected cells in the blood as well as more free virus in the plasma. A number of studies have confirmed that HHV-6 DNA can be amplified from serum / plasma during the primary infection in 50 to 86% of samples tested [Huang 1992a; Secchiero 1995a; Clark 1997]. Plasma samples from healthy adult controls have not amplified HHV-6 DNA [Huang 1992a; Cuende 1994; Secchiero 1995a]. In a PCR study which included children and adults plasma viral DNA was present: in nearly all children (6/7) with primary infection; in 23% (3 / 13) of BMT recipients, where it was associated with fever and respiratory infection;and in 22% (4/18) of HIV infected adult patients [Secchiero 1995a]. This semiquantative PCR could detect down to less than 10 molecules of HHV-6 template. The children with ES had $6 \times 10^2 - 6 \times 10^5$ DNA copies / ml plasma, all the other positive samples had $1.5 \times 10^2 - 1.5 \times 10^3$ DNA copies / ml plasma, except one of the BMT samples which had $1.5 \times 10^3 - 1.5 \times 10^4$ DNA copies / ml plasma. It was not possible to isolate virus from any of the PCR positive samples in this study. Immune dysfunction in the BMT patients and the HIV infected patients was considered to have allowed either re-infection or reactivation of infection with HHV-6. None of the immunosuppressed patients were children.

Quantitative PCRs for HHV-6 and HHV-7 has been developed, and used to detect virus in tissue samples,PBMCs and whole blood, but not plasma or serum [Secchiero 1995b; Clark 1996]. In a very large, prospective, longitudinal study of HIV infected adults, DNA PCR was carried out on whole blood samples for HHV-6, HHV-7 and CMV [Fabio 1997]. In total, 1001 samples were tested, a median of 3 samples from 247 patients. CMV was detected in 17% of patients, HHV-6 in 6%, and HHV-7 in 3%. The maximal median viral load per patient of CMV (10,300 genome copies / ml blood) was significantly higher than that of HHV-6 (400 genome copies / ml blood)or HHV-7 (400 genome copies / ml blood). In this study, as in others [Fairfax 1994; Gautheret 1995], a positive relationship between high CD4 count and presence of HHV-6 was also found. The authors suggest that quantitatively testing whole blood may give more sensitive longitudinal information about virus activity in the immunocompromised than testing plasma alone, since HHV-6 is not always detectable in serum or plasma even in primary infection [Clark 1997].
Multiplex PCR's have also been developed for the herpes viruses and can be useful to detect the presence of these several of these viruses in the same clinical sample, but their use has not yet been reported in immunocompromised children [Tenorio 1993; McElhinney 1995].

Subtype types of HHV-6 amplified

All the saliva samples which amplified HHV-6 in this study were found to be type B, the most common subtype of HHV-6 found in the PBMC's of children with primary infection [Dewhurst 1993]. The largest study of saliva from children and adults also demonstrated that the majority of samples were type B HHV-6 (97%) [Hall 1998]. Similarly in the PBMC's of HHV-6 infected children and their family members over 96% of nearly 3500 samples tested were type B [Hall 1998]. Where type A was amplified it was often in association with type B, and where one family member had type A it did not make it more likely that other family members would also have this type. The most interesting finding of this study was that HHV-6 positive CSF samples from children with primary infection demonstrated a significantly different pattern, with 14 % of samples HHV-6 type A positive. In these cases where HHV-6 could be amplified from PBMC's as well it was always type B. The significance of this is difficult to explain, type A virus has more frequently been found in immunosuppressed patients, and it is still not known whether it has a different pathogenicity from type B, but the authors here suggest that HHV-6 type A may be a more neurotropic strain of the virus. As it is not possible to differentiate serological responses to the two types it is not possible to ascertain if infection with the different types is happening simultaneously or at different times.
In a study of 44 healthy young adults, where 95% had HHV-6 in saliva, and in all cases this was type B HHV-6. In 25 cases type B HHV-6 was found in saliva and PBMC's, in 17 only in saliva, and in 1 only in the PBMC's. Seven of the individuals were infected with type A, but this was only in PBMC's and in 5 cases there was a dual infection with both types. Di Luca et al found all their HHV-6 positive samples (5/ 8 salivary gland biopsies and 3 / 85 salivas) to be

subtype B on restriction enzyme digestion [Di Luca 1995a]. In 71 HHV-6 DNA positive samples (most saliva and a few urine) from HIV infected patients and controls, all but one were type B, and the single type A was from the saliva of a healthy control [Gautheret-Dejean 1997].

Although none of the serum samples from paediatric oncology patients in this study amplified HHV-6 DNA, examination of the subtype of virus amplified from serum in other studies has demonstrated both subtypes A and B. In the plasma study of Secchiero et al, all the HHV-6 DNA from children with primary infection was type B, three HIV infected patients had type A and one type B, and all three BMT patients had type A HHV-6 [Secchiero 1995a], implying that both types can cause an active viraemia, and although again in this study the type A was more prevalent in the immunocompromised the pathogenic significance of this remains uncertain.

Summary

This study has demonstrated firstly, that HHV-6 DNA (type "B") occurs commonly in the mouths of normal healthy children without any obvious pathology and secondly, that children immunosuppressed on chemotherapy also frequently appear to secrete HHV-6 without evidence of ill health. Furthermore those who are febrile, neutropaenic and unwell less frequently secrete the virus. Whether this might be a local effect of chemotherapy on salivary production of HHV-6 was considered. HHV-6 DNA was not found in any of serum samples tested, suggesting no evidence of active HHV-6 viraemia was found in this small group. The possible role of HHV-6 virus as a serious pathogen in immunosuppressed patients remains to be fully elucidated, but in this limited study of a paediatric cohort of immunosuppressed oncology patients no evidence of deleterious virus activity was found.

Summing up of the Serological and PCR studies of Human Herpesvirus 6 infection in immunocompromised children

Criticism of techniques and methods

Numbers

The number of patients included in the serological study, both the cross sectional study of children (n =66 patients and 66 controls) presenting with malignancy and the longitudinal study of children on therapy (n = 45 patients, 3 -33 samples per patient) was considered quite adequate to identify any significant differences or changes in the serological responses of the patients. However, prospectively collected saliva samples were obtained from only 12 new patients (2-13 samples per child) as the number of children presenting that year with malignancy was fewer than the usual expected (30 -40 per year). Although 115 samples were obtained altogether from the children, and the patients did appear to fall in to two groups, those frequently HHV-6 DNA positive like the healthy controls and those rarely positive, this pilot study would need to be extended to a larger cohort for the findings to be confirmed. This is also the case for the serum study of HHV-6 DNA of which only a proportion of the samples were tested.

Techniques

Serological study

For the IFA test a well established technique was used, without problems. This required culture of HHV-6 infected lymphoblastoid cells and some batches did have a better level of viral infectivity than others, but it was possible to confirm this with the monoclonal antibodies (a gift from Dr P Coyle, Belfast). IFA is a subjective technique and was demonstrated to be more likely to miss low positive sera than the ELISA. It was not possible to titrate out sera for the IFA in this study, but this would have been an interesting comparison with the ELISA index. Testing for antibody avidity in the samples would also have been of interest to identify whether infection was recent or distant and whether there was any difference in the pattern in the oncology patients.

Several attempts were made during the study to culture Z29 strain , "B" type HHV-6 as well as the AJ strain, "A" type HHV-6, with the aim of identifying any difference in the antibody responses to the two virus strains. Unfortunately the cultures of this strain of the virus (carried out in another laboratory) were not successful and it was not possible to pursue this.

The ELISA developed for this study was a relatively crude test using a whole HHV-6 infected and comparison uninfected cellular lysate as the coating antigen. Although it performed consistently there are now more refined and indeed commercial ELISA kits for HHV-6

antibodies [Sloots 1996; Nielsen 1996]. The ELISA was only used for HHV-6 IgG antibody testing. Had there been time to make the developments required to adapt it for IgM antibody testing, then it would have been possible to test many more of the sequential patient samples for IgM. Instead as only the IFA test was used, it was necessary to restrict IgM testing arbitrarily to the cases where activity of other herpesviruses had been demonstrated.
With the development of more refined antigens from HHV-6 it will be possible to identify the pattern of evolution of the antibody response to this virus. The responses to early and late antigens maybe different between different groups. In one study, more patients with lymphoma and Hodgkin's disease than healthy controls had antibodies to an early HHV-6 antigen [Iyengar 1991], implying that these patients had evidence of viral activity leading to this continued response to the early antigen, similar to the different responses seen in patients with ongoing EBV activity. A study of the evolution of antibody responses to a number of the herpesviruses in immunosuppressed children might be helpful to identify differences associated with virus activity.

