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HUMAN HERPESVIRUS-6-INDUCED CYTOKINES AND LYMPHOCYTE ANERGY

By

Monika Nowak

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Department of Microbiology and Immunology
Faculty of Medicine
University of Ottawa

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ABSTRACT

Human Herpesvirus-6 (HHV-6) is a lymphotropic β-herpesvirus. Epidemiological studies have shown it to be a major etiologic agent of *roseola infantum*. HHV-6 has been linked to several illnesses and is also considered an important cofactor in AIDS. Although HHV-6 infects a variety of cells *in vivo* and *in vitro*, complete viral replication occurs most efficiently in T lymphocytes. Following primary infection, HHV-6 persists in a latent state and can be reactivated under conditions of acquired or induced immunosuppression.

The nature of HHV-6 immunosuppression remains unresolved. To address this, the cytokine repertoire expressed by T cells following HHV-6 infection and the T cell anergy induced by HHV-6 were studied. Results show that HHV-6 infected T cells secreted increased amounts of IL-1β, IL-6, IL-10, IFN-γ and TNF-α when compared to mock-infected T cells. The enhanced cytokine release was mediated by binding of the virus to cells and did not require viral replication. HHV-6 exposed T cells showed a significant impairment in their proliferative response to mitogen as well as to activation signals delivered through surface molecules or other transmembrane pathways. Some virus transcription was required to suppress lymphocyte proliferation. HHV-6 infected T cells express a soluble suppressive factor(s) which inhibits the lymphoproliferative response of uninfected cells. The suppressive factor requires its native conformation for activity and its suppressive activity is reduced when neutralized with anti-HHV-6 serum. NK cytolysis in PBMCs exposed to the suppressor factor could be enhanced by the addition of IL-15. These results suggest a mechanism whereby the immune system may respond to acute immunosuppressive HHV-6 infection.

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For Ben and Noémi.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

APC: Antigen presenting cell

CTL: Cytotoxic T lymphocyte

EBV: Epstein-Barr virus

HCMV: Human cytomegalovirus

HIV: Human immunodeficiency virus

HSV: Herpes simplex virus

IE: Immediate-early

IFN: Interferon

IL-: Interleukin-

IM: Infectious mononucleosis

LAK: Lymphokine activated killer cell

LTR: Long terminal repeat

mAb: Monoclonal antibody

NFκB: Nuclear factor kappa B

NK: Natural killer

ORF: Open reading frame

PBMC: Peripheral blood mononuclear cells

PDB: Phorbol 12, 13-dibutyrate

PHA: Phytohaemagglutinin

PKC: Protein kinase C

SD: Standard deviation

SEM: Standard error of mean

SRBC: Sheep red blood cell

TCR: T cell receptor

TNF: Tumor necrosis factor

UV: Ultraviolet

VSV: Vesicular Stomatitis Virus

I. Introduction

I.I. The Discovery of Human Herpesvirus-6

Human herpesvirus-6 (HHV-6) was first identified by Salahuddin et al. (1986) at the Laboratory of Tumor Cell Biology (National Cancer Institute, Bethesda, MD). The involvement of this lab with acquired immune deficiency syndrome (AIDS) research led to parallel studies on the malignancies associated with AIDS, such as AIDS-associated lymphoma and Kaposi's sarcoma. Although the human immunodeficiency virus (HIV) was known to affect T cells, the cell types and biology involved with the AIDS-associated malignancies were so different as to postulate the involvement of another virus. In early experiments, peripheral blood mononuclear cells (PBMC) from a group of patients with various lymphoproliferative disorders and AIDS were cultured and activated. A unique cytopathic effect (CPE) characterized by the formation of enlarged, rounded, refractile cells which survived for several days after the onset of cytomorphological changes was observed (Salahuddin et al., 1986). This CPE was morphologically distinct from that induced from potentially contaminating HIV. To determine if this new CPE was due to a new transmissible agent, cell-free culture supernatant was transferred from these cells to fresh cord blood leukocytes. The CPE was transmissible without the presence of HIV-1 antigens. Human T cell lymphotropic viruses I and II (HTLV I and II) were also ruled out as potential candidates and electron microscopic analysis showed the agent to be morphologically indistinguishable from members of the herpesvirus family. Monoclonal and well characterized human antibodies directed against the other human herpesviruses failed to react with the new virus in immunofluorescence assays. DNA hybridization

studies using cloned fragments from the new virus and other herpesviruses further indicated that the newly isolated virus was distinct (Josephs et al., 1986). The cells demonstrating the CPE were thought to be immature B cells which formed the basis for the early nomenclature, human B cell lymphotropic virus (HBLV) (Salahuddin et al., 1986). It was soon established that the infected cells were T cells rather than B cells (Lusso et al., 1987) and the virus was therefore renamed HHV-6 following previously established herpesvirus nomenclature (Ablashi et al., 1987). Coincident with the publication of the discovery of HHV-6 by Salahuddin et al. (1986), Downing et al. (1987) noted grossly enlarged cells when lymphocytes derived from a Ugandan AIDS patient were cocultured in the presence of IL-2 and PHA and fresh cord blood lymphocytes. Similar to Salahuddin et al., (1986) herpesvirus-like particles were also observed by electron microscopy but no antigenic cross-reactivity was seen with antibodies specific to other herpesviruses. Various other labs also isolated a new agent with similar characteristics (Tedder et al., 1987; Lopez et al., 1988). Although the T cell tropism of HHV-6 made it an unlikely agent for AIDS-associated B cell lymphomas, there was growing interest in knowing whether this new virus had any disease association and hence epidemiological studies and molecular characterization were pursued.

I.2. Classification

Human herpesviruses are classified into three subgroups: α-herpesviruses [herpes simplex virus-1 (HSV-1), HSV-2, Varicella Zoster Virus (VZV)], β-herpesviruses [human

cytomegalovirus (HCMV), HHV-6 (A, B), HHV-7], and γ-herpesviruses [Epstein-Barr virus (EBV), HHV-8 (also known as Kaposi's Sarcoma-associated Herpesvirus, KSHV)]

HHV-6 has been classified within the Herpesviridae family based on its ultrastructural and genetic characteristics (Biberfeld et al., 1987; Josephs et al., 1986). Based on the lymphotropism of HHV-6, it was initially proposed to be a γ-herpesvirus but was later included in the β-herpesvirus subfamily based on its genomic similarity to HCMV.

1.2.1. HHV-6 Variants A and B

HHV-6 isolates belong to one of two subgroups or 'variants' designated HHV-6A or HHV-6B. Laboratory strains U1102 and GS are representative of HHV-6A strains and Z29 is representative of HHV-6B strains. Virus strains U1102, Z29 and GS were initially isolated from AIDS patients from Uganda, Zaire and the United States of America, respectively. Strains A and B are differentiated based on restriction endonuclease digestion patterns, cell tropism and reactivity with specific antisera. The overall genetic divergence between the two variants ranges between 2 and 25% depending on the loci analysed (Aubin et al., 1991; Gompels et al., 1993). HHV-6A and B appear to have both a different epidemiological distribution in the human population with variant B being more prevalent than variant A, and possible differences in disease associations (Dewhurst et al., 1993; Frenkel et al., 1994; Carrigan and Knox, 1995).

I.3. Morphology

Morphologically, HHV-6 is a typical herpesvirus with a diameter of 160-200 nm. The nucleocapsid exhibits icosahedral symmetry and contains 162 capsomeres which enclose a core containing a double-stranded DNA genome. The nucleocapsid is surrounded by an amorphous tegument and externally, by a lipid-containing envelope containing spike-like projections.

I.4. Genetic Characteristics

The prototypic laboratory strain of HHV-6A, U1102, has been completely sequenced (Genbank accession number X83413) and from this sequence the overall structure, coding content and genetic relationships between HHV-6 and other herpesviruses have been compared (Gompels et al., 1995). HHV-6 is the fifth human herpesvirus genome to be sequenced, after HSV-1, VZV, EBV and HCMV. From sequencing data it has been established that 67% of the HHV-6 genes have a corresponding counterpart in HCMV. The genome of HHV-6 is colinear with HCMV and encodes products with closer similarity to HCMV than to the other sequenced herpesviruses. At the amino acid level there is an average of 30% identity. The relative arrangements of the HHV-6 herpesvirus conserved genes are identical to those of homologous genes in HCMV and HHV-7, as expected for a betaherpesvirus genome. Conserved gene blocks I to VII contain homologous genes and are orientated identically between HHV-6, HHV-7 and HCMV (Gompels et al., 1995; Nicholas, 1996). The

properties of proteins encoded by HHV-6 are derived in some cases from direct experimental data, or extrapolated from functions defined by similar HCMV or HSV proteins. Many HCMV genes have no HHV-6 counterpart and vice versa.

1.4.1. HHV-6 DNA

HHV-6 has a linear, double-stranded DNA genome. Its size is smaller than that of HCMV. HHV-6 genomes range from 160 kilobase pairs (kbp) to 170 kbp compared with the HCMV genome which is 230 kbp. Computer analysis of HHV-6 genome predicts 119 open reading frames (ORFs) with 102 likely to be distinct genes (Gompels et al., 1995). The genome of HHV-6 consists of a single, A+T rich, unique long (U_L) element of approximately 140 kbp flanked by two identical G+C rich direct repeat (DR) segments of approximately 8.000 bp each. Within the DRs, the ORFs are designated DR1-DR8. ORF in the U_L are designated U1-U100. DR1-8 are duplicated in the left DR (DRL) and right DR (DRR).

The overall G+C content was calculated to be 40-43% (Lindquester and Pellett, 1991; Gompels et al., 1995) which is the lowest G+C content found so far for human herpesvirus genomes. The genome has "local" rather than "global" suppression of CpG dinucleotide frequency which may be related in some way to a biological function (Martin et al., 1991). It is believed that these areas of suppression function in gene control and latency because they are observed only in the major immediate-early (IE) gene region. HHV-6 contains at least one origin for replication (ori-lyt) located within the U_L. There

are three clusters of major reiterations located at the right end of U_L which include SR repeats encoding a RNA binding motif, TG dinucleotide repeats and Kpn containing repeats. The Kpn containing repeats contain consensus binding sites for the transcription factors SP2 and nuclear factor kappa B (NFκB), and are thought to compose an immediate-early enhancer locus. The genome of HHV-6 has limited non-coding segments and minimal splicing occurs after transcription.

1.4.2. HHV-6 Proteins

HHV-6 encodes several membrane proteins including homologues of HSV glycoprotein B (gB) (U39) (Chou and Marousek, 1992) and gH/gL (U48/82) (Josephs et al., 1991). Members of these membrane glycoproteins appear to be conserved in all herpesviruses and are thought to play a role in virus infection and cellular spread. Both gB and gH/gL induce neutralizing antibodies however, anti-gB requires complement in order to neutralize. Other ORFs which encode glycoproteins unique to HHV-6 with unknown function are contained within U20-U24. U20 and U85 ORFs contain immunoglobulin domains. Since several members of the immunoglobulin superfamily have roles in cell-to-cell contact, it is postulated that these glycoproteins may function in the infected cell's adhesion to other uninfected cells. Six other membrane proteins have been identifed with single membrane spanning regions, one of which (U83) has predicted features of an intercrine cytokine. Three other ORFs encode proteins with predicted multiple membrane spanning regions. Two of these, U12 and U51, have homology to G-protein coupled

receptors (GCR). A third multiple membrane spanning protein encodes an integral membrane glycoprotein gM (U72) whose counterpart in HSV is a major component of the virion particle (Lawrence et al., 1995). Several other genes have been identified in the HHV-6 genome which may serve as transcriptional activators based on homology to similar HCMV genes located within IE loci. The HHV-6 genome also encodes sequence and functional homologues of other viral genes which are necessary for origin-dependent DNA replication. These are the DNA polymerase processivity factor (U27), DNA polymerase (U38) (Teo et al., 1991), single-stranded DNA binding protein (U41), helicase/primase complex (U43, U74, U77) and the origin binding protein (U73).