PCR Study

The PCR developed for this study was across a segment of genome known to have a restriction site present in type "B" and not type "A" HHV-6 and it appeared to perform relatively well. However, its sensitivity may have been affected by the addition of Betaglobin primers to the first round and the sensitivity experiments were not repeated with the added betaglobin primers. It might also have been useful to carry out the sensitivity experiments with an HHV-6 DNA dilution series in saliva, but the difficulty arises of having a base line definitely HHV-6 negative saliva sample.
Developments at all the stages from extraction of DNA to visualisation of amplification products of PCR have been rapid over the last decade. More sensitive and refined PCRs for HHV-6 are now available which can detect very low copy numbers of virus in different clinical tissues and have the advantage of being quantitative [Secchiero 1995b; Clark 1996]. These more refined techniques enable quantitative comparison between presence of virus in patients and controls in different settings. Quantitative PCR would have been useful in this study to examine more closely the differences in saliva results between patients and controls, as well as between frequently and rarely HHV-6 secreting patients. Quantitative PCR would also enable comparisons between the load of virus in the saliva, the PBMC's and the serum in individual patients.
One of the main aims of this study was to look for evidence of virus activity in the host, but in retrospect too few sites in too few patients were examined. Saliva was initially selected as a secretion easily obtainable from patients and controls for comparison. Other clinical samples which could have been examined in the patients include: nasopharyngeal aspirates, PBMC's, urine and CSF (lumbar punctures are regularly carried out on children with leukaemia for administration of treatment).
The only technique developed here was PCR for HHV-6 DNA, but other techniques also

give very useful information about virus activity. Presence of viral antigen or mRNA imply the virus is replicating, important additional information when considering latent DNA viruses. A number of HHV-6 antigen detection tests have now been developed and used on clinical samples [Marsh 1996; Yoshida 1996]. It had been hoped as part of this study, using the monoclonal antibodies to HHV-6, to develop a fluorescent method of staining PBMC's for HHV-6 antigens suitable for analysis by flow cytometry. Early experiments with infected lymphoblastoid cells were promising but it was not possible to extend to clinical samples. Since there has elapsed considerable time between the completion of this study and the writing up, many new techniques have been developed which could examine more sensitively the activity of HHV-6 in immunocompetent and immunocompromised children. However, although some of these tests are commercially available, most clinical laboratories are still not set up to regularly measure either antibody responses to HHV-6 or for evidence of viral activity. Clinically available, rapid diagnostic techniques for HHV-6 would appear useful. This common infection in infancy can be clinically impossible to differentiate from other more sinister causes of fever, rash, or CNS disturbance. Acute identification of the virus would enable clinicians to discontinue searching for other causes and would be generally clinically reassuring. Delay in acute viral diagnosis remains a problem for paediatricians.

Assessment of Findings of the Serological and PCR studies

Serological study

The findings of this study were as might have been expected, almost all children presenting with malignancy had an antibody response to HHV-6, similar to controls. For the few who did not, young age was the likely important factor. There was one 9 year old child who had leukaemia who remained IgM antibody negative on all samples tested over 60 months, and IgG antibody negative on almost all samples (those weakly positive, were after blood products). This child later had a relapse of his leukaemia and died. It could be speculated that he had some kind of immune deficiency leading to his development of leukaemia but also potentially his lack of response to this very common virus.

The longitudinal decline in ELISA index for HHV-6 IgG is most likely to represent decreased lymphocytic function with chemotherapy. The ability to look at this more closely with responses to different HHV-6 antigens, e.g. early or late antigens, might have given more useful information as to the activity of the virus or the nature of the remaining antibody response. The finding that older children with leukaemia had lower ELISA indexes than the solid tumour patients might also represent some defect / altered immune function in these children with malignancy affecting the lymphocytic system. This would need to be investigated further, in more cases, with more sensitive techniques, as discussed above.

It would also be of interest to examine the cytotoxic T cellular immune responses to HHV-6 in immunosuppressed children as judging by evidence from the other herpesviruses, this is probably the most important host protective response.

PCR Study

This study and others would appear to concur that HHV-6 is frequently to be found in the saliva of seropositive children. The most interesting finding in the saliva study was the difference in secretion of HHV-6 in the supernatants as compared to the pellets, presumed to contain the oral cellular material. It would appear that secretion of HHV-6 into the saliva is common in healthy children as well as the majority of the oncology patients. However in a minority of patients virus was rarely found in the supernatant. This could be due to: the effects of chemotherapy on the salivary glands; different phenotypes of saliva e.g. pH etc; or effects of other infections in the mouth. It remains a paradoxical finding, that evidence of an active infection should be less common in the ill / immunosuppressed than the healthy. To examine this further it would again be necessary to look for direct evidence of virus replicative activity (e.g. antigen or mRNA) in addition to presence of DNA , which may still be due to latent infection.

That others have found a similar paradoxical relationship in the PBMC's, although not saliva, of patients with HIV tends to back up the possibility that this may be a real phenomenon [Gopal 1990; Fairfax 1994; Fabio 1997]. With HHV-6 DNA more easily identified in more intact immune system. Further investigation of this would again require detection of viral antigens / mRNA's.

Too few serum samples were examined to give a true impression of whether this virus is active in the blood of these patients. A much larger cohort requires to be tested, and this should include samples from children with primary HHV-6 infection as positive controls.

No cases of HHV-6 infection were identified in this small study which would have been likely to benefit from anti-viral treatment.

Further studies

As this study only examined a small number of children who in current terms were only mildly immunosuppressed, subsequent work could examine HHV-6 activity in other groups of more severely immunosuppressed children. Although there are some small studies which include children, the activity of this virus in children with congenital immunodeficiency as well as those undergoing bone marrow transplantation could be further explored. In future studies it would seem worthwhile to examine the activity of families of herpesviruses rather than individual viruses as their interactions may be very important. The most sensitive techniques should be used to examine for viral activation and differences relating to clinical symptoms, including graft failure / delayed engraftment in BMT patients should be sought. As always

with clinical studies a considerable problem will be one of sufficient numbers and such a study should be multi-centre.

The activity of HHV-6 in HIV infected children has not been examined longitudinally over time. This would appear to be important as some small adult studies have demonstrated that this virus may disseminate in patients with advanced HIV disease. Clinical problems with herpesviruses have not always been the same in children as adults, e.g. with EBV related lymphoma and kaposi's sarcoma, both much less common in childhood. Theoretical interactions between HHV-6 and HIV could lead to progression of HIV related disease and this has not been pursued in children. The fact that most adults infected with HIV already have HHV-6 on board and have previously mounted an immune response to it is quite different from the situation in children where HHV-6 infection follows the vertical acquisition of HIV. Studies of herpesvirus activity in children are now more difficult as most children are on highly active anti-retroviral therapy which is likely to affect immune responses to the herpesviruses as well as suppress HIV replication.

The findings of the Zambian study of primary HHV-6 and HHV-8 infection in infants should be confirmed and extended [Kasolo 1997]. This study demonstrates activity of more than one herpesvirus simultaneously and also, unlike all other studies of primary HHV-6 infection type A HHV-6 infection was found in 44%. This may be of significance to later development of virus associated disease such as Kaposi's sarcoma. It is also notable that EBV associated Burkitts lymphoma is prevalent in sub-Saharan Africa, a possible link with the increased prevalence of HHV-6 sub type A in children and activation of EBV with subsequent development of lymphoma should be examined. The interactions of these herpesviruses with HIV in children in the African setting in also worthy of further study. Such studies which require sensitive and complex virological techniques are not easy to establish in a developing world setting.

The presence of HHV-6 in salivary glands, and the likelihood that this is a site of latency for the virus suggests that examination of HHV-6 activity could be undertaken in conditions associated with salivary gland dysfunction. No relationship has been found with HHV-6 and Sjogren's syndrome [Ranger Rogez 1995]. However, it is of interest that up to 20% of children with HIV infection have quite marked salivary gland swelling, particularly affecting the parotid glands, often in association with lymph node enlargement. A relationship between this sometimes distressing symptom to herpesvirus activity has not been demonstrated. Saliva from HIV infected children has not been examined for HHV-6 or other herpesviruses. Parotid swelling in HIV infected children is also often found in association with the syndrome of lymphoid interstitial pneumonitis. The aetiology of this syndrome also remains obscure, whether it is related to a lymphocytic response to HIV itself or to other infections, such as the herpesviruses, deserves further investigation.

CNS persistence of HHV-6 and other herpesviruses has been demonstrated in a number of small studies and the possibility that this can lead to later problems in the immunosuppressed has been suggested. However the numbers are small and larger scale studies with better

controls are required. A nation wide study of HHV-6 and HHV-7 in childhood encephalitis in the UK has started this Autumn (Dr K Ward and Prof E Ross, with the Surveillance unit of the Royal College of Paediatrics and Child Health), long term follow up of these children could be of interest in terms of the potential on going, or later activity of this virus in the CNS.

Lessons learnt

Most of what we currently understand about HHV-6 implies that as a herpesvirus, it is of relatively low grade pathogenicity to humans, both immunocompetent and immunocompromised. Perhaps this is because it has an ancient relationship with its host leading to a useful level of mutual symbiosis. Never the less, there remain areas of activity of this virus which do need further investigation, in particular its interactions with the other herpesviruses within the host which could be detrimental.

The small studies described in this thesis, small because of the numbers, time constraints and finances, have added only the tiniest pieces to the jigsaw of understanding of HHV-6. They could not have been expected to deliver much more, but in personal terms they have taught important lessons. The opportunity to work alongside scientists and learn the patience required to develop methods and apply them was greatly appreciated. Clinicians always desire interpretable results urgently. The scientist has knowledge of the development and problems of a method and the gaining of results maybe of secondary importance. This study has helped to me to understand better the dilemma of application of the scientific to the clinical, and as ever in the process has raised more questions than it answered.

Human Herpesvirus Virus -6 (HHV-6) infection in Immunocompromised children

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Appendix

Published Work

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Serological Response of Paediatric Oncology Patients to Human Herpesvirus-6

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The serological response of paediatric oncology patients to human herpesvirus-6 (HHV-6) was investigated at presentation and during treatment. Sera from 66 patients presenting with malignancy and 66 controls were examined for anti-HHV-6 IgG by indirect immunofluorescence test (IFA) and enzyme linked immunosorbent assay (ELISA), and for anti-HHV-6 IgM by IFA. Serial samples from 45 children on chemotherapy were examined for anti-HHV-6 IgG by ELISA and sera from selected patients on chemotherapy were examined for IgM by IFA. The response of these patients to four other herpesviruses was also investigated.