Like other herpesviruses, HHV-6 appears to replicate by a rolling circle mechanism, generating head to tail concatemers which are subsequently cleaved to unit length linear genomes during viral packaging (Pellett and Black, 1996). HHV-6, like HCMV, lacks a thymidine kinase and hence is resistant to acyclovir but does encode a kinase that can modify nucleosides (U69). HHV-6 encodes the major subunit of ribonucleotide reductase (U28), and a putative deoxyuridine triphosphate nucleotidylhydrolase (dUTPase) homologue (U45). HHV-6 encodes DNA packaging and virus assembly proteins (U53, U29, U30 and U60/66), tegument proteins (U11, U48, U54) and capsid proteins (U56, U57, U29, U33, U58, U53a) (Littler et al., 1990). Although HHV-6 encodes proteins which are similar to HCMV or to other herpesviruses, genes unique to HHV-6 have also been found. HHV-6 encodes a protein with striking similarity to the *rep* 68/78 gene product of the human adenovirus helper-dependent parvovirus AAV-2 (Thomson et al., 1991), a defective parvovirus. The Rep protein has

trans-regulatory properties, which inhibit cellular transformation by papillomaviruses. Araujo et al (1995) showed the Rep68/78 homologue to suppress H-ras transformation of NIH-3T3 cells and inhibit transcription from the HIV-1 long terminal repeat (LTR) promoter in human T cells. It is unclear what role this protein plays in the interaction of HHV-6 with its host cells and organism. This homologue does not exist in any other known herpesvirus.

I.5. Biological Features

I.5.1. Cell Tropism

The cellular receptor(s) for HHV-6 is not known. Extensive analysis of *in vitro* infected cells of different origin (e.g. peripheral blood, umbilical cord blood, bone marrow, tonsil, thymus) led to the demonstration that HHV-6 preferentially replicates in CD4+ T cells, resulting in cytopathic effects and eventual cell death (Lusso et al., 1991b). Although it is generally accepted that HHV-6 replicates most efficiently in CD4+ T cells, HHV-6's host range also includes CD8+ T cells (Lusso et al., 1991b), γ/δ T cells (Lusso et al., 1995), macrophages (Kondo et al., 1991), natural killer cells (Lusso et al., 1993), megakaryocytes and possibly epithelial cells (Krueger et al., 1990), and B cells immortalized by EBV (Ablashi et al., 1988; Cuomo et al., 1995).

PHA-stimulated primary lymphocyte cultures can be used to isolate both HHV-6A and HHV-6B subgroups. Some differences have been documented in the cellular tropism of these subgroups when adapted for growth in cell lines. The ability to grow in various

continuous cell lines is one method used to differentiate isolates. Generally, HHV-6A isolates grow in HSB-2 cells and HHV-6B in Molt-3 and MT-4 T cell lines.

1.5.2. Latency

Like other herpesviruses, HHV-6 can establish a latent infection which remains for the lifetime of the host but can be reactivated during the induction of host immunosuppression. It has been suggested that circulating monocytes (Kondo et al., 1991) and the epithelia of salivary and bronchial tissue (Krueger et al., 1990) may serve as sites for HHV-6 latency *in vivo* following primary infection. The establishment of latency implies the virus is subjected to tight immunological control and/or has developed strategies to evade defence mechanisms. HHV-6 DNA has been detected in latently infected lymphocytes by PCR in up to as many as 90% of specimens from healthy donors (Jarrett et al., 1990; Cone et al., 1993; Cuende et al., 1994). These frequencies are not likely to be due solely to reinfections. *In vitro*, HHV-6 behaves as a cytopathic virus in lymphocytes and to date there is no culture system for the establishment of latent infection in lymphocytes. A culture system exhibiting latent HHV-6 infection in monocyte/macrophages has been established (Kondo et al., 1991). In this system HHV-6 can be reactivated after phorbol ester treatment. This study also showed that HHV-6 DNA can be detected predominantly in monocytes from HHV-6 infected patients during the convalescent phase and in both monocytes and lymphocytes during the acute phase of infection. These results suggest that the monocyte is the site of latent infection *in vivo* and *in vitro* and may be reactivated by certain factors. It is not known if HHV-6 infection

resembles the latent infections of HSV-1 and EBV where the virus does not enter the lytic phase of its replication cycle but expresses only those genes required for latency maintenance.

1.5.3. Virus-Cell Interactions

Diverse interactions have been demonstrated between HHV-6 and its host cells. HHV-6 infection results in the downregulation of the γ, δ and ε chains of CD3 on both CD4+ and CD8+ T cells (Lusso et al., 1991b). This effect required virus replication as ultraviolet (UV)-inactivated virus had no effect on CD3 expression. Given the major role of the CD3/TCR complex in the physiology of antigen recognition and activation of T cells, its downregulation may cause important alterations of the normal cellular immune responses and have an immunosuppressive effect. Another feature of HHV-6 infected T cells is related to the ability of HHV-6 to transcriptionally activate the expression of CD4 in CD4 nonexpressing cells such as CD8+ T cells, NK and γ/δ T cells (Lusso et al., 1991a, 1995). The induction of CD4 expression appears to be regulated by immediate early (IE) or early HHV-6 gene products, as inferred from studies with the viral DNA polymerase inhibitor phosphonoformic acid. The effect on CD4 and CD3 by HHV-6 infection was contested by another study where Northern blot analysis and immunoprecipitation showed that infection with HHV-6 did not affect the transcription and protein synthesis of CD3 or CD4 in CD4+ T cells (Furukawa et al., 1994). These

discrepancies can possibly be explained by different target cells, HHV-6 isolates and culture systems used.

A characteristic of α-herpesvirus infected cells is the rapid shut-off of host macromolecular metabolism early in infection. As a result, host DNA synthesis is shut off (Roizman and Roane, 1964) and host protein synthesis declines very rapidly (Roizman et al., 1965; Summers et al., 1975). Acute infection by HHV-6 was shown to dramatically decrease cellular DNA synthesis (Di Luca et al., 1990) in spite of an apparent increase in cellular protein synthesis (Black et al., 1991). This gross stimulation of host cell protein synthesis in the absence of detectable cell division has not been reported for other herpesviruses.

An important biological effect of virus infection is the ability to stimulate the release of cytokines. This was demonstrated in HHV-6A acute infection of PBMC (Flamand et al., 1991) where IL-1β and Tumor necrosis Factor-α (TNFα) were shown to be induced at both the transcriptional and protein levels. This cytokine induction was mediated by a thermolabile viral structural protein or required a conformationally intact virion as heat inactivated HHV-6 and HHV-6 antibody positive serum blocked this cytokine induction. UV-irradiated HHV-6 retained IL-1β and TNFα induction potential; therefore, viral replication was not required for this effect. Kikuta et al (1990) demonstrated that HHV-6 induced the secretion of α-interferon from the non T cell fraction of PBMC. Exogenous α-interferon (α-IFN) partly suppressed replication of

HHV-6 in cord blood cells *in vitro* (Kikuta et al., 1990, Takahashi et al., 1992) indicating that HHV-6 was sensitive to the anti-viral effect of IFN on cells.

HHV-6 can induce a humoral immune response in infected individuals, but there are little data to demonstrate a cellular immune response. HHV-6 infection of PBMC inhibited mitogen-, IL-2- and antigen-stimulated proliferative responses (Horvat et al., 1993). This suppression of T cell function was further investigated (Flamand et al., 1995) and found to be associated with a downregulation of IL-2 mRNA and protein synthesis which accompanied the diminished cellular proliferation. Adding exogenous IL-2 did not correct the inhibition of mitogen-driven proliferation. The precise mechanism by which HHV-6 represses IL-2 transcription is not known.

1.5.4. Clinical Manifestations of HHV-6

Primary HHV-6 infection has been shown to be the cause of *roseola infantum* (also known as *exanthem subitum* or sixth disease). The etiologic link between HHV-6 and roseola was made by Yamanishi et al (1988) based on viral isolation and serology. This has since been supported by further studies (Takahashi et al., 1988; Enders et al., 1990). Roseola is the most common exanthem seen in infants under 2 years of age, with peak prevalence from 7 to 13 months of age. The presence of maternal antibody to HHV-6 probably is protective since the disease is rarely, if ever, seen before the age of six months. HHV-6 infection typically results in a benign, febrile, self-limiting illness. The disease is characterized by a transient high fever (febrile phase) followed by the

appearance of a rash mainly in the trunk (exanthem phase) as the fever subsides. Seroepidemiologic studies have shown seroconversion to strain B occurs by 2 years of age, and the seroprevalence in the healthy adult population exceeds 90% (Okuno et al., 1989). The A variant has not been linked to any human disease but A variants are most frequently isolated from immunocompromised patients while subgroup B has been associated with roseola.

In addition to roseola, HHV-6 has been associated with several other illnesses (reviewed in Pellet et al., 1992; Lusso and Gallo, 1995a,b). HHV-6 has been linked to EBV-negative mononucleosis (Steeper et al., 1990 Akashi et al., 1993), persistent lymphadenopathy (Niederman et al., 1988), fulminant hepatitis (Sobue et al., 1991), autoimmune disorders such as Sjogrens syndrome (Fox et al., 1990) and chronic fatigue syndrome (Levine et al., 1992; Buchwald et al., 1992). It is noteworthy that the above mentioned disorders are associated with some immunological abnormalities suggesting that HHV-6 may be enabled to replicate and further promote pathological consequences as immunological controls are either weakened or disrupted.

There is increasing evidence suggesting that HHV-6 may act as an opportunistic agent and cause severe morbidity in patients with immune deficiencies, particularly HIV-infected individuals (reviewed in Lusso and Gallo, 1995a) and in transplantation recipients (reviewed in Singh and Carrigan, 1996). Various studies have suggested a role for HHV-6 in the suppression of bone marrow function in bone marrow transplantation recipients (Drobyski et al., 1993a). Further support for HHV-6 being involved in bone marrow suppression came from *in vitro* studies showing suppression, prevention or inhibition of

bone marrow progenitor cell differentiation after infection (Knox and Carrigan, 1992) and a reduced ability to respond to growth factors (Burd et al., 1993) when exposed to HHV-6. HHV-6 has been detected in some human tumors (Jarrett et al., 1988; Josephs et al., 1988b) but in a less consistent fashion and the association with cancer has an uncertain significance.

HIV-infected patients constitute another category of immunosuppressed individuals for whom HHV-6 infection may have important pathological effects. The first suggestion of a possible role for HHV-6 in AIDS came from the demonstration that HHV-6, like HIV, has a CD4+ T lymphocyte as the principle target both *in vivo* and *in vitro*. Together with its ability to co-infect CD4+ T lymphocytes with HIV (Lusso et al., 1989) and to induce expression of the major membrane receptor for HIV-1,CD4, in CD4-negative lymphoid cells (Lusso et al., 1991a, 1993, 1995), various other viral interactions have been demonstrated. HHV-6 has been shown to transactivate the HIV-LTR (Zhou et al., 1994, Kashanchi et al., 1994, Di Luca et al., 1991) and to increase the expression of HIV lytic-promoting cytokines, TNF-α and IL-1β (Flamand et al., 1991). One study claimed co-infection of HHV-6 and HIV-1 inhibited HIV replication resulting in prolonged survival of CD4+ cells in culture (Levy et al., 1990). Thus, HHV-6 may act as a cofactor in HIV infection either by expanding the range of susceptible cells and/or by regulating the expression of HIV-1 genes.

I.6. Immune Responses to Herpesvirus Infection

In general, virus infection triggers a cascade of events leading to immune cell activation which eventually results in the control of the invading pathogen. The nonspecific arm of the immune response represented by NK cells, macrophages, granulocytes and cytokines is thought to play an important early role in limiting the progression of infection by viruses. Mechanisms such as inhibition of virus multiplication by IFNs and lysis of infected cells by NK cells limit viral spread, enabling viral specific B and T lymphocytes to mount an effective response for the eventual resolution of viral infection.

I.6.1. NK Cells

Studies using depletion and adoptive transfer of selected subpopulations of NK cells, macrophages, and CD4+ and CD8+ T lymphocytes indicate that each of these is important in protection against infection with herpesviruses. The importance of natural immunity via NK cells in herpesvirus infections can clearly be seen in murine experimental models. Transfer of NK cells to NK-deficient animals or, conversely, elimination of NK cells through anti-NK serum treatment resulted in either protection from or promotion of the HSV-1 infection, respectively (Ranger-Zisman et al., 1987; Staats et al., 1991). In humans, decreased NK activity precedes a recurrent HSV-I outbreak, such that NK cells from patients with recurrent herpesvirus infection have diminished ability to lyse either the NK sensitive cell target K562 or autologous HSV-infected targets (Rola-Pleszczynski and

Lieu, 1984). Thus NK cells serve an important role in limiting the severity of virus infection in the period preceding the development of T cell mediated immunity.

The ability of NK cells to lyse autologous HHV-6 infected PBMC has been reported (Malnati et al., 1993). Takahashi et al (1992) reported an increase in NK cytotoxicity during the acute phase of HHV-6 infection when compared to the NK activity observed during convalescence. One study looked at the consequences of HHV-6 infection on NK cell lysis of K562 targets during *in vitro* infection of PBMC (Flamand et al., 1996). They observed an enhancement of NK cytolysis and attributed this early immune activation to IL-15 which was simultaneously induced with HHV-6 infection. IL-15 has been reported to have potent NK stimulatory activity (Bamford et al., 1994).

1.6.2. Cellular Immunity

Herpesvirus infection can result in suppression of cellular immunity. There is a large body of literature dating back to the 1970's demonstrating the importance of antigen-specific T cells in protection against experimental and natural herpesvirus infection. The majority of these studies used murine models and HSV-1 or HSV-2. From this data, several key cellular immune responses have been shown to be dysfunctional after HSV infection.

Decreased delayed type hypersensitivity (DTH) reactivity was reported by Nash and Gell (1983) in a HSV murine model and was thought to be mediated by enhanced suppressor T cell activity. One study looked at the DTH response from infectious mononucleosis patients using an EBV antigen skin test and saw no significant difference

when compared to controls (Perez-Blas et al., 1992). There is no evidence to date for a role for DTH response, or suppression of DTH reactivity in controlling infection of humans with α-herpesviruses.

Along with cytokine assays, the measurement of mitotic activity by ^{3}H-thymidine incorporation into newly synthesized DNA is one of the most frequently used assays for T cell activation. Generally, proliferation assays measure CD4+ T helper cell response to recall antigens (e.g. influenza or tetanus toxoid). Loss of T helper cell responses to either mitogen or recall antigens is indicative of a general or specific immunologic defect (Schnittman et al., 1990; Clerici et al., 1989; Schulick et al., 1993). The proliferation assay cannot differentiate the type of T cells responding, i.e. T cells that support the humoral (antibody) or cellular arm of the immune response. Proliferation assays have been used to measure lymphoproliferative responses to various stimuli in herpesvirus infected cells. *In vitro* studies of lymphocyte reactivity from HSV-positive patients to HSV antigens, mitogens and non-HSV microbial antigens showed a lack of suppressive effects on lymphocyte blastogenesis. This differs greatly from the suppressed lymphocyte response caused by CMV (Rinaldo et al., 1980; Rinaldo and DeBiasio, 1983) and EBV (Tosato et al., 1979) infection. However, *in vitro* infection of PBMC with HSV does inhibit lymphocyte responses to mitogens, allogeneic lymphocytes and microbial antigens (Plaeger-Marshall and Smith, 1978; Singal and Rawls, 1980). These suppressed T cell responses were attributed to HSV complexing with IL-2 (Wainberg et al., 1984) and/or to a decrease in IL-2 production (Wainberg et al., 1985). Proliferative responses to mitogen were found to be impaired in EBV infected patients (Perez-Blas et al., 1992) and the

ability to stimulate T cells through the T cell receptor (TCR)/CD3 pathway in patients suffering from acute CMV infection was also impaired (Timon et al., 1993). Both impairments were associated with a defect in IL-2 synthesis and exogenous IL-2 restored lymphocyte responses to normal levels. HHV-6 impaired the proliferative response of PBMC to mitogen and antigen (Horvat et al., 1993) which was attributed to a suppression of IL-2 mRNA and protein synthesis; however, exogenously added IL-2 did not correct this defect (Flamand et al., 1995). In this case, the defect may lie in the IL-2 receptor signaling pathway leading to the inability of cells to respond to IL-2 as there was no difference in IL-2 receptor expression levels in infected cells compared to controls. Taken together, these studies on lymphocyte proliferative responses to various stimuli after infection with different herpesviruses suggest that there is not a common mechanism used by herpesviruses to suppress lymphocyte responses.

Cytotoxic T cell function is also affected by herpesvirus infection. A reduced sensitivity of HSV-infected cells to lysis by cytotoxic T lymphocytes (CTL) has been observed and thought to be mediated by a HSV-DNA encoded function (Carter et al., 1984). Reduced sensitivity of these cells may be due in part to the ability of HSV to inhibit MHC class I and minor histocompatibility antigen expression (Jennings et al., 1985; Kuzushima et al., 1990). Another factor related to the decreased susceptibility of HSV-2 infected cells to CTL-mediated lysis is the reduced CTL precursor frequency found *in vivo* using a mouse model (Jennings et al., 1985). An unidentified soluble factor elaborated into supernatants of HSV infected mouse spleen cells inhibited induction of CTL *in vitro* and may represent another mechanism of protection against CTL (Horohov et al., 1985).

HSV-specific. MHC class I restricted CTL responses have been demonstrated in humans following *in vitro* incubation of PBMC with HSV antigen (Yasukawa et al., 1983; Sethi et al., 1980; Torpey et al., 1989). CD4+ T lymphocytes have also been reported to have cytolytic function against HSV (Torpey et al., 1989; Schmid, 1988) and Yasukawa et al (1990) confirmed that there are more CD4+ CTL precursors than CD8+ CTL precursors suggesting that CD4+ cells are the dominant CTL in the human response to infection. Both these CTLs may function to decrease viral titers at the lesion site and limit local spread; however, there is very litle information on CTL activity during natural infection of HSV in humans. The frequent isolation of HHV-6 from patients with cell-mediated immune deficiencies provides evidence that cellular immunity is important in protection against and recovery from HHV-6 infection. There is virtually no knowledge of the CTL responses elicited by HHV-6 *in vivo* except that CD4+ T cell clones recognizing HHV-6 antigens have been raised from peripheral blood of healthy seropositive adults (Yakushijin et al., 1992; Yasukawa et al., 1993).

1.6.3. Monocyte/Macrophage

Decreased antigen-presenting function has been described in HSV-1 infections and this was associated with a decrease in IL-1 production (Enk et al., 1991; Hayward et al., 1993). *In vitro* CMV infection of monocytes has been shown to result in decreased phagocytosis, oxidative metabolism and production of inflammatory mediators (Rouse and Horohov, 1986). Suppressive effects on monocyte/macrophage function have also been observed in HHV-6 infection. Infection of normal human bone marrow with HHV-6

resulted in suppression of growth-factor-induced outgrowth of macrophages (Burd et al., 1993). These authors also described a suppression of the respiratory burst response of blood monocytes (Burd and Carrigan, 1993). Suppression of monocyte/macrophage function could compromise an important effector cell required early in infection and exacerbate any underlying immunodeficiency.

I.7. Actions and Roles of Cytokines in Virus Infection

The immune system is regulated by a complex network of pleiotropic and redundant cytokines, which are secreted at varying degrees even when the system is apparently quiescent, and further expressed to varying degrees in response to antigen. Viruses that directly infect cells of the immune system, like HHV-6, may be important and persistent sources of immune activation or immune suppression. This activation, or lack of it, may partly be linked to cytokine secretion.

The cytokine responses required to support appropriate defence mechanisms are currently being extensively researched. These studies try to link particular cytokine profiles with induction of different protective components of the immune response. For the purpose of this introduction, the discussion of cytokines will be focused primarily on those affecting cells involved in cell-mediated immune responses, particularly T cells, in the context of a virus infection. The discussion of these cytokines will be based on their function as mediators of either natural immunity, lymphocyte activation or immune regulation.

I.7.1. Mediators of Natural Immunity (α/βIFN, TNFα, IL-1β, IL-6)

Natural immunity plays a crucial role in host protection during acute primary viral infections, such that its suppression can have profound effects on the ability of the host to control disease progression. This is especially relevant in those illnesses or conditions, such as in AIDS or post-transplant immunosuppressive therapy, for which there is already an underlying acquired or induced immunodeficiency. The principal mechanisms of natural immunity against virus infection are the activation of NK cells and the induction of type I IFN and proinflammatory cytokines (IL-1, IL-6 and TNFα) from infected cells.

Type I IFNs include leukocyte-derived αIFN and fibroblast-derived βIFN. They cause cells to synthesize enzymes that interfere with replication of viral RNA or DNA. Virally infected cells secrete IFN to protect neighboring cells from viral infection. Type I IFNs also activate NK cells, resulting in increased NK cytotoxicity and NK-cell proliferation (Trinchieri, 1989). The major function of NK cells is to kill virally infected cells before specific immune responses have developed, therefore it is timely that virus infection induces a cytokine needed to boost the ability of NK cells to lyse infected target cells. Another function ascribed to Type I IFN is the upregulation of MHC class I molecule expression. Most CTLs recognize foreign antigens bound to class I MHC molecules and by increasing the expression of MHC class I molecules, Type I IFN boosts the effector phase of cell-mediated immune responses by enhancing the efficiency of CTL-mediated killing. The activities of type I IFN all act concurrently to help eradicate viral infections. αIFN has been shown to be elevated in plasma from exanthem subitum patients

during the acute phase of the ailment accompanied by enhanced NK activity (Kikuta et al., 1990; Takahashi et al., 1992).

TNF plays a role in the response to various infectious organisms and is the principal mediator of the host response to gram-negative bacteria, specifically to lipopolysaccharide (LPS) molecules derived from the bacterial cell wall. The major cellular source of TNF is the LPS-activated mononuclear phagocyte, although antigen-stimulated T cells, activated NK and mast cells can also secrete this protein. Many TNF responses involve increased rates of transcription of particular target genes, often through activation of NF-κB or AP-1 transcription factors. TNF stimulates mononuclear phagocytes and other cell types to produce cytokines, including IL-1, IL-6, itself and chemokines. TNF shares many nonimmunological effects with IL-1 and IL-6, including induction of acute phase proteins, activation of the coagulation system, suppression of bone marrow stem cell division, and cachexia. TNF exerts an interferon-like protective effect against viruses and augments expression of class I MHC molecules (Israel et al., 1989) thus potentiating CTL-mediated lysis of virally infected cells. In addition to its inhibition of the replication of various viruses (e.g. VSV, adenovirus), TNF can enhance the replication of some viruses. Most notably is the activation of HIV expression induced by TNF, sometimes in concert with other cytokines (Folks et al., 1989; Rosenberg and Fauci, 1990). The mechanism responsible for HIV activation was demonstrated to be the induction of NF-κB family proteins that interact with recognition sequences in the HIV LTR (Osborn et al., 1989). This finding coupled with the analysis of TNF production in HIV-infected patients,

led to the widely accepted belief that TNF and related cytokines play an important role in the progression of HIV disease (Rosenberg and Fauci, 1990).

IL-1 shares overlapping biological properties with TNF and IL-6. IL-1, like TNF and IL-6, has the ability to stimulate T and B lymphocytes, to augment cell proliferation and to initiate or suppress gene expression for several proteins (Dinarello, 1992). Circulating levels of IL-1 are elevated in a variety of clinical situations and, together with similarly elevated levels of TNF and IL-6 correlate with the severity of some diseases, suggesting that these cytokines participate in the host response to or in the development of disease. The principal function of IL-1, similar to TNF, is that of mediator of the host inflammatory response in natural immunity. The major cellular source of IL-1, like that of TNF, is the LPS-activated mononuclear phagocyte. The two forms of IL-1 (IL-1α and β) are products of two different genes but bind to the same cell surface receptors and their biological activities are similar. Both IL-1 polypeptides are synthesized as approximately 33 kD precursors that are proteolytically cleaved to generate the mature 17 kD proteins. The 33 kD IL-1α precursor is biologically active and may represent the "membrane bound" form of IL-1 (Kurt-Jones et al., 1985). IL-1β must be processed to the 17 kD form before it can exert biologic effects. This is mediated by an IL-1-specific protease, IL-1β-converting enzyme (ICE) (Cerretti et al., 1992; Thornberry et al., 1992). Most of the IL-1 activity found in the circulation is IL-1β (Demczuk et al., 1987). Many IL-1-induced transcriptional effects, like those of TNF, involve NF-κB (Shirakawa and Mizel, 1989) and AP-1 (Muegge et al., 1989). IL-1 has many nonimmunological effects but does participate

in T and B cell activation. IL-1 amplifies T cell activation by inducing IL-2 and IL-2R gene expression.