Ninety percent of presenting patients and controls were IgG positive for HHV-6 by IFA and ELISA. Anti-HHV-6 IgG as measured by the HHV-6 ELISA index declined over time in patients on chemotherapy. Two presenting controls and one leukaemic child with a primary cytomegalovirus seroconversion were anti-HHV-6 IgM positive. In the patient group seropositivity to herpesviruses (types 1–6) increased with age, the mean age of those with IgG to HHV-6 alone was 3.7 years compared to 6.8 years for those with antibodies to all five viruses.

At the time of presentation paediatric oncology patients have a similar serological response to HHV-6 as age-matched controls and this IgG response wanes with treatment. Whether this is significant in terms of viral pathogenicity is not known and will require investigation of viral activity in these patients.

KEY WORDS: serology, children, malignancy

INTRODUCTION

Children with malignancy are immunosuppressed as a result of their disease and therapy, and lymphopaenia with altered cell mediated immunity and decreased antibody production increase their susceptibility to viral infection [Van Der Meer, 1989]. They are also well known to be at risk from primary and reactivated infection with herpesviruses [Hirsch, 1989]. In view of this susceptibility to herpesvirus infection and the lack of information concerning human herpesvirus-6 (HHV-6) infection in this group of patients an investigation of the possible pathogenic role of HHV-6 was undertaken.

HHV-6 is a ubiquitous virus known to infect almost all children, with more than 90% showing an IgG response to HHV-6 by 2 years of age [Asano et al., 1990, Ward et al., 1993a]. Placental transfer of anti-HHV-6 IgG antibody occurs and as levels decline within the first 6 months of life, the infant becomes susceptible to primary infection [Yoshikawa et al., 1989]. Following primary infection latency is established probably in the salivary glands or the peripheral blood mononuclear cells [PBMC; Fox et al., 1990; Luppi et al., 1993].

HHV-6 was originally isolated from the PBMC of immunosuppressed patients with lymphoproliferative diseases and with acquired immunodeficiency syndrome [AIDS; Salahuddin et al., 1986]. It appears that in immunosuppressed patients HHV-6 can reactivate more easily and that the patients may carry an increased load of viral DNA [Luppi et al., 1993]. HHV-6 can be pathogenic in the immunocompromised population, and primary and reactivated infections have been described [Ward et al., 1989, 1993b; Yoshikawa et al., 1992.] The virus has been responsible for causing pneumonitis [Carrigan et al., 1991], hepatitis [Ward et al., 1989], and bone marrow failure [Drobyski et al., 1993]. There is little information specifically relating to immunocompromised children, although a study of 25 Japanese paediatric bone marrow transplant recipients demonstrated reactivation of HHV-6 [Yoshikawa et al., 1991].

Several techniques have been used to examine the serological response to HHV-6 including the indirect fluorescent antibody test [IFA; Tedder et al., 1987], anticomplement immunofluorescence test [Okuno et al., 1989], neutralisation test (NT) [Yoshikawa et al., 1990], enzyme linked immunosorbent assay [ELISA;

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Asano et al., 1990], radioimmunoprecipitation [Coyle et al., 1992], and immunoblotting (IB) [Coyle et al., 1992]. Early reports of the serological response to HHV-6 tended to underestimate the seroprevalence and this was probably related to the dilutions of sera examined. The more recent consensus is that the IgG response to HHV-6 is positive in greater than 90% of people. The slightly lower rates found in fluorescence assays may relate to the more subjective nature of these tests and the difficulty in establishing clear cut offs for low-positive specimens. Young children have the highest geometric mean titres of antibody to HHV-6 and antibody levels wane with age [Brown et al., 1988].

This paper describes a retrospective serological investigation of the IgG and IgM responses to HHV-6 in paediatric oncology patients using ELISA and IFA tests. Samples were tested from children at the time of presentation with malignancy and compared with controls. During the period of treatment with chemotherapy sequential samples were also tested. The response of these patients to the other four herpes viruses (herpes simplex virus [HSV], varicella zoster virus [VZV], cytomegalovirus [CMV], and Epstein Barr virus [EBV]) was also examined.

MATERIALS AND METHODS

Patients and Samples

The approval of the local paediatric ethical committee was obtained for this study. Samples from paediatric oncology patients seen at the Royal Hospital for Sick Children are routinely sent for viral serology at presentation before administration of blood products, and at regular intervals during treatment. These samples are tested at the Regional Virus Laboratory (RVL), City Hospital, Edinburgh, and any surplus serum is stored long term at −20°C. Computer search was used to identify serum samples taken from oncology patients at presentation, from January 1987 to December 1991. In some cases the samples taken at presentation were exhausted. Sixty-six sera were identified (31 from acute leukaemics [AL] and 35 from patients with solid tumours [ST]). These samples were age and season matched to 66 stored sera from patients who presented to the hospital in the same year with minor illnesses (upper respiratory tract infection, pyrexia, or viral infection). The age range of patients was 1 month–13 years with a bimodal distribution with peaks at 3 and 10 years of age, representing the age range of children presenting with malignancy. These sera were tested for anti-HHV-6 IgG by IFA and ELISA, and for anti-HHV-6 IgM by IFA.

All the sera from patients and controls were tested for anti-VZV IgG and anti-CMV IgG by commercial ELISA kits (Sigma). Previous results were available from some patients for EBV and HSV antibody responses. Anti-EBV IgG was tested by IFA on infected EB3 cells and antibodies to HSV were titrated by the complement fixation test (RVL standard methods, unpublished).

Sequential sera from 45 patients (26 AL and 19 with ST) on continuing chemotherapy who presented between January 1989 and December 1991 were examined for anti-HHV-6 IgG by ELISA. Three to thirty-three samples were tested per patient depending on the length of therapy. These samples were obtained approximately monthly and there were more from the leukaemics who underwent a longer course of therapy. In 13 of these patients where the sample taken at presentation was exhausted (9 AL and 4 with ST), samples were available from within the first month of treatment, usually but not always, before administration of blood products. The age range of the patients was the same as for the presenting group with a similar bimodal distribution. Sequential sera from eight cases were selected and tested for anti-HHV-6 IgM by IFA (two asymptomatic seroconversions to CMV, five cases of primary varicella or shingles, and one case who remained IgG negative to HHV-6 throughout treatment.

Cell Culture

Cells of the lymphoblastoid cell line JJhan were grown in culture medium (RPMI with Hepes buffer, foetal calf serum [10%], glutamine [2 mM], and penicillin [100 IU/ml] and streptomycin [100 μg/ml]). The cells were activated with phytohaemagglutinin (5 μg/ml) and then infected with the AJ strain of HHV-6 from an infected aliquot of cells (a gift from Prof. R. Tedder [Tedder et al., 1987]). At the time of infection the maximal ratio of uninfected to infected cells was 10:1. After 7 days, successful infection of the culture was confirmed with mouse anti-HHV-6 monoclonal antibodies (kindly donated by Dr PA Coyle) and the cultures were harvested for the IFA and ELISA tests. For IFA cells were spotted onto slides, air dried, fixed in acetone, and then stored at −20°C until required. On each slide well the ratio of infected to uninfected cells per well was always less than 50%.

Serological Tests

Indirect immunofluorescent-antibody test. A positive and diluent only control was run on each slide. Sera were tested for anti-HHV-6 IgG at a dilution of 1 in 10 in phosphate buffered saline (PBS), pH 7.2, with 0.2% bovine serum albumin (BSA). Slides were incubated in a moist chamber at 37°C for 1 hour, washed three times with PBS, and dried. Fluorescein isothiocyanate (FITC)-conjugated, anti-human IgG (Scottish Antibody Production Unit-SAPU) was applied to slides at a dilution of 1 in 80 and incubated for 30 minutes at 37°C. Slides were then washed twice in PBS and once in phosphate buffer (PB), pH 8.4, dried, and mounted for examination under ultraviolet light. Large bright dots of fluorescence, mostly intracytoplasmic but also intranuclear, particularly apparent in ballooned cells and syncytia, were observed with anti-HHV-6 IgG positive sera.

Sera tested for anti-HHV-6 IgM were diluted 1 in 10 with IgG blocking agent (Incstar), incubated for 15

TABLE I. Seroprevalence of HHV-6 IgG in Patients and Controls by ELISA and IFA (Including All Patients and Controls and Those Under 1 Year)

	Positive (%)	Negative (%)	Equivocal (%)	Not suitable (%)	Total
ELISA patients (ALL)	64 (97)	2 (3)	—	—	66
IFA patients (ALL)	61 (92.5)	4 (6)	—	1 (1.5)	66
ELISA controls (ALL)	61 (92.5)	3 (4.5)	2 (3)	—	66
IFA controls (ALL)	58 (88)	4 (6)	3 (4.5)	1 (1.5)	66
ELISA patients and controls (<1 year)	6 (60)	4 (40)	—	—	10
IFA patients and controls (<1 year)	5 (50)	5 (50)	—	—	10

minutes at room temperature, and after pulse centrifugation, the supernatant was applied to slides for 3 hours at 37°C. The slides were washed three times with PBS, dried and FITC conjugated, and anti-human IgM (SAPU) diluted 1 in 32 was applied for 1 hour at 37°C. Slides were then washed and mounted, as above, for examination under ultraviolet light. The pattern of fluorescence with IgM positive sera was of finer cytoplasmic and surface brightness, often most marked in the ballooned cells.