IL-6 is a multifunctional cytokine that is produced by both lymphoid and nonlymphoid cells and regulates immune responses, acute-phase reactions and haematopoiesis (Hirano and Kishimoto, 1990; Sehgal et al., 1989). IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, some activated T cells in response to IL-1 and to a lesser extent, TNF. The two best described targets of IL-6 are hepatocytes and B cells. IL-6 causes hepatocytes to synthesize plasma proteins that contribute to the acute phase response. IL-6 also serves as a growth factor for activated, differentiated B cells. IL-6 has been demonstrated to be a paracrine or autocrine growth factor for EBV-immortalized B cells, resulting in increased immunoglobulin production and B cell immortalization. IL-6 is involved in T cell activation, growth and differentiation (reviewed in Van Snick, 1990) in part mediated by endogenously produced IL-2 leading to IL-2Rα expression (Noma et al., 1987; Garman et al., 1987). IL-6 also induces the differentiation of CTLs in the presence of IL-2 (Takai et al., 1988; Okada et al., 1988; Smyth et al., 1990).

1.7.2. Mediators of Lymphocyte Activation (IL-2, IL-15).

The induction of cytokine production in response to antigen recognition is a hallmark of T cell activation. Activated T lymphocytes produce numerous cytokines required to activate effector cells against the invading pathogen. IL-2, originally called T cell growth factor (TCGF), is the principal cytokine responsible for this activation leading

to the progression of T lymphocytes from the G_1 to S phase of the cell cycle (Stern and Smith, 1986). IL-2 is produced by CD4+ T cells, and in lesser quantities by CD8+ T cells. IL-2 acts on the same cells that produce it, i.e. in an autocrine manner, and on nearby T lymphocytes in a paracrine manner. Normally, IL-2 is transcribed, synthesized, and secreted by T cells only upon activation by antigens.

Originally, IL-2 was shown to promote proliferation of T cells in culture and allow long-term growth and responsiveness of antigen-specific CTL clones (Morgan et al., 1976; Gillis and Smith, 1977). Subsequently, IL-2 was shown to induce its receptor on T cells (Depper et al., 1985; Smith and Cantrell, 1985), activate CTLs (Watson and Mochizuki, 1980) and induce growth and activation of B cells into antibody-secreting cells (Blackman et al., 1986). IL-2 has a wide range of effects upon many cell types that express the IL-2 receptor including NK and monocytes (O'Garra et al., 1988). It is therefore not surprising that the inability to produce IL-2 can lead to profound immune dysfunction. This is clearly demonstrated by one of severe combined immune deficiency (SCID) in which the genetic defect affects IL-2 production (DiSanto et al., 1990; Weinberg and Parkman, 1990). Treatment of some of these patients with recombinant IL-2 restored immune function (Pahwa et al., 1989).

The action of IL-2 on T cells is mediated by binding to IL-2 receptor proteins (reviewed by Karnitz and Abraham, 1996). Two distinct cell surface proteins on T cells bind IL-2. The first to be identified, called IL-2Rα or CD25, is a 55 kD polypeptide that appears upon T cell activation (Leonard et al., 1984). Binding of IL-2 to cells expressing only IL-2Rα (the low-affinity receptor) does not lead to any detectable biologic response.

The second IL-2 binding protein, IL-2Rβ, is about 70-75 kD (Hatakeyama et al., 1989). IL-2Rβ is expressed with a 64 kD polypeptide, IL-2Rγ (Takeshita et al., 1992). Co-expression of IL-2Rβγ constitutes the intermediate-affinity receptor for IL-2 and causes growth of cells. Expression of all three receptors, IL-2Rαβγ, results in binding of IL-2 with high affinity. IL-2 binding and growth stimulation can be blocked by antibodies to either α or β IL-2R subunit. Resting T cells express only IL-2Rβγ. T cell activation by antigen leads to IL-2Rα expression, thereby increasing the affinity of IL-2Rβγ and allowing growth stimulation. IL-2 produced by the activated T cell further increases IL-2Rα expression and stimulates IL-2 synthesis, thereby providing a positive amplification system.

IL-15 is a recently discovered cytokine that shares many of the biological activities associated with IL-2 (Grabstein et al., 1994; Burton et al., 1994). It was initially isolated from the supernatant of a simian kidney epithelial cell line which supported the proliferation of the IL-2-dependent cell line CTLL (Grabstein et al., 1994). It was simultaneously described by Burton et al (1994) that the adult T cell leukemia cell line, Hut-102, secretes a lymphokine that stimulates T cell proliferation and induces activation of large granular lymphocytes into LAK cells. It was discovered that IL-15 interacts with the β and γ subunits but not with the α-chain of the IL-2 receptor (Giri et al., 1994). The β and γ subunits are required for ligand internalization and signal transduction. The equivalant of an α-chain specific for IL-15 has recently been cloned and designated as IL-15Rα (Giri et al., 1995b). The fact that IL-15 has a different receptor component suggests that IL-15 has biological activities not shared by IL-2.

Although IL-15 mRNA is expressed in several human tissues such as placenta, skeletal muscle, heart, lung, liver, kidney, as well as LPS-activated monocytes (Grabstein et al., 1994), the active protein has not yet been detected in supernatants of most cells that express RNA for this cytokine. In contrast to most other cytokines, the synthesis and secretion of IL-15 may be controlled predominantly by regulation at the level of translation or post-translation rather than at the transcriptional level.

The principal biological activities of IL-15 include stimulation of the proliferation of activated CD4+ and CD8+ T cell subsets (Grabstein et al., 1994), proliferation and differentiation of B cells (Armitage et al., 1995) and proliferation of NK cells and induction of cytolytic effector cells including CTL and LAK cells (Carson et al., 1994, Grabstein et al., 1994). Exogenous IL-15 was also found to augment T cell proliferative response to PHA, tetanus toxoid and HIV peptides in HIV positive patients (Seder et al., 1995).

I.7.3. Immunoregulatory Cytokines (IL-10, IL-12, γIFN)

Patterns of cytokine secretion by CD4+ T helper (Th) subsets, Th1 and Th2, have been linked to a polarization of the cell mediated and humoral immune responses (Romagnani, 1995). The Th1 subset is characterized by secretion of IL-2 and γIFN which provide minimal help for humoral responses but strongly activate cell-mediated immunity involving macrophages, granulocytes, and cytotoxic T cells. In contrast, the Th2 subset, which secretes interleukins-4, -5, -6, -9, -10, and -13, is reported to stimulate antibody production and modulate or inhibit cell-mediated responsiveness. These two types of

responses appear to be mutually exclusive, due to the observed cross-regulatory patterns in which the Th2 cytokines IL-4 and IL-10 inhibit the differentiation and cytokine synthesis of Th1 cells, and γIFN secreted by the Th1 subset inhibits the proliferation of Th2 clones (Mosmann and Moore, 1991). IL-12 acts on precursor Th cells to differentiate into Th1 type secreting T cells which support cellular immune responses (Manetti et al., 1993; 1994). These Th cytokine responses have best been identified in experimental bacterial and parasitic infections and are well defined in the mouse system but less so in humans. As different cells are involved in viral infections, it is likely that cytokine responses to viral infections will not directly parallel responses to other pathogens.

"Cytokine synthesis inhibitory factor" or IL-10 was initially described as a factor produced by Th2 clones which specifically inhibited cytokine synthesis by Th1 clones (Howard and O'Garra, 1992). Subsequent studies have shown IL-10 to be an immunosuppressive agent which inhibits the lymphocyte cytokine production from CD8+ cells (Yssel et al., 1992; Hisatsune et al., 1994) and NK cells (Mosmann, 1994). IL-10 has been shown to inhibit the activation of NF-κB (Wang et al., 1995), an important transcription factor which promotes the transcription of many cytokine and cellular activation genes. Furthermore, IL-10 plays a role in the development of immune unresponsiveness by regulating the expression of the costimulatory molecules such as B7 (Ding et al., 1993; Chang et al., 1995) and MHC Class II expression, thereby inhibiting the antigen-presenting capacity of monocytes (de Waal Malefyt et al., 1991). It has also been shown to have a direct effect on CD4+ T cells by suppressing IL-2 secretion (Taga and Tosato, 1992). IL-10 has been shown to inhibit HIV replication in acutely infected

monocyte/macrophages by blocking secretion of the HIV-inducing cytokines TNFα and IL-6 (Weissman et al., 1994). In contrast, IL-10 demonstrates immunostimulatory activity for mast cells (Thompson-Snipes et al., 1991), cytokine-activated T cells (Suda et al., 1990; MacNeil et al., 1990), and activated B cells (Rousset et al., 1992). IL-10 inhibits the antigen-driven activity of both Th1 and Th2 subsets (Del Prete et al., 1993; de Waal Malefyt et al., 1991). IL-10 produced by different cell types is differentially regulated, with IL-12 and IL-6 inducing IL-10 production by T cells and TNFα enhancing IL-10 production from monocytes (Daftarian et al., 1996).

IL-10 bears strong DNA and protein similarities to open-reading frames found within two herpesviruses: the BCRF1 locus in EBV (Moore et al., 1990) and ORF10 in equine herpesvirus, type 2 (Rode et al., 1993). It has been suggested that the viral IL-10 genes were originally acquired from mammalian sources. The protein encoded by the viral IL-10 gene is biologically active and has many of the activities of its cellular counterpart (Moore et al., 1993). Viral IL-10 is thought to aid the pathogenicity of EBV in two ways: by suppressing the production of immunostimulatory cytokines such as IL-2 and by acting as a B cell growth factor to promote growth and EBV cellular transformation (Miyazaki et al., 1993).

IL-12, originally known as natural killer stimulatory factor, is a heterodimeric cytokine composed of two covalently linked chains, a heavy chain (p40) and a light chain (p35). IL-12 biological activity has been demonstrated to only be associated with the p75 heterodimer. The major target cells for IL-12 biological functions are T cells and NK cells for which IL-12 augments cytolytic activity, enhances proliferation and induces γIFN

production (Kobayashi et al., 1989; Perussia et al., 1992; Gately et al., 1992; Hsieh et al., 1993). Phagocytic cells (monocytes, macrophages and dendritic cells) appear to be the primary producers of IL-12 (D'Andrea et al., 1992). IL-10 can inhibit IL-12 production by phagocytic cells which in turn inhibits γIFN production by T lymphocytes (D'Andrea et al., 1993). IL-12 is considered to be the link between the nonspecific immune response and adaptive immunity by its ability to induce γIFN production from Th1 and NK cells. The IL-12 induced γIFN and other cytokines then act on phagocytic cells potentiating their bacteriocidal activity.

In viral infections, exogenously added IL-12 has been shown to have a protective effect in LCMV and MCMV infection of mice, depending on the dose. Low doses enhance CD8+ T cell responses, while high doses induce circulating levels of γIFN and TNF leading to immunotoxicity and increased viral replication (Orange et al., 1994). In a model of encephalomyocarditis (EMC) virus infection, a single injection of IL-12 prior to lethal injection of virus protected mice (Ozmen et al., 1995). IL-12 exerted its protective effect through the induction of γIFN as mice lacking the γIFN receptor gene were not protected by IL-12. Adding exogenous IL-12 to PBMC from HIV infected individuals restores *in vitro* T cell proliferation and IL-2 production in response to influenza and HIV antigens as well as to PHA-induced γIFN production (Clerici et al., 1993). As IL-12 increases the mediators of defence against viral infection, i.e. γIFN, activated NK cells and activated T cells, it may have an immunoregulatory role during viral infections; however, there is no definitive evidence that IL-12 has any intrinsic anti-viral properties (Biron, 1994).