ELISA. The method of Chou and Scott [1990] was adapted and used to detect anti-HHV-6 IgG with an alkaline phosphatase detection system [Cubie et al., 1993]. Antigen was prepared by the same method from infected and uninfected cells. Cells (3×10^7) were centrifuged at 1,200*g*, suspended in 4 ml of chilled glycine buffer (pH 9.5), and sonicated on ice for 30 seconds. The sonicate was centrifuged at 5,000*g* for 20 minutes and the supernatant stored at −70°C.

Antigen was diluted in glycine coating buffer (pH 9.5) to give an optimal working dilution of 1 in 160 (protein content of 5 μg/ml) and used to coat microtitre plates. Alternating 8 well rows of a 96 well microtitre plate (Nunc) were coated with either 100 μl of infected or uninfected antigen and incubated overnight at 4°C. The plate was washed three times with PBST washing buffer (PBS, pH 7.2, with Tween [0.05%] and BSA [0.1%]). Remaining binding sites were blocked with 200 μl of PBST (with 5% BSA) at 37°C for 1 hour and the plate was then washed three times. Coated and blocked plates could be stored at 4°C for at least 1 week before use. An automated ELISA plate washer was used for all washes.

One hundred microliters of sera diluted 1 in 100 in PBST (with 1% BSA) was applied to pairs of wells and incubated for 2 hours at 37°C. After three washes, 100 μl of diluted (1 in 1,000) anti-human IgG conjugated with alkaline phosphatase (Sigma) was added to each well for 1 hour at 37°C. After five washes, 100 μl of the substrate para-nitrophenyl phosphate (1 mg/ml in freshly made diethanolamine buffer, pH 9.8) was added to each well and incubated at room temperature, in the dark, for 30 minutes. The reaction was stopped with 50 μl of 3 M sodium hydroxide and the absorbance read at 405 nm.

As the antigen was a crude cell lysate, it was necessary to test each serum sample on both infected and uninfected antigen. Some sera had a stronger background reaction with the uninfected antigen than others and this was compensated for by using the difference between the two absorbances to calculate the ELISA index: (absorbance infected cells − absorbance uninfected cells)/(negative cut off).

The following control sera were included on each microtitre plate: a strong positive; a low positive; and three negatives. The plate was blanked on a well incubated with diluent only. The negative cut off for the test was the mean difference in absorbance of the three negative controls plus 3 standard deviations, but if this figure was less than 0.1 then 0.1 was used. The ELISA index was considered to be positive if greater than 1.1, negative if less than 0.9, and equivocal between 0.9 and 1.1.

Statistical Methods

The results from the ELISA of the presenting cohort of patients were converted to positive, negative, or equivocal for comparison with the IFA results. The data were analysed by the chi-squared test or Fisher's exact test for groups of data with very small numbers. A repeated measures model which allows correlation between the repeated values from the same patient was applied to the sequential data from patients on treatment so the effects of time, age at presentation, and diagnosis on the trends in the ELISA values could be analysed [Jennrich and Schlucher, 1986]. The statistical package used was SAS (PROC MIXED).

RESULTS

Comparison of IFA and ELISA for Anti-HHV-6 IgG

There was no significant difference in the results obtained by these two tests in the presenting cohort of patients, although there were more equivocal and negative sera by IFA (Table I). The high degree of concordance between the tests is demonstrated in Figure 1. In particular all of the sera which tested negative by ELISA also did so by IFA. Haemolysis of these small paediatric samples did not alter the pattern of results in either test.

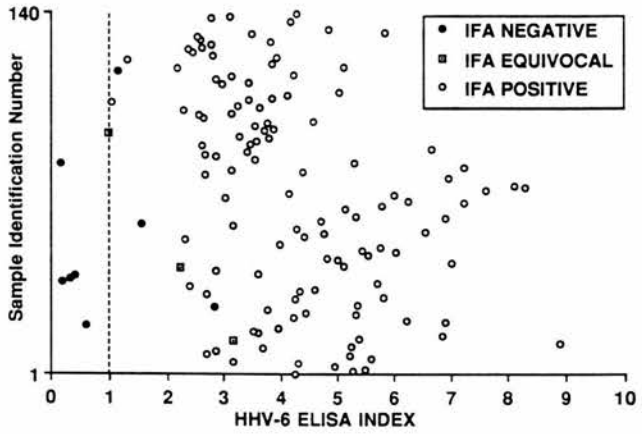

Fig. 1. Comparison of ELISA index and IFA test results for HHV-6 IgG from all presenting sera, patients, and controls. The dotted line represents the cut off between positive and negative ELISA results.

Presenting Samples From Oncology Patients and Controls

Antibodies to HHV-6 at presentation. Around 90% of patients and controls were anti-HHV-6 IgG positive by both tests and there was no significant difference in seropositivity for anti-HHV-6 IgG between patients or controls (Table I). ST patients were as likely as leukaemics to develop antibody. The proportion of seropositive cases in children under 1 year was lower (50–60%), but there was no significant difference between the patients or controls in this small group. By ELISA only five children had no antibody to HHV-6: two were patients, a 6-month old with neuroblastoma and a 9-year-old with AL (see below); all three controls were infants aged 4, 6, and 7 months.

IgM antibodies to HHV-6 were found in two control patients, an 8-year-old with a febrile illness and a 13-month-old with an upper respiratory tract infection. One leukaemic patient was also positive for IgM at presentation (see below).

Antibodies to five herpesviruses at presentation. There was no significant difference in response to VZV or CMV between patients or controls. Two thirds of patients and controls had IgG to VZV and one third of patients and controls had IgG to CMV (Table II). Evidence of past EBV infection was found in 33 (62%) of the 53 patients' samples available for testing while HSV antibodies were detected in only 9 (15%) of 61 patients tested. Again there was no significant difference in response between patients with ST or leukaemia.

In the patient group complete results of serological response to the five herpesviruses were available in 52 (79%) patients, and demonstrated an IgG response to an increasing number of herpesviruses with increasing age. Where the child only had antibody to one herpesvirus this was usually HHV-6. The mean age of children who only had IgG to HHV-6 was 3.7 years compared to 6.6 years for the remainder who had antibodies to two or more herpesviruses, including HHV-6.

Sequential Sera From Patients on Chemotherapy

Anti-HHV-6 IgG in sequential sera. Six hundred seventy-six sequential specimens from 45 patients were examined for anti-HHV-6 IgG. Five hundred forty-four samples were obtained from the 26 leukaemia patients (9–33 samples per child). One hundred thirty-two samples were tested from the 19 patients with ST (3–15 per child). The majority of samples were positive for anti-HHV-6 IgG. One 9-year-old leukaemic child had no antibody to HHV-6 in the first sample tested, 23 further samples from this child were tested, 3 had an ELISA index of just over one, however each time this subsequently became negative and he remained negative 2 years later when his leukaemia relapsed. All sera from this child were negative for anti-HHV-6 IgM.

A repeated measures model was fitted to all the data from these patients to analyse the effects of time, age at presentation, and diagnosis (AL or ST) on any trends in the ELISA Index values obtained. There was no overall significant difference between AL or ST patients with both groups showing a decline in ELISA index over time. The slope of this decline was not significantly different between the two groups of patients when compared over a 1-year time period. The relationship between age and ELISA index was different between the two groups, in the ST patients there was little difference in ELISA Index values with age, but older AL patients tended to have lower values ($P = 0.039$).

Anti-HHV-6 IgM in sequential sera. Sequential sera from six patients with changes in activity against other herpesviruses were examined for anti-HHV-6 IgM. There were two children who seroconverted to CMV asymptomatically. One produced a fourfold rise in anti-CMV IgG titre and was strongly positive for anti-CMV IgM at the time of seroconversion, again 3 months later, but in no other later sample. None of the 29 samples from this child had anti-HHV-6 IgM. In contrast the other patient had anti-HHV-6 IgM at presentation, but insufficient sample remained to test for anti-CMV IgM. Two months after starting treatment he had a fourfold rise in anti-CMV IgG titre and was strongly positive for anti-CMV IgM, at the same time demonstrating IgM to HHV-6. Over the next 21 months of therapy nine samples were positive for anti-HHV-6 IgM, five of these were also positive for anti-CMV IgM, one was negative, and three could not be tested (Fig. 2). None of the cases of varicella zoster infection were associated with production of IgM to HHV-6.

DISCUSSION

More than 90% of the presenting patients and controls in this study had IgG to HHV-6 by ELISA and slightly less by IFA (Table I). The interpretation of the fluorescent antibody test, being visual, is more subjective and the differentiation of low positives from negatives is difficult. Although the ELISA used a crude cell lysate as antigen, lysates of both infected and uninfected cells were used in parallel and the difference in absorbance was used to calculate an HHV-6-specific

TABLE II. Seroprevalence for HHV-6, CMV, VZV, EBV, and HSV IgG in Presenting Patients and Controls

	CMV			VZV			EBV		HSV	
	Positive	Negative	Equivocal	Positive	Negative	Equivocal	Positive	Negative	Positive	Negative
HHV-6										
Positive patients[a]	18	39	7	41	22	1	32	19	9	50
Positive controls	21	38	4	40	20	3	NA	NA	NA	NA
Negative patients	—	2	—	2	—	—	1	1	—	2
Negative controls	1	2	—	—	2	1	NA	NA	NA	NA

[a]Equivocal results added to positive results.