γIFN is the sole representative of the Type II interferons. It is a 34 kDa glycoprotein that is well conserved among animal species. In humans, it is apparently produced exclusively by NK cells and T cells in response to mitogenic or antigenic stimulation (DeMaeyer and DeMaeyer-Guignard, 1991). Recent studies suggest that the T cells expressing the activation-dependent CD30 membrane antigen are responsible for the majority of γIFN production by T cells following stimulation with IL-12 (Alzona et al., 1994). The immunoregulatory activity of γIFN is particularly crucial in the area of T helper cell development in mice. γIFN secreted by the Th1 subset inhibits the proliferation of Th2 clones. Through its enhancement of various immune effector functions, γIFN provides a first line of anti-viral defense. Effects of γIFN on immune cells are numerous and include: induction/augmentation of MHC class I and II antigens on macrophages (Skoskiewicz et al., 1985; Momberg et al., 1986) and T and B cells (Wong et al., 1983, Capobianchi et al., 1995), induction of B cell proliferation, differentiation, and secretion (Sidman et al., 1984; Brunswick and Lake, 1985; Huang et al., 1993), and induction of cytolytic activity in LAK and CTL cells (Siegel, 1988; Giovarelli et al., 1988). γIFN also inhibits IL-10 production by monocytes (Chomarat et al., 1993). γIFN has been identified as a mediator of suppression of immune responses. Graft-versus-Host disease is associated with suppression of cellular immune responses, as evident from reduced proliferative responses of lymphocytes to mitogens. Using anti-γIFN neutralizing antibodies can relieve the suppression (Wall et al., 1988; Huchet et al., 1993). Recently, mice with a targeted disruption of the γIFN (Dalton et al., 1993; Graham et al., 1993) and γIFN receptor gene (Huang et al., 1993) have been generated and used for analysis of susceptibility to virus

infections. Mice lacking the γIFN receptor were shown to have a defect in resistance to vaccinia virus resulting in increased vaccinia titers, but no significant change in their susceptibility to other viruses; therefore, γIFN may participate in defence against some but not all viral infections (Huang et al., 1993). NK cell and CTL responses to either vaccinia virus or LCMV were normal in γIFN receptor negative mice indicating that γIFN may mediate anti-viral effects against certain viruses *in vivo* but that γIFN signals are not required for the activation of cytotoxic effector cell functions.

I.8. Viral Modulation of Host Defence Mechanisms

Herpesviruses have evolved various mechanisms of evading the immune system. They encode genes that are homologous to host immune modulators that could be implicated in viral evasion of the host immune response (Spriggs, 1996; Davis-Poynter and Farrell, 1996). Apoptosis is a key element of a host's defence against viral infection by inhibiting viral spread and persistence (reviewed in Shen and Shenk, 1995). Several herpesviruses encode genes that antagonize apoptosis, namely, γ34.5 of HSV (Chou and Roizman, 1994), CMV's immediate-early 1 and 2 proteins (Zhu et al., 1995), and EBV's BHRF1 and LMP-1 (Henderson et al., 1993; 1991). HSV-1 may also evade the immune system by expressing two types of IgG Fc receptors on the cell surface. One, a complex of gE and gI (Johnson et al., 1988), binds monomeric IgG, while the other, consisting of gE alone, binds polymeric IgG (Dubin et al., 1991). The Fc receptors appear to play a role in protecting infected cells or virions from the host antibody response. Another component

of the immune system known to interact with HSV proteins is complement. C3b has been shown to bind to both gC1 and gC2 (McNearny et al., 1987). The HSV α47 gene (ICP47 protein) causes a resistance to lysis by CD8+ CTLs due to retention of the MHC class I molecules in the cytoplasm (York et al., 1994). ICP47 associates with the TAP1/TAP2 complex resulting in inhibition of transport and association of peptide with MHC class I (Fruh et al., 1995; Hill et al., 1995). Loci found in HCMV have also been shown to be responsible for decreased MHC class I expression (Ahn et al., 1996; Jones et al., 1995, 1996; Wiertz et al., 1996a,b).

In contrast to CD8+ T cells, NK cells are inhibited, rather than activated by target cell expression of MHC class I (Karre, 1995; Gumperz and Parham, 1995). Expression of low levels of MHC class I is therefore often associated with increased susceptibility to NK cell-mediated lysis. Recently, the murine CMV MHC class I homologue, m144, was shown to mimic cellular MHC class I and inhibit the host NK cell response *in vivo* (Farrell et al., 1997). Similarly, a human CMV gene product, UL18 (also a MHC homologue) was shown to aid transfected cells evade destruction by NK cells (Reyburn et al., 1997). The viral MHC homologues contribute to immune evasion through interference with NK cell-mediated clearance. Herpesviruses use these DNA-encoded products to counteract the host's immune response and to ensure survival of the infected cell and consequently the virus itself.

Some herpesviruses have also developed elaborate forms of molecular mimicry whose only purpose appears to be to circumvent cytokine-mediated functions in the host defences. This ability is based on the acquisition of genes for cytokines or cytokine

receptors from host cells, allowing viruses to direct the synthesis of proteins that either neutralize cytokines or inhibit their synthesis. Examples include the EBV BCRF-1 protein which is highly homologous to human IL-10 (Moore et al., 1990), the M-T7 gene in myxoma virus that codes for a soluble γIFN receptor (Upton et al., 1992), poxvirus genes T2 and B15R which encode soluble cytokine receptors specific for TNF and IL-1, respectively (Smith et al., 1991; Alcami and Smith, 1992; Spriggs et al., 1992), and cowpox virus which encodes the proteinase inhibitor, crmA, which specifically blocks the enzyme, ICE, required to generate the biologically active form of IL-1β (Ray et al., 1992).

I.9. Anergy

T cell activation results from a series of intracellular signals involving tyrosine kinase activation, membrane phospholipid breakdown, elevated protein kinase C activity, and rises in cytoplasmic calcium. These signals stimulate the transcription of genes required for the proliferative and effector responses of the T cell. Many of these genes increase following activation of T lymphocytes, including those which encode for cytokines and their respective receptors and cellular transcription factors. A lack of any of these elements can result in T cell anergy.

Anergy can be described as functional cell inactivation without cell death which is potentially reversible. In T cells, the biochemical mechanisms leading to the induction of anergy are only partially understood. Lack of costimulator molecules (B7-CD28) have been shown to induce anergy. T cells survive but are rendered incapable of responding to the antigen. Anergic T cells fail to secrete IL-2 and, therefore, to proliferate in response to

antigen stimulation. The block of IL-2 transcription in anergic T cells may be due to a failure to activate nuclear factors that bind to the IL-2 promoter and stimulate transcription (Kang et al., 1992; Go and Miller, 1992; Sundstedt et al., 1996). In some experimental models, anergy can usually be prevented if IL-2 is provided exogenously (Timon et al., 1993; Perez-Blas et al., 1992; Yoo et al., 1996). Other explanations of virus induced anergy include viral protein(s) binding to or perturbing a specific cell receptor that is necessary for activation of lymphocytes (Linette et al., 1988; Timon et al., 1993; Perez-Blas et al., 1992). Instead of activation, virus infection may induce apoptosis rather than proliferation, as shown for HIV (Groux et al., 1992). Alternatively, viral protein(s) could trigger inhibitory signals in T cells or could activate the secretion of factors from lymphocytes or monocytes that can inhibit lymphoproliferation (Groux et al., 1996). It is possible that some form of suppression is mediated by cytokines.

The mechanism(s) of HHV-6-induced inhibition of cell proliferation remains unknown. As noted, various members of the herpesvirus family are noted for their immunosuppressive abilities (Rinaldo and Torpey, 1993). Depending upon the particular herpesvirus, immune cell hypo-responsiveness or anergy may involve the expression of immunosuppressive cytokines such as viral or cellularly encoded IL-10 for EBV, or transforming growth factor β (TGFβ) as is the case with CMV (Michelson et al., 1994). Several studies have suggested that a virion component (Horvat et al., 1993) or a viral-induced factor (Burd and Carrigan, 1993) is responsible. To date, the nature or origin of the factor(s) involved in immunosuppression by HHV-6 has not been described. The nature of HHV-6 induced immunosuppression remains unresolved.

I.10. Statement of Objectives

Although HHV-6 has previously been shown to induce several cytokines from PBMC, including IFN-α, IL-1β and TNF-α, a comprehensive study of the *in vitro* cytokine response of HHV-6's principal target cell (i.e. the T-lymphocyte) following infection, as well as the role these cytokines play in the resolution of the virus infection and/or promotion of T-cell anergy has not been investigated. Such information would contribute not only to our understanding of HHV-6 and T-cell interaction, as well as T-cell anergy induced by HHV-6, but may also shed light on how the immune system resolves HHV-6 infection, even when confronted with the strong immunosuppressive capability of HHV-6.

The aim of this thesis was (I) to determine the cytokine repertoire expressed by T cells following HHV-6 infection, (II) to examine the cellular pathways which are involved in T-cell anergy induced by HHV-6 and (III) to characterize the suppressor factor(s) associated with the induction of anergy.

II. MATERIALS AND METHODS

II.1. Cell culture

PBMC, purified T cells, HSB-2 cells (CCL120.1, American Type Culture Collection, Rockville, MD), Jurkat E6-1(TIB 152, ATCC), H9 (HTB 176, ATCC), Hut-78 (TIB 161, ATCC) and K562 cells (CCL 243, ATCC) were maintained at 37°C in a 5% $C0_2$ humidified incubator in Iscove's Modified Dulbecco Medium (IMDM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS, Immunocorp Sciences, Montreal, Que. Canada), 50 U/ml penicillin and 2 μg/ml gentamicin.

II.2. Virus stock preparation

HHV-6 viral stocks were produced by inoculating 10 ml of logarithmically growing HSB-2 cells ($1x10^6$/ml) with 0.5 ml HHV-6 containing culture supernatant (GS strain, NIH AIDS Research and Reference program, Rockville, MD), and incubating the mixture for 3 hours at 37°C. After the initial 3 hour incubation, cells were diluted to $5x10^5$ cells/ml and further cultured for 4 or 5 days. The infected cells were removed by centrifugation and the virus pelleted from the culture supernatant by centrifugation at 15,000 x g for 3 hours at 4°C. The virus pellet was resuspended in 1:100th volume of 10% FBS/IMDM (100X viral stock), and stored at -80°C until use. Mock-HHV-6 was obtained using an identical procedure to that for isolating virus, but was derived from uninfected HSB2 cells.

II.3. UV Irradiation of Virus

HHV-6 100X stock was UV-irradiated by a 30 minute treatment under a laminar flow hood germicidal lamp placed 60 cm above the HHV-6 sample (Tanner et al. 1996). UV-treated virus expressed less than 98% of its original infectivity, as determined by positive reactivity to anti-HHV-6 monoclonal antibody cell immunofluoresence on freshly infected HSB-2 indicator cells. Virus stocks contained $2x10^8$ infectious virions/ml, as determined by anti-HHV-6 monoclonals 2E2, H-AR8, H-AR4 and P14C5 using fixed-cell immunofluorescence on serially diluted virus and HSB-2 indicator cells as described below (II.5.).

II.4. Psoralen Inactivation of Virus

HHV-6 100X virus stock was inactivated by psoralen cross-linking by initially mixing 50 µl virus stock (representing $5x10^6$ infectious particles) with 0.26 ng of psoralen (Sigma) and then subjecting the mixture to psoralen cross-linking using a long-wave (365nm) UV lamp (Spectroline, Westbury, NY) placed 3 cm above the virus sample for 30 minutes. The virus mixture was kept on ice during this time under sterile conditions in a laminar flow hood.