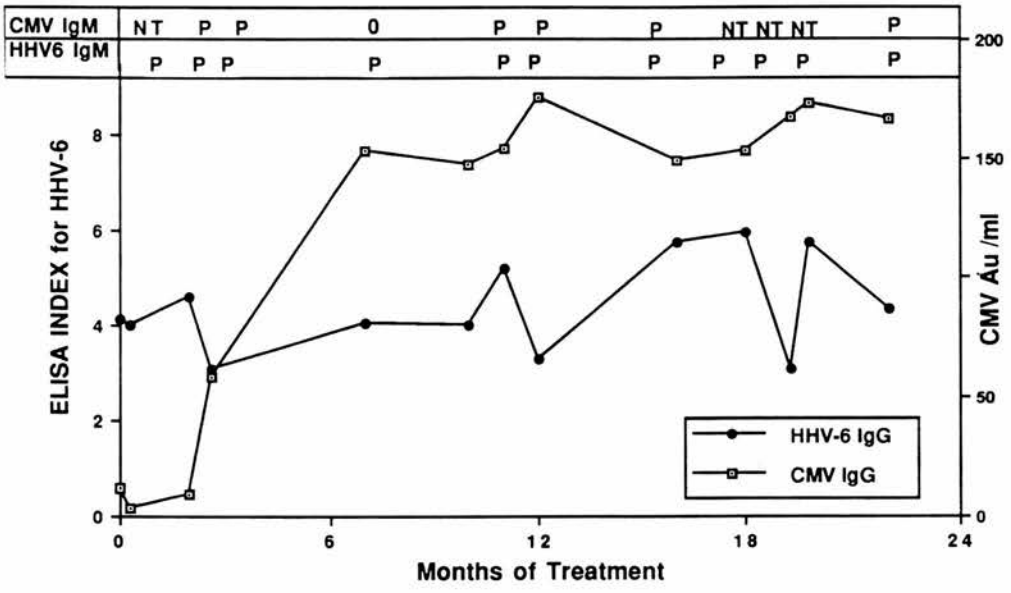

Fig. 2. Sequential serology for HHV-6 and CMV from a leukaemic patient who seroconverted to CMV. IgG to HHV-6 (ELISA Index) and CMV (antibody units/ml) is recorded on the graph and IgM production to HHV-6 and CMV is recorded in the table. NT, not tested; P, IgM positive; O, IgM negative.

ELISA index. The ELISA test results demonstrated a relatively clear separation between positive and negative sera with few equivocal results and all the samples which were negative by ELISA were also negative by IFA (Fig. 1). The percentage of anti-HHV-6 IgG seropositivity in previous studies has varied according to the tests used, the dilution of serum tested, and the age range of the subjects. In paediatric populations studied beyond the age of 1 year, the anti-HHV-6 IgG seropositivity is up to 100%, with the highest seropositive rate obtained by ELISA and NT [Asano et al., 1990; Yoshikawa et al., 1990].

Antigenic cross-reactivity with other herpesviruses might affect the apparent response to HHV-6, although the work of several investigators would refute this. Absorption of antibodies to other herpesviruses does not reduce titres to HHV-6 by IFA or ELISA [Saxinger et al., 1988; Buchbinder et al., 1989; Linde et al., 1990]. There is no correlation between antibody titres to different herpesviruses and HHV-6 by IFA, ELISA, or IB (Salahuddin et al., 1986, Saxinger et al., 1988, Asano et al., 1990), and the majority of individuals seronegative for CMV and EBV are HHV-6 seropositive [Linde et al., 1988].

This study confirms that most paediatric oncology patients have encountered HHV-6 at presentation and appear to have a similar response to the virus as age-matched controls. The findings are in agreement with a recent study which examined sera from 50 patients with acute lymphoblastic leukaemia (age range 1–52 years, mean age 17.6 years), in which there was no significant difference in titres for anti-HHV-6 IgG between cases and controls, by IFA or ELISA [Levine et al., 1992a]. In contrast earlier IFA test studies of adult patients with leukaemia and lymphoma had suggested the seroprevalence and antibody titres to be greater in patients than controls [Ablashi et al., 1988; Clark et al., 1990]. Further investigation of the serological response of paediatric oncology patients could include examination of the pattern of avidity of the IgG response to HHV-6 as well as the response to different HHV-6 antigens. It has been shown that low avidity IgG is produced in the first few months after primary HHV-6 infection, followed by high avidity antibodies

later [Ward et al., 1993a]. In a study by Iyengar et al. [1991] most normal sera tested positive for a late HHV-6 antigen and only one third for an early HHV-6 antigen. Seropositivity for the early antigen was increased in patients with lymphoproliferative disease, which could indicate increased activity of the virus and possibly an altered immune response of the host.

Five presenting sera had neither IgG nor IgM to HHV-6 by ELISA or IFA, three were from control patients, and one from a ST patient all under the age of 1 year. The fifth serum was from a 9-year-old leukaemic patient who was seronegative at presentation but tested IgG positive in 3 samples out of 23, and who was negative at relapse 2 years later. No samples from this child were IgM positive making it unlikely that he underwent primary seroconversion. It is much more likely that these unsustained rises in IgG antibody were related to blood products received. At presentation there was IgG to VZV but no other herpesviruses.

Examination of the sequential sera from patients on chemotherapy for anti-HHV-6 IgG demonstrated no significant difference between ST and AL patients when compared over the same time period. Although the trend in anti-HHV-6 IgG was downward with time, there was little evidence of large fluctuations in IgG levels which might be expected if reactivation of HHV-6 had occurred. In a longitudinal study of 37 patients with Hodgkin's disease (age range 5–72 years) IFA titres increased significantly in patients who relapsed and decreased significantly over time in those who did not [Levine et al., 1992b]. It was not possible to examine any relationship between changes in ELISA indices and relapse of disease in the current study. The finding that older children had lower ELISA Indexes for HHV-6 reflects that of other workers who have shown that the highest geometric mean titres for HHV-6 IgG are found in young children [Brown et al., 1988; Yoshikawa et al., 1990].

It was not possible to test all the sera for anti-HHV-6 IgM and so sera from five patients who had demonstrated laboratory and/or clinical evidence of herpesvirus activity were selected. In this small group VZV primary and reactivated infection did not appear to cause reactivation of HHV-6 with IgM production. Primary CMV infection can be associated with rising titres of IgG to HHV-6 and also IgM to HHV-6 [Chou and Scott, *et al.*, 1990; Irving et al., 1990; Ward et al., 1991]. One of the two children in this group with primary CMV infection made no IgM to HHV-6 at all and the ELISA index for anti-HHV-6 IgG remained unchanged. The other child produced anti-HHV-6 IgM on several occasions, including at presentation, but again the ELISA index for anti-HHV-6 IgG remained steady with no significant change in relation to the rise of anti-CMV IgG (Fig. 2). HHV-6 and CMV are closely related genetically with shared immunogenic surface glycoproteins and yet there does not appear to be significant IgG antibody cross-reactivity [Chou and Scott, 1990; Irving et al., 1990]. Avidity studies have shown that immunocompetent and immunosuppressed patients with primary CMV infection produce anti-CMV IgG of low avidity and anti-HHV-6 IgG of high avidity, implying reactivation of HHV-6 infection with a different antibody response [Ward et al., 1993b]. IgM production without changes in IgG antibody level to HHV-6 has been demonstrated during reactivation [Enders et al., 1990].

While the serological response to all five herpesviruses was examined for 52 (79%) of the patients data were only available for CMV and VZV for the control patients. However, in this group of children aged from less than 1–13 years the overall seropositivity for patients and controls was similar for VZV (65% and 60%, respectively) and CMV (27% and 33%, respectively). In the patient group the acquisition of herpesvirus infection increased with time even in this population, and not surprisingly it appeared that when a child had antibodies to only one herpesvirus this was usually HHV-6.

This study has demonstrated that paediatric oncology patients, whether ST or leukaemic patients, have a similar response to HHV-6 as age-matched controls and that almost all have encountered the virus at the time of presentation with disease. The seroprevalence for the two herpesviruses, VZV and CMV, is also similar between controls and patients. Sequential samples from patients during immunosuppressive treatment showed a gradual decline in ELISA index for IgG to HHV-6 with time but did not give any helpful information as to whether reactivation of HHV-6 was occurring. IgM to HHV-6 was produced by one child with a primary CMV seroconversion but again this did not conform reactivation and the possibly of antibody cross-reactivity could not be excluded.

Assessment of serological response in the immunocompromised will always give an incomplete picture of the host response. To ascertain further whether this virus is pathogenic in immunosuppressed children the next phase of this study will examine for evidence of viral activity in such patients.

ACKNOWLEDGMENTS

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Human Herpesvirus-6 DNA in the Saliva of Paediatric Oncology Patients and Controls

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Children with malignancy are immunosuppressed and susceptible to serious infections with herpesviruses. The majority of children on chemotherapy for malignancy are seropositive for human herpesvirus-6 (HHV-6), and although HHV-6 has been demonstrated to be a pathogen in severely immunocompromised patients, whether this is the case for paediatric oncology patients is unknown.

HHV-6 is secreted in saliva and in this study samples were examined prospectively for HHV-6 DNA in healthy children and those with malignancy. In a nested polymerase chain reaction (PCR), a 287 bp outer fragment and 163 inner fragment of HHV-6 DNA were amplified. The resulting amplimer contained a Hind III restriction site present only in "B" type HHV-6 and this was used to identify the type of HHV-6 amplified. In saliva from healthy control children, 74% (28/38) of samples were HHV-6 DNA-positive in either the supernate, pellet or both. In the patients, 58% (45/77) of all samples were HHV-6 DNA-positive. When sequential samples from twelve patients were examined the children appeared to fall into two groups: those who were frequently HHV-6 DNA-positive (60% of samples or more) and those who were rarely HHV-6 DNA-positive (33% of samples or less) ($P < 0.0001$). The only apparent difference between these two groups was that the less frequently HHV-6-positive group was more often febrile and unwell with neutropaenia. Hind III digestion demonstrated all the positive samples to be "B" type HHV-6. Possible explanations for this difference in HHV-6 secretion between the patient groups are discussed.

KEY WORDS: HHV-6 DNA, saliva, malignancy, children

INTRODUCTION

Human herpesvirus-6 (HHV-6) is an ubiquitous herpesvirus transmitted by saliva, and most infants will be infected by the virus by two years of age [Prukasananonda et al., 1992]. The classical infectious syndrome of exanthem subitum does not always occur and many infections are sub-clinical. In common with the other herpesviruses, once primary infection has occurred, latency is established in salivary glands and blood mononuclear cells [Fox et al., 1990; Luppi et al., 1993].

Children with malignancy are immunosuppressed as a result of their disease and also their treatment and are well known to be susceptible to herpetic infections [Hirsch, 1989]. HHV-6 can be a pathogenic virus for immunosuppressed patients including bone marrow transplant patients, liver transplant patients and others [Drobyski et al., 1993, Ward et al., 1989].

Whether it is pathogenic for paediatric oncology patients on chemotherapy is not known. The majority of such children have IgG for HHV-6 at presentation and levels of antibody wain with treatment, but detection of IgG or IgM antibodies to HHV-6 is not a reliable indicator of current virus activity in these patients [Lyall, 1994].

The aim of this study was to develop a nested polymerase chain reaction (PCR) for HHV-6 which could be used to examine the saliva of paediatric oncology patients and controls for HHV-6 DNA, giving direct evidence that HHV-6 is present in the saliva. Saliva was chosen for examination as a source for HHV-6 because it has frequently been shown by PCR to contain HHV-6 DNA in adults, but to date has only been examined in a few children [Cone et al., 1993]. Saliva is also an acceptable secretion to obtain voluntarily from healthy children. The study aimed to compare findings in the saliva of normal healthy children with sequential samples from paediatric oncology patients.

METHODS

Patients and Controls

Ethical committee approval and consent of parents and children were obtained. Twelve new patients aged 3 to 13 years who attended the oncology service of the

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Royal Hospital for Sick Children between August 1992 and September 1993 were enrolled. Seven had leukaemia and five, solid tumours. The first patient sample was obtained before starting chemotherapy and thereafter samples were obtained when the patients attended for treatment or were febrile and unwell. Seventy-seven saliva samples were obtained from the 12 patients. Healthy siblings and local volunteer children acted as normal controls. Thirty-eight control samples were obtained from 27 healthy volunteer children and 10 siblings (two samples from one sibling), age range 4–16 years. The saliva supernate and cell pellet from each sample was examined for HHV-6 DNA. At presentation, serum from each patient was tested for anti-HHV-6 IgG by fluorescent antibody test [Lyall, 1994].

Preparation of Saliva for PCR

Saliva samples were obtained after a fifteen second gargle with five ml of normal saline and processed as soon as possible. After vortexing, the saliva was centrifuged at 1,000 rpm for 8 min and the supernatant separated from the cell pellet. Foetal calf serum (1.5%) and ampicillin were added to the supernates and 0.5 ml of viral transport medium was added to the pellets. The samples were stored at −70°C and were tested together in batches. Two hundred μl of the samples were mixed with an equal volume of double strength lysis buffer to give a final concentration of 100 μg/ml proteinase K, 0.02 mM Tris HCl pH 7.5, 0.01 mM EDTA and 1% Tween 20. The samples were incubated for 90 min at 55°C and 10 min at 95°C. DNA was then extracted from the sample using phenol chloroform and precipitated over night at −20°C in pure ethanol [Sambrook et al., 1989]. The DNA was resuspended in 100 μl of water and 10 μl added to the first round PCR.

Sequence of Genome Chosen for Amplification

A sequence of the large tegument protein gene (LTP gene) [Josephs et al., 1991] which contains HHV-6 strain variations demonstrated by restriction enzyme digestion [Aubin et al., 1991, 1993] was chosen for amplification. A Hind III digestion site at position 2945 of the LTP gene was shown to be present in "B" but not "A" type strains of HHV-6. This has been used to discriminate between the two types of HHV-6 [Dewhurst et al., 1992, 1993]. To increase sensitivity and specificity, a nested PCR was designed to amplify a 287 bp outer segment and the 163 bp inner segment across the Hind III restriction enzyme site (position 2817–3103). The following primers were used: **1 outer,** 2817–2840, 5'-agt cat cac gat cgg cgt gct atc-3'; **2 outer,** 3103–3081, 5'-tat cta gcg caa tcg cta tgt cg-3'; **3 inner,** 2879–2902, 5'-tcg act ctc acc cta ctg aac gag-3'; **4 inner,** 3041–3018, 5'-tga cta gag agc gac aaa ttg gag-3'. Any samples which amplified HHV-6 DNA were then digested with Hind III and the product run on an agarose gel stained wth ethidium bromide (Fig. 1).

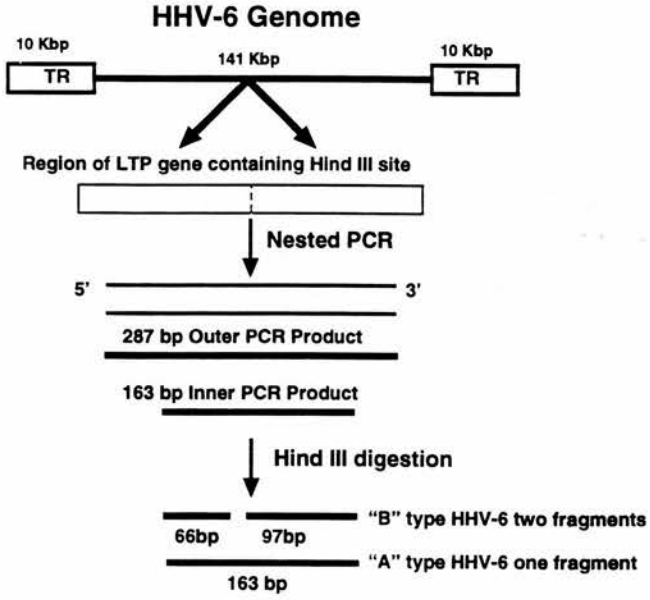

Fig. 1. Diagram of the plan for nested PCR for HHV-6 and identification of any amplified products as either HHV-6 type "A" or "B" according to the effect of Hind III digestion.

Nested PCR Method

A series of experiments was undertaken to optimise the nested PCR for the detection of low copy numbers of HHV-6 genome. A hot start (80°C) was used for the first round PCR to minimise non-specific priming and increase sensitivity. In the first and second rounds of amplification 30 cycles were used, the first denaturation, was for 5 minutes at 94°C and all subsequent denaturations for 1 minute, annealing was for 1 minute at 60°C, and extension for 1 minute at 72°C, except for the last cycle where extension was for 8 minutes. In the first round reaction, 10 μl of extracted sample was added to 90 μl of mix and in the second round 2 μl of first round product was added to 48 μl of mix. The same reaction mix was used for each round. Two units of Taq polymerase (Perkin Elmer, U.K.) with the manufacturer's buffer, 1 μM of primers, 0.2 mM of d-Nucleoside triphosphates, and 1.5 mM of magnesium chloride were used per reaction. Great care was taken to avoid contamination at all stages of the PCR process.

All saliva supernate and pellet samples were subjected to nested PCR twice. Samples were amplified in batches of 28: including three water controls; one uninfected lymphoblastoid cell line negative control; and three positive controls containing 30, 300, and 3,000 HHV-6 amplified genome segments of type "A" from the AJ strain of the virus (a kind gift from Professor R. Tedder). The products of the amplification were run on a 3% agarose gel containing ethidium bromide. Positive samples were digested with the restriction enzyme Hind III for one hour at 37°C. Products of digestion were run on a further ethidium bromide-stained gel.

Any sample which never amplified DNA on any occasion was checked for inhibition of PCR by "spiking" an

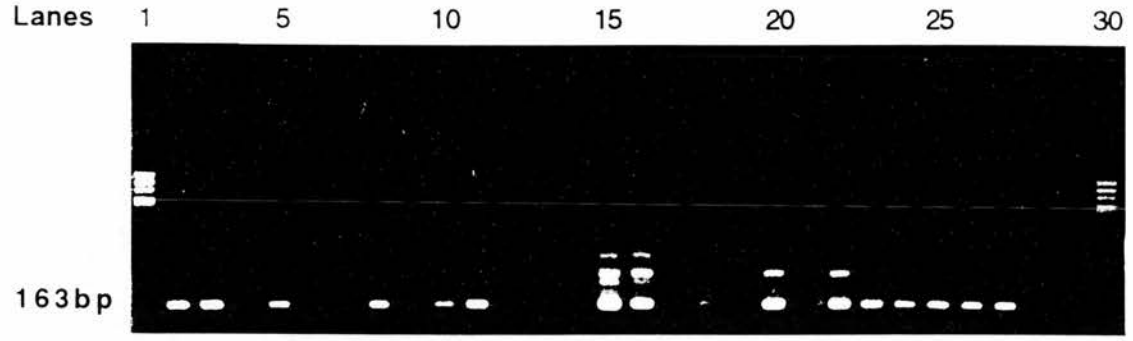

Fig. 2. Agarose gel of patient saliva samples for nested PCR for HHV-6 DNA. **Lanes 1 and 30:** DNA molecular weight marker; **lanes 9, 17, 29:** water controls; **lanes 25–27:** HHV-6 amplimer controls; **lane 28:** uninfected lymphoblastoid cell line control; **lanes 2, 3, 5, 8, 10, 11, 15, 16, 18, 20, 22–24:** HHV-6-positive samples; **lanes 4, 6, 7, 12–14, 19, 21:** HHV-6-negative samples.

aliquot with 600 HHV-6 amplimers as a template and then repeating the nested PCR.