II.5. Immunofluorescence

HHV-6 infected or mock infected cells were washed twice with PBS (Sigma) and adjusted to 10^6/ml and 5 µl of this suspension was spotted per slide well (corresponding to 5000 cells/well). The slide was air-dried, and fixed overnight in methanol at -20°C. Before

staining, the slides were washed in PBS for 15 minutes at room temperature. All subsequent incubations and washings were performed at room temperature. Fixed cells were incubated with 10 µl of blocking buffer [PBS containing 5% FBS and 2% goat-anti-human α-macroglobulin serum (Cappel, Malvern, PA)] for 30 minutes in a humidified chamber. The slides were washed for 10 minutes by immersion in a coplin jar containing PBS. 10 µl of a 1:30 dilution of four anti-HHV-6 monoclonals, 2E2, H-AR8, H-AR4 and P14C5 (kind gift of Dr. D.V. Ablashi, Georgetown University, Washington, DC), were added per well and slides were incubated for 2 hours in a humidified chamber. The slides were washed 3 times with PBS. Slides were then incubated with 5 µl of a fluoroscein isothiocyanate (FITC) conjugated goat anti-mouse-IgG (Ortho Diagnostics, Raritan, NJ) for 45 minutes. The slides were again washed 3 times with PBS. The slides were mounted with a 1:1 ratio of glycerol to PBS and scored for positive fluorescence using a Zeiss Axiophat fluorescence microscope (Oberkochen, Germany).

II.6. Isolation of human T cells and monocytes

T cell isolation: Enriched peripheral blood T cells were obtained from healthy donors following separation on Ficoll-hypaque density gradients and 2-aminoethylisothiouronium (AET)-treated sheep red blood cell (SRBC) rosetting. AET-treated SRBC were prepared by washing 20 ml of SRBC (SRBC in Alsevers, PML microbiologicals, Tualatin, OR) with 30 ml PBS three-times. SRBC were pelleted by centrifugation at 2000 rpm for 10 minutes between washes. AET (Sigma) stock was prepared by dissolving 0.8 g of AET in 20 ml dH_20 and adjusting the pH to 8.4 with 10N NaOH. AET solution was filter sterilized using

a 0.2 μm filter (Gelman Sciences, Ann Arbor, MI). 12.5 ml of filtered AET solution was added to the washed SRBC and cells were incubated for 20-30 minutes at 37°C in a water bath. The cells were then washed 5-times with PBS as before and resuspended in IMDM to a final concentration of 2%. PBMC were obtained from healthy donors by centrifugation of whole blood in a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient. Following three PBS washes, the PBMC were adjusted to $1x10^7$ cells/ml in IMDM and mixed at a ratio of 2:1 (v/v) with freshly prepared 2% AET-SRBC. The cells were pelleted by a 10 minute centrifugation at 1000 x g and incubated overnight on ice. The rosetted cell pellet was gently resuspended and overlaid on 10 ml of Ficoll-Hypaque, and centrifuged at 2200 rpm for 30 minutes. The nonrosetted cells and the remaining Ficoll-Hypaque layer were aspirated off and the pelleted T cells were lysed with 2 ml of ACK red-blood-cell-lysing reagent (0.15M NH_4Cl, 1.0M $KHCO_3$, 0.1mM Na_2EDTA, pH 7.3). Upon complete hemolysis, T cells were washed twice with serum-free IMDM. The enriched T cell preparations contained on average: 84% ± 2.4 (SEM) CD3+ T cells, 9.5% ± 1.5 CD16+/CD56+ NK cells, 1.5 % ± 0.25 CD14+ monocytes, and 1.5% ± 1.5 CD19+ B cells, as determined by flow cytometric analysis.

Monocyte isolation: Monocytes were isolated by plastic adherence. T_{25} tissue culture flasks (Corning, Corning, NY) were coated with 5 ml of FBS by incubation for 1 hour at 37°C. The flasks were rinsed with IMDM and the non-rosetted fraction from a T cell isolation was added at $3x10^6$/ml. Cells were incubated for 1 hour at 37°C. The nonadherent cells were aspirated off and the flask washed thrice with PBS. Monocytes were detached by incubation with Versine (PBS; 1mM EDTA) on ice for 20 minutes with

frequent pipetting to dislodge the adherent cells. Monocytes were washed and adjusted to 10^6/ml.

II.7. Infection protocol

$2x10^6$ T cells in a 1 ml volume were inoculated with $3x10^6$ infectious virus particles or an equal volume of mock preparation, and incubated for 2-3 hours on ice with frequent mixing. Cells were then washed twice with complete medium to remove nonadsorbed virus and resuspended to $2x10^6$/ml in complete medium for subsequent culture in 24-well plates (Becton Dickinson Labware, Lincoln Park, NJ) or in 200 µl of complete medium at $5x10^5$/ml for culture in 96-well round bottom plates (Becton-Dickinson Labware). For T cells that were PHA-stimulated and infected, cells ($2x10^6$/ml) were first cultured in the presence of PHA (1:200 v/v) for 5 hours before adding virus stock at the above concentrations. Cells were not washed after addition of virus, hence cells were exposed to PHA and virus during the whole culture period. For some experiments, cells were first infected, washed, then cultured in the presence of PHA (1:200 v/v).

II.8. Cytokine mRNA analysis by reverse transcriptase (RT)-Polymerase Chain Reaction (PCR)

Cytokine mRNA was measured by RT-PCR. Cells were seeded in 1 ml of complete medium at $2x10^6$/ml in 24-well plates. Total RNA was harvested at 24, 48 and 72 hours post-infection. Briefly, total RNA was isolated by lysing cells in TRI Reagent (MRC, Cincinnati, OH) followed by isopropanol precipitation of the upper aqueous phase as

recommended by the manufacturer. RNA was then pelleted by centrifugation, washed with 75% ethanol, air-dried and resuspended in DEPC-treated water to a final concentration of 1 μg/μl. Resuspended RNA was stored at -80°C until use. For cytokine RT-PCR analysis, all reagents, unless otherwise stated, were obtained from Perkin-Elmer (Norwalk, CT). Reverse transcription was performed in a total volume of 20 μl and contained 2 μg total RNA, 1.25 μM of random hexamer, 1.25 μM oligo dT, 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM each of the four dNTPs, 1 U of RNase inhibitor and 2.5 U of Moloney Murine Leukemia Virus reverse transcriptase. Reactions were processed for 30 minutes at 42°C followed by heating at 99°C for 5 minutes to inactivate the enzymes. PCR was performed using 2 μl of the RT reaction product in a 50 μl sample volume using a Perkin Elmer 480 Thermocycler. Primers for IL-4, IL-5, IL-8, IL-10, and IFN-γ were obtained from Stratagene (La Jolla, CA) generating 456, 295, 225, 204 and 501 bp PCR products respectively. All other cytokine primers used were synthesized (National Biosciences Inc., Plymouth, MN) and were based on previously reported cDNA sequences (see Table I for list of primers). The PCR amplification conditions for the Stratagene primers were 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 0.2 mM each of the four dNTPs. The Stratagene primers were used at a final concentration of 1 μM. The thermocycling conditions began with a 5 minute denaturation step at 94°C, followed by a 5 minute annealing step at 60°C, followed by 35 cycles each consisting of 1.5 minutes at 72°C, 45 seconds at 94°C, and 45 seconds at 60°C. After the 35th cycle a final extension step for 10 minutes at 72°C was performed. Thermocycling conditions for IL-1β, -2, -3, -

6, -7, -9, -15, TNF-α, TGF-β and β-actin were identical to the Stratagene primer protocol, with the substitution of a denaturation temperature at 94°C for 1 minute, a 55°C annealing temperature for 1 minute, and a 2 minute extension at 72°C. IL-11 primers were used at a 1.2 μM final concentration, and thermocycler conditions were as outlined by Suen et al (1994). IL-12 (p35) primers were used at 0.4 μM with 5 mM $MgCl_2$, and thermocycling conditions were as described by Gubler et al (1991). IL-13 PCR primers were used at 1.2 μM, and the 35 cycles each consisted of a 40 second step at 94°C, a 1.5 minute step at 65°C, and a 2 minute step at 72°C (Wickenhouser et al, 1995). IL-14 RT-PCR was performed as described by Ford et al (1995). TNF-β or lymphotoxin (LT) RT-PCR was performed as described by Yamamura et al (1991). PCR reactions were resolved on 1-2% agarose gels containing ethidium bromide (0.2 μg/ml), photographed, and the intensity of the PCR product was determined by laser densitometry using an Imaging Densitometer, Model GS-670 and Molecular Analyst Software (Bio-Rad, Richmond, CA). The cytokine mRNA levels were measured in a semi-quantitative manner. For each sample, the amount of cytokine mRNAs was quantitated relative to the respective level of β-actin mRNA (Kawasaki, 1990). The relative cytokine mRNA induction was calculated by comparing this ratio to mock-treated cells using the following formula: (OD of cytokine mRNA transcript of the sample/OD of β-actin transcripts of the sample)/(OD of cytokine mRNA transcripts of mock-treated cells/OD of β-actin mRNA transcripts of mock treated cells) (Murphy et al., 1990).

TABLE I
Oligonucleotide primers used for RT-PCR

Cytokine	Sequence (5'-3')	gel size (bp)	ref.
IL-1β	ATGGCAGAAGTACCTGAGCTC	540	Flamand
	TTCCTTGAGGCCCAAGGCCAC		et al., 1991
IL-2	ATGTACAGGATGCAACTCCTGTCTTG	462	Orschescek
	TCAAGTCAGTGTTGAGATGATGCTTTG		et al., 1994
IL-3	ATGAGCCGCCTGCCCGTCCTG	459	Orschescek
	CTAAAAGTACGCGAGGTCCAAAG		et al., 1994
IL-6	CAAAGAATCTAGATGCAATAA	201	Tanner
	GCCCATTAACAACAACAATCTG		et al., 1996
IL-7	ATGTTCCATGTTTCTTTTAGGTATATC	680	Orschescek
	TGCATTTCTCAAATGCCCTAATCCG		et al., 1994
IL-9	TCACCATGGTTCTGGCCATGGTCCTTACCTC	487	Orschescek
	ATAATATTTCATCTTCATATCTTCCCTCTCA		et al., 1994
IL-11	ACTGCTGCTGCTGAAGACTCGGCTGTG	322	Suen
	ATGGGGAAGAGCCAGGGCAGAAGTCTG		et al., 1994
IL-12	GACCCAGGAATGTTCCCATGC	501	Gubler
	ATGAAGAAGTATGCAGAGC		et al., 1991
IL-13	CAGGGATCCATGCATCCGCTCCTCAATCCTCTCCTGTTG	459	Wickenhouser
	GTAGGGATCCTCAGTTGAACCGTCCCTCGCGAAAAAGTT		et al., 1995
IL-14	TCCTGACCAGCACAT	1371	Ford
	AGCAAGGACCTACAACAGCAGCCATCC		et al., 1995
IL-15	AGGATTTACCGTGGCTTTGAG	568	Grabstein
	AAGCAGCAGAGTGATGTTCGT		et al., 1994
TNFα	CCCTCAAGCTGAGGGGCAGCTCCAG	412	Flamand
	GGGCAATGATCCCAAAGTAGACCTG		et al. 1991
TGFβ1	GCCCTGGACACCAACTATTGC	100	Yamamura
	GCTGCACTTGCAGGAGCGCAC		et al., 1991
LT	CCTCACACCTTCAGCTGCCC	350	Yamamura
	GAGAAACCATCCTGGAGGAA		et al., 1991
β-actin	CCTTCCTGGGCATGGAGTCCT	202	Tanner
	GGAGCAATGATCTTGATCTTC		et al., 1996