Statistics

Nonparametric tests were used to compare these small groups of patient samples including the Chi-squared test and the Mann-Whitney U-test.

RESULTS

Specificity and Sensitivity of the Nested PCR for HHV-6

The sensitivity of the method was assessed by using a dilution series of extracted DNA from Type "A" HHV-6 (AJ strain). Positive bands were observed on agarose gel down to an input of 16 fg of DNA (equivalent to 93 copies of HHV-6 genome). Using DNA after a single round of amplification as input, the nested PCR had a detection limit of 1–10 ag on agarose gel, equivalent to 3 HHV-6 amplimers. The specificity of the primers chosen was demonstrated by lack of amplification of DNA extracted from six other herpesviruses. HSV-1, HSV-2, CMV and VZV were all obtained as laboratory isolates. EBV was cultured from the EB3 cell line and HHV-7 DNA was a kind gift from Dr. D. Clark, Royal Free Hospital, London (data not shown).

Performance of PCR

In 65% of runs, dilutions of A type virus were positive down to 30 genome copies, while in the remaining runs the detection limit was 300 copies. No amplified HHV-6 DNA was even found after PCR in any of the water or lymphoblastoid cell controls. Agreement in duplicate PCR runs was obtained in 74% of supernate samples and 76% of cell pellets, the discrepancy in results suggesting that some PCR runs were more sensitive than others. Samples were interpreted as positive if they were PCR-positive on at least one occasion. Spiking experiments demonstrated only one saliva pellet and two supernates were inhibitory for the PCR.

PCR Results for All Samples

When the total number of samples obtained was considered and the results obtained for the pellet and supernate were summed, then 28/38 (74%) of control saliva samples and 45/77 (58%) patient saliva samples were PCR-positive for HHV-6 DNA (Fig. 2). When the supernate and pellet results were considered separately then 23/38 (61%) of control supernate samples and 30/77 (40%) patient supernate samples were PCR-positive for HHV-6, and 21/38 (55%) of control pellet samples and 31/77 (40%) patient pellet samples were PCR-positive for HHV-6 (Table I). All the PCR-positive samples digested with Hind III and gave two fragments on agarose gel confirming them as DNA from "B" type HHV-6 (Fig. 3). No "A" type HHV-6 was identified in this group of patients or controls.

TABLE I. Results of HHV-6 PCR for All Samples Tested

	Patients no. (%)	Controls no. (%)	Statistics Chi-square
HHV-6 PCR			
Supernate	30/77 (40)	23/38 (61)	$P < 0.05$
Pellet	31/77 (40)	21/38 (55)	NS[a]
Both	45/77 (58)	28/38 (74)	NS

[a]NS, not significant.

PCR Results for Individual Patients

All the patients were seropositive for HHV-6 IgG at presentation. The samples were obtained at regular intervals from the time of diagnosis until the end of the collection period, when the children were either well or febrile and unwell and the number of samples obtained per child varied from 2 to 13 (Table II). The ratios of positive/negative samples for each child were compared and revealed two distinct groups of patients, those with mainly HHV-6-positive saliva and those with mainly HHV-6-negative saliva (Chi-square $P < 0.0001$). There was no difference between these two groups for age, total white blood count or absolute lymphocyte count. However, in the mainly HHV-6-negative group, there were more febrile children (10/30 HHV-6 PCR-negative versus 7/47 HHV-6 PCR-positive Chi-square $P = 0.05$) and they had lower absolute neutrophil counts (Mann-Whitney U-test $P = 0.02$).

TABLE II. Details of Patients' Sequential Saliva Samples and PCR for HHV-6

Patient group and no.[a]	Diagnosis ST/AL[b]	Age yrs	Weeks followed[c]	HHV-6 PCR[d] positive	HHV-6 PCR negative	HHV-6 pos/ total samples	Febrile[e] Pos	Febrile[e] Neg	Well[e] Pos	Well[e] Neg
A1	ST	3	9	—	4	0	—	—	—	4
A2	AL	4	4	—	2	0	—	2	—	—
A4	AL	8	24	1	6	0.14	1	2	—	4
A5	ST	13	30	1	4	0.2	—	—	1	4
A10	AL	6	20	2	4	0.33	1	4	1	—
A12	AL	5	11	2	4	0.33	—	—	2	4
B3	ST	7	47	4	1	0.8	—	—	4	1
B6	ST	5	59	10	3	0.77	1	—	10	2
B7	ST	10	32	6	2	0.75	2	—	4	2
B8	AL	8	36	12	1	0.92	3	—	9	1
B9	AL	12	6	3	2	0.6	—	1	3	1
B11	AL	4	5	3	—	1	—	—	3	—

[a]Group A: Rarely HHV-6 DNA positive; group B: Frequently HHV-6 DNA positive.
[b]Diagnosis: ST, solid tumour; AL, acute leukaemia. Age at presentation, in years.
[c]Weeks followed: i.e., the number of weeks over which samples were obtained from the patient.
[d]HHV-6 PCR: Number of positive or negative samples and the ratio of positive sample to the whole number.
[e]PCR results according to whether the patient was well or febrile at the time.

(29/47, 61%), very few supernate samples in the rarely HHV-6-positive group contained HHV-6 DNA (2/30, 6%). This could imply that healthy children and paediatric oncology patients who are well are more likely to secrete HHV-6 into the saliva.

Finding HHV-6 more frequently in the more healthy children appears at first surprising, as latent herpes viruses are more often recurrent and more likely to cause problems in immunosuppressed patients. We considered whether the children who were less well were not so good at producing saliva specimens, but this was not noted when specimens were collected. If the children who were less well had been receiving antiviral therapy this might have affected secretion of HHV-6, but only 3 samples were collected from children on acyclovir and 2 of these were HHV-6 PCR-positive. We suggest, therefore, that immunosuppressed and unwell patients on chemotherapy secrete less HHV-6 due to the effects of chemotherapy, with a depleted lymphocyte population reducing the reservoir for HHV-6 and affected salivary gland cells less able to support replication of HHV-6.

This small study has demonstrated firstly, that HHV-6 DNA occurs commonly in the mouths of normal healthy children without any obvious pathology and secondly, that children immunosuppressed on chemotherapy frequently secrete the virus without evidence of ill health. Furthermore those who are febrile, neutropaenic and unwell less frequently secrete the virus. HHV-6 latency is not completely understood and the role of this virus as a serious pathogen in immunosuppressed patients remains to be fully elucidated. Further studies, including quantitative PCR, will be required to confirm and extend this finding.

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Human herpesvirus 6: primary infection and the central nervous system

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HUMAN HERPESVIRUS 6 AND THE HISTORY OF EXANTHEMA SUBITUM

The clinical syndrome of exanthema subitum (ES), also known as roseola infantum, was first described in 1913 and in greater detail in the 1950s when an association with an infectious pathogen was suggested by the fact that the infection could be passed from one infant to another.[1–3] ES is a condition of infants in whom several days of high fever and malaise were followed by defervescence of the fever and appearance of a rash. The rash was erythematous, maculopapular and usually over the head and trunk and could last for a few hours to several days. These observant clinicians also documented that this condition was associated with febrile convulsions.

In 1988 with elegant use of virologic techniques Yamanishi et al.[4] identified human herpesvirus 6 (HHV-6) as the cause of ES. In a series of infants with primary infection he demonstrated: seroconversion with production of antibody to HHV-6; culture of the virus from peripheral blood lymphocytes (PBL); and using PCR, amplification of the HHV-6 genome from PBL. Further studies by others identified a relationship between the viral load of HHV-6 and the severity of the infection in terms of days of fever and malaise.[5] It is also possible to have primary HHV-6 infection with fever without rash or rash without fever.[6, 7] After the discovery of HHV-6 as the cause of ES, seroepidemiologic studies across the world soon confirmed that an IgG response to HHV-6 was found in almost 100% of children tested, and the peak period for seroconversion occurred during the second half of the first year of life.[8, 9]

Human herpesvirus 7 (HHV-7), which is genomically closely related to HHV-6, was first isolated in 1990 from the PBL of a healthy adult.[10] This virus, like HHV-6, is secreted in the saliva. Most children also have a serologic response to HHV-7, although it develops later than that to HHV-6. It has now been reported that HHV-7 can also cause the classical syndrome of ES.[11] Whether it has other pathogenicity is not yet known.

CHARACTERISTICS OF HHV-6

HHV-6 was first identified in cultures of PBL from patients with malignancy and AIDS.[12] This sixth member of the herpesvirus family and HHV-7 are most closely related genetically to cytomegalovirus. These three viruses belong to the beta subgroup of herpesviruses, and the genomic similarity between HHV-6 and cytomegalovirus is equivalent to that between the alpha subgroup herpesviruses, herpes simplex virus and varicella-zoster virus.[13] There is cross-reactivity in the host IgM response to HHV-6 and cytomegalovirus.[14] HHV-6 is T cell-lymphotrophic *in vivo* and *in vitro* and can coinfect cells that are infected with HIV, although it does not use the CD4 surface protein to enter these cells.[15] In common with other herpesviruses after primary infection latency is established and HHV-6 genomic material can be found in PBL, salivary glands and bronchial glands. Under certain conditions HHV-6 infection can reactivate.[16, 17] HHV-6 DNA is frequently secreted in the saliva of healthy children.[18]

The two subtypes of HHV-6, A and B, are very closely related with >90% homogeneity in the areas of genome examined.[13] The two subtypes have different *in vitro* cell tropism and can be identified by panels of monoclonal antibodies.[19] Type A, which grows more easily *in vitro*, was the first HHV-6 subtype to be discovered, but only a few isolates of this subtype have since been identified. Most subtype A isolates have come from immunosuppressed patients. Many more subtype B strains have now been isolated from both immunocompetent and immunosuppressed patients. Almost all the isolates from children with primary infection have been subtype B strains.[20] Whether there are important pathogenic differences between the two subtypes of virus is unknown.

PRIMARY HHV-6: THE FIRST FEBRILE ILLNESS

The largest Japanese study of the clinical features of primary HHV-6 infection, in which infection was diagnosed in 176 of 688 children admitted to hospital with

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fever, rash or both, tended to confirm the classical syndrome of ES.[21] This study found that 94% of infants were infected by 1 year of age, with a mean age of infection of 7.3 months (range, 3 weeks to 18 months); for the majority of infants this was the first febrile illness. Virtually all children (98%) had high fever to a maximum of 39.4°C, lasting for a mean of 4.1 days. A maculopapular rash appeared on the face and trunk in 98% of cases, most commonly at the resolution of the fever. The rash lasted for a mean of 3.8 days and there was no desquamation. Mild diarrhea occurred in 68% of cases and erythematous papules were seen in the pharynx in 65%. Cervical lymphadenopathy was present in 31% of cases and 50% had a cough. A bulging anterior fontanel was present in 26% of the children and convulsions occurred in 8%. The convulsions were all of short duration and occurred before the rash developed, during the febrile stage of the illness. The mean age of infants having convulsions was 10.9 months (range, 5 to 17 months).

HHV-6: RARELY DIAGNOSED CLINICALLY

The most exhaustive studies of primary HHV-6 in children have been performed in the US. The first of two very large studies[22] examined 243 children <2 years of age presenting consecutively to hospital with fever (≥38°C) for evidence of HHV-6 infection by culture, PCR and antibody response. Primary HHV-6 infection was identified in 34 cases (14%), with a mean age of 9.5 months. Features of this group included high fever (mean, 39.7°C) and inflamed tympanic membranes. Three cases had a rash after the fever defervesced and 3 had a rash with the fever. One child had a febrile seizure. The mean white blood cell count on presentation was 8.9×10^9/l with 39% lymphocytes and 51% neutrophils. All but one of the strains of HHV-6 cultured were subtype B and the other culture included subtypes A and B.[20]

A further study examined 2587 children <3 years of age for adverse effects of HHV-6 primary infection. There were 586 infants with acute nonfebrile illness and 356 healthy controls; none of these control patients had HHV-6 primary infection. Of 1653 infants with acute febrile illness, 160 had HHV-6 primary infection (9.7%). The mean age of this group was 9.4 months (range, 2 weeks to 25 months). Considering only infants ages 6 to 12 months, 21% (75 of 365) of febrile illnesses were caused by HHV-6.

In only 17% of the 160 cases was the correct clinical diagnosis made; 30% were diagnosed as fever caused by otitis media and 29% as fever of uncertain cause. The incidence of infection did not vary with the seasons. The majority had high fever(> 39°C) and 15% remained febrile for >6 days. Only 11% had a rash at defervescence and 6% had a rash at presentation. Seizures occurred in 13% (21 of 160), compared with a 9% seizure rate in the 1394 febrile children withc HHV-6 infection ($P = 0.18$). HHV-6 primary infecti accounted for one-third of first febrile seizures (medi age, 14 months). Primary infection with HHV-6 occ ring later, in the second year of life, was associat with febrile seizures in 30% of cases. Some seizu were prolonged or recurrent.

Cerebrospinal fluid (CSF) collected from 29 childr with primary HHV-6 infection, including 7 with cc vulsions, was examined. The cell count, protein a glucose concentrations were normal in all specime and no HHV-6 could be cultured. HHV-6 genome w amplified from CSF by PCR in 7 cases, including 2 wi convulsions.

In the UK there have been no large scale prospecti studies of the role of HHV-6 as a pediatric pathoge Ward and Gray[24] examined a retrospective collection sera from 248 children submitted for viral diagnosis f anti-HHV-6 IgG antibody avidity. Low avidity antibo implies recent infection. A group of 25 children h febrile seizures and in 1 case clinical ES was diagnos at the time. Five of this group had low avidity an HHV-6 IgG implying primary infection, whereas were seronegative on acute phase serum and no conv lescent sample was obtained. The remaining 11 ch dren had high avidity antibodies to HHV-6, almc certainly excluding primary HHV-6 as a cause of t infection and seizures. This study might also suppc the possibility that primary HHV-6 can be a cause f febrile seizures. Again in the majority of cases prima HHV-6 infection was not considered in the differenti diagnosis.

DIAGNOSIS OF HHV-6 INFECTION

The diagnosis of primary ES is not straightforwar Culture of HHV-6 from PBL is a specialized techniq available only as a research tool. A few laboratori offer HHV-6 PCR of CSF and plasma as special test Serology, although an indirect method of diagnosis, more widely available and primary infection can b diagnosed in paired samples by seroconversion, pre ence of IgM or low avidity early IgG antibodies. Paire serum and CSF antibody titers could also be used diagnose CNS infection.

As well as the technical limitations to diagnosis HHV-6, it is clinically difficult to make the diagnosis ES. Children with this infection are probably mo often diagnosed as having otitis media or a "virus" wit fever. They may or may not develop a rash, and if the do it will most likely occur a few days after visiting th doctor. Indeed it is possible that many of these infan are unnecessarily prescribed antibiotics and then if th rash appears are erroneously described as "allergic t penicillin." Because the American study demonstrate that 21% of infants ages 6 to 12 months presenting t the "emergency room" with fever had primary HHV-

infection, we could do better if we considered this viral infection higher in the differential diagnosis of fever in this group of our patients.

HHV-6 AND THE CENTRAL NERVOUS SYSTEM (CNS)

Early clinical evidence described a relationship between ES and febrile seizures, now confirmed by these large studies. Although HHV-6 genome can be identified in the CSF, the lack of inflammatory cellular changes makes it difficult to understand the mechanism by which this virus affects the CNS. Since the discovery of HHV-6 there have been several case reports of more serious CNS complications including fatal encephalitis with primary HHV-6 infection.[25–29] In only one of these cases was brain tissue examined for evidence of HHV-6 infection, and in this case a needle biopsy of brain was negative for HHV-6 antigens at a late stage, 21 days, after presentation.[25] HHV-6 genome and antibodies to HHV-6 have been found in the CSF; inflammatory changes have been seen on computerized tomography and single photon computerized tomography scans; and abnormal encephalograms have been reported. No adequate examination of brain tissue by pathologic or *in situ* virologic techniques has been undertaken to ascertain the role of HHV-6 as a possible acute CNS pathogen. Because HHV-7 can cause ES with febrile convulsions, the role of this virus in the CNS also needs to be considered.[30]

In an extension of the American study CSF and PBL from children who had experienced primary HHV-6 infection were examined for persistence of HHV-6 DNA. HHV-6 was shown to persist in the CSF and PBL after acute infection, and in some patients it was detected only in CSF.[31] In a small study of autopsy brain specimens, HHV-6 DNA was found in frontal cortex and/or basal ganglia of 11 of 13 immunocompetent adults.[32] The authors suggest that the high frequency of HHV-6 DNA in the CNS implies that HHV-6 can invade the CNS and remain latent with the possibility of subsequent reactivation. Detailed virologic investigation of the brains of children who die from encephalitis and other causes for evidence of HHV-6 infection would help to ascertain whether this virus does act as a CNS pathogen, or whether it persists as a bystander.

Investigation of the brains of patients with multiple sclerosis (MS) and controls for HHV-6 DNA has also demonstrated that HHV-6 is frequently present in patients and controls.[33] Expression of HHV-6 virion proteins in the nuclei of oligodendrocytes was demonstrated only in patients with MS. This usually occurred in association with MS plaques, and it has been suggested that HHV-6 may have an etiologic role in the pathogenesis of MS. It is interesting to speculate whether patients who later develop MS had a primary HHV-6 infection with CNS manifestations such as febrile seizures. More information on the CNS load of HHV-6 and the immune response to the virus in the CNS of these patients will help to elucidate any relationship.

The immunocompromised patient could be considered at particular risk; whether HHV-6 is a significant CNS pathogen for children or adults immunosuppressed iatrogenically, congenitally or by infection is unknown. In a preliminary study of five adult patients dying with AIDS, HHV-6 was demonstrated by PCR in different areas of the brains of all patients but in no brain tissue from two patients with accidental deaths.[34] A case of fulminant HHV-6 encephalitis has also been reported in an infant with HIV infection.[35] CNS demyelination associated with HHV-6 infection has also been reported in adults with AIDS.[36]

Because febrile convulsions are common and there is evidence that HHV-6 and perhaps HHV-7 infection may play an etiologic role, clinicians must include these viruses in the differential diagnosis of the cause of the fever and attempt to make a virologic diagnosis. Because there are long term sequelae of febrile convulsions, including hippocampal sclerosis that can cause subsequent epileptic problems, the role of these possible neuropathogenic viruses should be considered in the etiology of such complications. Where cases of encephalitis have occurred, even a retrospective study of necropsy brain tissue from children could give some useful information about the CNS pathogenicity of these herpesviruses. Diagnosis of infection could also have treatment implications, for although HHV-6 is relatively resistant to acyclovir, it is susceptible to ganciclovir.[13] Thus if HHV-6 was found to be a significant CNS pathogen, antiviral therapy could be considered in severe cases.

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