II.9. Cytokine protein or biological activity measurement

Cells were either mock-, HHV-6-infected or PHA-stimulated and cultured ($2x10^6$/ml) in 24-well plates. At 24, 48 and 72 hours post-infection supernatants were harvested, aliquoted and stored at -80°C until assayed for cytokine protein. IL-1β (R&D Systems), IL-12 (R&D Systems), TNF-α (R&D Systems) and IL-2 (Becton-Dickinson) protein levels in culture supernatants were measured by a sandwich ELISA following the manufacturer's recommendations. These assays were sensitive to 0.3 pg/ml, 5 pg/ml, 4.4 pg/ml, and 3 BRMP units/ml, respectively. IL-10 was measured essentially as described (Mossmann, 1992) using a sandwich ELISA with two different mAb which recognise two distinct epitopes. ELISA strips (Nunc Immunomodules, Denmark) were coated overnight at 4°C with 100 µl of a capture anti-human IL-10 monoclonal antibody 18551D (PharMingen, San Diego, CA) at a concentration of 4 µg/ml in coating buffer (0.1 M $NaHCO_3$, pH 8.2). The plates were washed twice with 300 µl 0.05%Tween-20 (Sigma)/PBS and blocked for 2 hours with 200 µl 10% FBS/PBS at room temperature. After washing twice with 0.05% Tween-20/PBS, 100 µl of IL-10 containing tissue culture supernatants and IL-10 standards were incubated for 4 hours at room temperature. After washing four times with with 0.05% Tween-20/PBS, captured IL-10 was detected by incubating with 100 µl of a biotin-labeled rat anti-human IL-10 18562D (4 µg/ml, PharMingen) diluted in 10% FBS/PBS. Plates were washed six times with 0.05% Tween-20/PBS, and samples were incubated with 100 µl streptavidin-peroxidase (Jackson ImmunoResearch, West Grove, PA) used at a final dilution of 1:1000. For color development, a 4 mg tablet of o-phenylenediamine dihydrochloride (OPD, Sigma) was

diluted in 10 ml 0.1M citrate buffer with 4 μl of 30% hydrogen peroxide (Sigma). 100 μl of this substrate were added to wells and the reaction allowed to develop at room temperature for 20 minutes after which samples were immediately read at 450 nm using a microplate reader (Bio-Rad). Recombinant human IL-10 (R&D systems) was used as a standard at a range of 1000 to 8.5 pg/ml. The sensitivity of the IL-10 ELISA was 16 pg/ml. For IFN-γ detection, a protocol identical to that for IL-10 ELISA was used, except that the primary capture antibody was the anti-human IFN-γ monoclonal M701 (Cedarlane, Hornby, ON) and the biotin-labeled secondary antibody, used at 3 μg/ml, was the anti-IFN-γ monoclonal AB 138 (The Binding Site, Birmingham, U.K.). Recombinant human IFN-γ was used as a standard (Endogen, Cambridge, MA). The sensitivity of the IFN-γ ELISA was 30 pg/ml. IL-15 protein from tissue culture supernatant was tested in three different ELISAs each coated with a different capture anti-IL-15 monoclonal antibody (H. Gaylord, R&D Systems, personal communication). After incubating samples and standards with the primary antibody, the wells were washed and incubated with a cocktail of secondary biotinylated anti-IL-15 monoclonal antibodies. After washing, samples were incubated with horseradish peroxidase-conjugated avidin. Samples were developed with 3,3',5,5'-tetramethylbenzidene (Sigma) and read at 450 nm. rIL-15 expressed in *E. coli* was used as standard (R&D Systems). The IL-15 ELISA was sensitive to 15 pg/ml. IL-15 was also measured by a commercially available ELISA (Genzyme Diagnositics, Cambridge, MA) which had a detection limit of 10 pg/ml.

IL-6 biologic activities in the culture supernatants were determined using the IL-6 dependent cell line 7TD1 (ATCC CRL 1851), as described (Tanner et al., 1990). 7TD1

cells ($2x10^3$/well of 96-well plate) were seeded in IMDM supplemented with 10% FBS and $5x10^{-5}$ M β-mercaptoethanol (Sigma) and cultured with serial dilutions of IL-6 containing supernatant for 72 hours at 37°C. During the final 4 hours of culture, 7TD1 cells were pulsed with 1μCi [^{3}H]thymidine and harvested with a TomTec cell harvester and assayed for [^{3}H]thymidine incorporation with a 1405 Microbeta plate liquid scintillation counter (Wallac, Turku, Finland). One unit of bioactivity in this assay was defined as the activity inducing half-maximal proliferation of 7TD1 cells. Recombinant IL-6 (R&D) was used as a positive standard.

II.10. Detection of HHV-6 DNA

HHV-6 DNA was detected using a sandwich capture molecular hybridization assay that utilizes colorimetric detection (Digene, Beltsville, MD). Briefly, an aliquot of a HHV-6 specific PCR reaction containing 5'-biotinylated products is hybridized with a specific single-stranded RNA probe. The resultant RNA:DNA hybrids are captured through biotin onto the surface of streptavidin-coated microwells. Immobilized hybrids are then reacted with an anti-hybrid antibody conjugated to alkaline phophatase and detected with a colorimetric substrate (*para*-nitrophenylphenol). Absorbance was read on a microplate reader (Bio-Rad) set at 405 nm. The intensity of the color generated is proportional to the amount of biotinylated PCR product in each reaction. Positive and negative assay controls and negative PCR controls were included in each run of the assay. Positive and negative assay controls consisted of DNA extracted from HHV-6 infected and uninfected HSB2 cells, respectively. DNA was extracted using TRI Reagent (MRC, Cincinnati, OH).

Negative PCR control consisted of a PCR reaction with no DNA template. Oligonucleotides were used as primers with the sequences 5` GTTCCAGGCGGCATGAATTC 3` (sense strand) and 5` biotin-ACACGGCCTCTCTACATCAC 3` (antisense strand). These primers amplify a 385 base pair amplicon and were selected from a region (first open reading frame) with no known sequence similarity to other herpesviruses (Drobyski et al., 1993b). PCR was performed in 100µl reaction mixtures containing 10 µl of genomic DNA, 200 µM (each) deoxynucleotide 5`triphosphate, 1 µM (each) primer, 50 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 250 mM KCl, 1% (wt/vol) gelatin. Samples were boiled for 3 minutes and then transferred to a 94°C heat block and 1 U of Taq DNA polymerase was added. Reactions consisted of 30 cycles of denaturation at 98°C for 15 seconds, annealing at 58°C for 20 seconds, and polymerization at 72°C for 20 seconds in a programmable thermocycler (Perkin-Elmer Corp., Norwalk, Conn.) followed by a 7 minute extension phase at 72°C. 5 µl of PCR sample was used in the assay. The RNA probe had a sequence of 5` GGACCATTTGGA 3` and was designed to bind to amplified DNA from both HHV-6A and B variants but did not bind to PCR-amplified DNA from HSV-1, HSV-2, CMV-, VZV or EBV infected cell cultures.

II.11. Flow cytometric analysis of cell surface markers

Cells ($0.5x10^6$) to be stained were washed with 0.1% sodium azide in PBS. The cell pellet was resuspended in 100 µl PBS. Monoclonal antibody directly conjugated with a fluorochrome was then added to the cells and allowed to incubate for 20-30 minutes on

ice. The cells were then washed twice in 0.1% sodium azide in PBS to remove unbound reagents, then resuspended in 1% paraformaldehyde and kept at 4°C in the dark until analyzed using a FACScan flow cytometer (Becton and Dickinson, Mountain View, CA). Negative control antibodies were IgG_1-fluorescein isothiocyanate (FITC) and IgG_{2a}-PE labeled murine monoclonal antibodies that react specifically with keyhole limpet hemocyanin (Becton Dickinson, San Jose, CA). The results were reported as the percentage of positive cells for each monoclonal antibody (displaying fluorescence intensities above the upper limit of a negative control) within the lymphocyte population that was electronically gated by size and complexity criteria.

II.12. Detection of apoptosis by cytofluorometry after Propidium Iodide (PI) staining

Apoptotic nuclei were monitored by flow cytometric analysis after (PI) staining in hypotonic buffer using a modified technique from Nicoletti et al. (1991). HSB-2 cells were either mock-, virus- or uv-irradiated virus-infected and put into culture for four days after which 10^6 cells were washed 3 times in PBS for 3 minutes at 2000 rpm. After the final wash, the cell pellet was gently resuspended in 0.5 ml hypotonic PI buffer (PI 50 µg/ml, 0.1% sodium citrate, 0.1% Triton X-100, Sigma). The tubes were incubated at least 6 hours in the dark at 4°C after which the cells were fixed in 1% paraformaldehyde/PBS before flow cytometric analysis. The PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson) using the red (FL3) fluorescence channel (600 nm band pass filter and 35 nm bandwidth). PI intercalates into double stranded nucleic acid and the endonucleosomal cleavage of DNA in apoptotic

nuclei results in a hypodiploid DNA peak that is easily quantified by measuring the number of events falling within an operator-defined region corresponding to apoptotic cells. The intensity of fluorescence was proportional to the cellular DNA content. 10^4 cells of each sample were analyzed and all measurements were done under the same instrument settings.

II.13. Proliferation assay

The proliferative response of HHV-6- or mock-infected cells was measured in response to a variety of stimuli. Cell proliferation is determined by estimating incorporation of [^{3}H]-Thymidine into DNA. T cells ($5x10^5$/ml), PBMC ($5x10^5$/ml), cord blood ($5x10^5$/ml), or T cell lines ($1x10^5$/ml) were seeded at 200 µl/well with or without a variety of stimuli and cultured for 4 days in a 96-well round-bottom plate and pulsed with 1 µCi [^{3}H]-Thymidine (25 Ci/mmole, Amersham, Mississauga, Ont. Canada) during the final 18 hours of culture. Cells were harvested using a TomTec cell harvester (Hamden, CT) which aspirates and lyses cells and transfers DNA onto filter paper while allowing unincorporated [^{3}H]-Thymidine to wash through. [^{3}H]-Thymidine incorporation into DNA was counted using a 1450 Microbeta PLUS liquid scintillation counter (Wallac). The following stimuli or their combinations were used on HHV6-infected or mock-infected cells: PHA (1:200 v/v) (Gibco BRL), anti-CD3 monoclonal antibody (1:200 dilution of culture supernatant from OKT3, ATCC CRL8001), anti-CD2 monoclonal antibody (5 µg/ml, Becton-Dickinson, Bedford, MA) or anti-CD28 monoclonal antibody (1:400 v/v, Research Diagnostics Ind., Flanders, NJ), or 5 ng/ml phorbol ester 12, 13-dibutyrate (PDB, Gibco BRL) and 0.5

μg/ml ionomycin (Sigma). These stimuli were incubated with the cells for the duration of the culture period. For some proliferation assays, cells were exposed to suppressor supernatants. Triplicate wells for each experimental condition were prepared.

II.14. Viral antigen-coated ELISA plate

In experiments involving protein-coated plates, concentrated UV-irradiated viral or mock-derived protein was diluted 1:10 in 0.1M sodium bicarbonate buffer (pH 8.2) and coated for 18 hours onto sterilized ELISA plates (Nunc, Denmark). Following several washes with PBS and blocking of the plate with complete medium, T cells ($5x10^5$/ml), PBMC ($5x10^5$/ml), or T cell lines ($1x10^5$/ml) were seeded at 200 μl/well in the presence or absence of either PHA (1:500 v/v), IL-2 (50 U/ml, Cetus, Emeryville, CA), IL-12 (10 ng/ml, R&D systems, Minneapolis, MN) or IL-15 (10 ng/ml, R&D systems), and cultured for 4 days. At the end of 3 days, cells were pulsed with 1μ Ci/well [^{3}H]-Thymidine and harvested as described above.

II.15. Suppression of "bystander" PBMC following addition of HHV-6 infected PBMC

PBMC were infected with UV-inactivated HHV-6 following the infection protocol. These infected cells (10^6/ml) or non-infected PBMC (10^6/ml) were then gamma-irradiated for 30 minutes (3,000 rads). The irradiated cells were then mixed with non-irradiated syngeneic PHA (1:500 v/v)-stimulated PBMC at a 1:1 ratio. Cell mixtures were then treated with either IL-2 (50 U/:ml), IL-12 (0.1 μg/ml), IL-15 (0.1 μg/ml) or a combination of all three cytokines and allowed to incubate for 5 days at 37°C. During the final 18 hours of culture,

cells were pulsed with 1 μCi [^{3}H]-Thymidine and harvested with a cell harvester and assayed for [^{3}H]thymidine incorporation with a microbeta plate liquid scintillation counter as described.

II.16. Generation of "suppressor-factor-containing culture supernatant"

Suppressor supernatant was defined as a supernatant derived from HHV-6 infected cells that when added to fresh cells, inhibited basal-, cytokine- or mitogen-driven proliferation. This virus-conditioned supernatant was generated by standard infection of PBMC, T cells or HSB-2. Control supernatants were derived from mock-infected cells. Cells (10^6/ml) were put into culture and 72 hours post-infection, supernatants were collected, filtered through a 0.2μm filter, aliquoted and stored at -80°C until used.

II.17. Inactivation of mock- or virus-infected cell culture supernatant

II.17.1. Heat Inactivation: Mock or HHV-6 infected cell supernatants were heat-inactivated by incubating culture supernatants for 1 hour at 56°C.

II.17.2. UV Irradiation: Mock or HHV-6 infected cell supernatants were UV-irradiated by incubating cell culture supernatants in a 60 mm petri dish on ice for 30 minutes under UV light in a laminar flow hood.

II.18. Generation of cytokine-activated killer cells and cytotoxicity assay

PBMC obtained from healthy donors were cultured in complete medium for 72 hours in the presence or absence of HHV-6 suppressor factor-containing culture supernatant (1:10

volume) or mock supernatant. HHV-6 suppressor factor culture supernatant, which was filtered through a 0.2 μm filter to eliminate HHV-6 virus particles, was derived from autologous PBMC previously cultured with HHV-6 for 72 hours. Mock and HHV-6-suppressor factor supernatant were supplemented with either IL-2 (50 U/ml), IL-12 (10 ng/ml) or IL-15 (10 ng/ml). Activated PBMC were harvested and tested for cytotoxicity or cell proliferation, as described above. K562 cells were used as ^{51}Cr-labeled (Amersham) targets. K562 target cells were labeled with 100 μCi of sodium chromate for 3 hours at 37°C. After three washes, the target cells (10^4/well) were mixed with cytokine-activated effector PBMC at a 1:50 ratio and incubated for 6 hours of co-culture. 100 μl of culture supernatant were removed and free ^{51}Cr measured. Spontaneous release was measured from wells containing medium and ^{51}Cr-labeled target cells. Maximum chromium release was determined by lysing target cells in 1% NP-40 (Sigma). The percent specific lysis was calculated as 100 x [(experimental release - spontaneous release) / (maximum release - spontaneous release)].

II.19. Absorption of HHV-6 suppressor factor from infected cell supernatant using anti-HHV-6 human serum coupled to Protein G-Sepharose

Protein G-Sepharose (Pharmacia LKB, Piscataway, NJ) was rehydrated for 15 minutes at room temperature with PBS prior to washing three times with PBS to eliminate preservative. The Protein-G Sepharose pellet was resuspended with an equal volume of PBS. To 60 μl of Protein G-Sepharose, an equal volume of pre-cleared (by centrifugation) anti-HHV-6 human serum or control serum was added and incubated with continuous

mixing for one hour at 4°C. The antibody-bound Protein G-Sepharose beads were washed 3 times with PBS to remove unbound serum components and resuspended in 10% FBS/IMDM and further incubated for 15 minutes at room temperature. For viral protein absorption, the antibody-bound protein G slurry was divided into two aliquots, spun and the final wash removed and 100 µl of media or 100 µl of HSB-2-derived suppressor supernatant were added. The mixture was incubated for 1 hour with continuous mixing at 4°C, beads pelleted, and supernatant filtered through a 0.2 µm filter. Absorbed culture supernatant was added to HSB-2 cells at a 1:20 final dilution. Cells were incubated for 3 days after which their proliferation was assayed by ^{3}H-Thymidine incorporation. HHV-6 anti-sera was obtained from Dr. J.M. Weber (Bureau of Microbiology, Laboratory Centre for Disease Control, Health Protection Branch, Health Canada, Ottawa, Ontario). These serum specimens were tested using an ELISA (Parker and Weber, 1993) to detect IgG and IgM antibodies to HHV-6. Details of the HHV-6 anti-serum samples provided follow:

Sample	IgG (O.D.)*	IgM (O.D)*	Sex	Age	Clinical Symptoms
V64221	0.45	negative	female	10 years	no data
V67595	negative	negative	female	10 years	cervical adenitis
V66655**	2.57	0.14	male	2 years	fever, vomiting, diarrhea, neutropenia

* Cutoff values for ELISA test:
IgG: positive > 0.10
IgM: absorbance values less than 0.10 are in the range of sera from healthy individuals.
** The IgG and IgM results are consistent with a recent infection (or reactivation) of HHV-6.

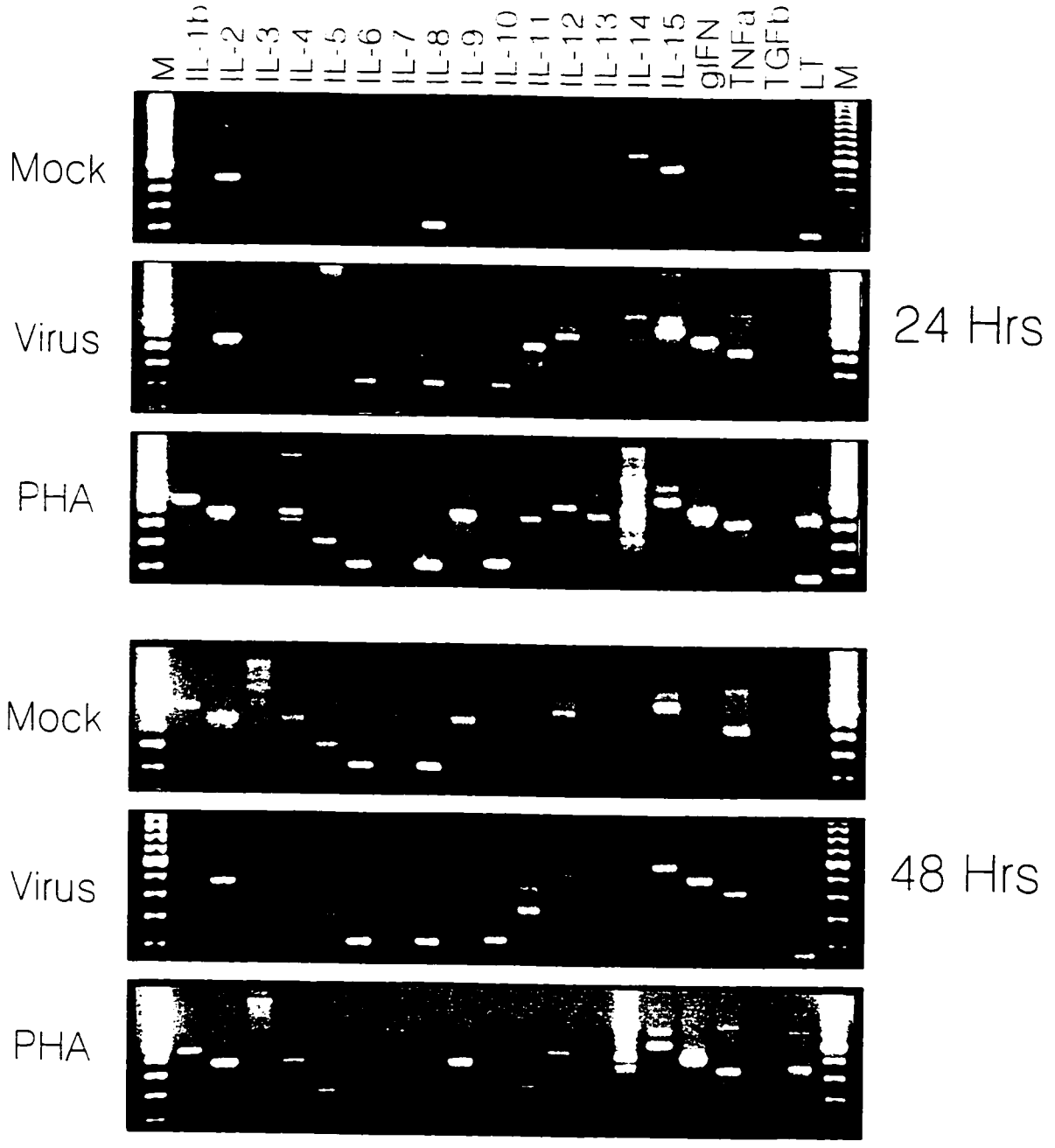
RESULTS

III.1. Cytokine Response of Immune Cells to HHV-6 Infection

Cytokines serve many functions that are critical to host defence against pathogens. Cytokines regulate the magnitude and nature of immune responses by influencing the growth and differentiation of lymphocytes. They also provide important amplification mechanisms that enable small numbers of antigen-specific lymphocytes to activate a variety of effector mechanisms to recognize that antigen. Excessive or dysregulated cytokine expression can result in tissue injury and/or act as a potential cofactor in disease pathogenesis and progression.

HHV-6 has previously been shown to induce IL-1β, TNFα and IFNα from PBMC (Flamand et al., 1991, Kikuta et al., 1990). A comprehensive study of the *in vitro* cytokine response of T cells following acute HHV-6 infection has not been performed. A study of the cytokine repertoire produced by T cells following HHV-6A (GS) infection was undertaken to determine which cytokines were expressed in T cells following HHV-6 infection. Purified human T lymphocytes were used, as they represent the major *in vitro* target for HHV-6 infection (Takahashi et al., 1989). RT-PCR analysis was performed on mock- and HHV-6-infected peripheral blood T cells from a minimum of two donors per cytokine. PHA-stimulated T cells were used as a positive control of cytokine RNA induction. Figure 1 shows results from one donor. When compared with mock infected cells, infection of T cells with HHV-6 resulted in the induction of IL-6, IL-10, IL-12 (p35

Figure 1. RT-PCR analysis of cytokine RNA in T cells following mock or HHV-6 infection. T cells which were seeded at $2x10^6$/ml following exposure to HHV-6 ($3x10^6$ infective virus particles), an equal volume of mock virus preparation, or to PHA (1:200 v/v) were cultured for 24 and 48 hour intervals prior to collection of total cellular RNA. 2 μg of total RNA was reverse transcribed and PCR amplified as detailed under Materials and Methods using cytokine primers listed in Table I or obtained from Stratagene (La Jolla, CA). PCR products were resolved in a 1% agarose gel. The 100 bp size markers (Gibco BRL) are indicated in lane M.


M
IL-1b
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IL-10
IL-11
IL-12
IL-13
IL-14
IL-15
gIFN
TNFa
TGFb
LT
M
Mock
Virus
PHA
24 Hrs
Mock
Virus
PHA
48 Hrs

subunit), IL-15, IFN-γ, and TNF-α mRNA by 24 hours post-infection. Analysis of cytokine RNA induction at 48 and 72 hours revealed no new cytokine mRNAs in infected cells, but an increase in cytokine transcription in mock infected cells. The donor shown in Figure 1 is not representative of the results when other donors were tested. If there was a discrepancy seen between the two donors' PCR results, more donors were tested. The IL-11 RT-PCR product (lower band) noted in Figure 1 was not reproducibly demonstrated in three other donors tested, and was therefore not listed as induced. The IL-14 lane, although containing multiple bands, did not contain the expected 1371 bp product, and was therefore also not considered induced. IL-1β mRNA was induced in three of four donors (see Table II), although not in the donor shown in Figure 1. The IL-12 PCR product was not detectable in two other donors and was therefore not listed as induced. The other cytokine mRNAs tested were either not significantly induced, or were found to remain at levels which were equal to those seen in mock-infected cells. The induction of some cytokine mRNAs with mock infection at 48 hours is likely due to stimulation of cells by culturing and/or from antigens present in the mock and HHV-6 stock. The lack of induction of several cytokine RNAs by HHV-6 infection could not be attributed to an inability of the T cells to respond, since all of the cytokines, with the exception of IL-3 were markedly induced in T cells following PHA stimulation (Fig. 1). IL-3 is a stem cell growth factor and was not expected to be expressed by T cells. Densitometry readings for the 24-hour cytokine RT-PCR profile from HHV-6-infected T cells in parallel experiments using three or more blood donors gave relative cytokine induction values of 2-, 6-, 2-, 2-,

6- and 3-fold for IL-1β, IL-6, IL-10, IL-15, IFNγ and TNFα RNAs respectively (Table II).

TABLE II: Induced Cytokine RNA levels in HHV-6 Infected T Cells

Cytokine	no. positive donors/ no. donors evaluated	Average Induction Index[a]
IL-1β	4/5	2
IL-6	2/3	6
IL-10	3/5	2
IL-11	1/4	ND
IL-12	1/4	ND
IL-15	3/4	2
IFN-γ	5/5	6
TNF-α	2/4	3

[a] RT-PCR products were scanned using procedures described in Materials and Methods. Cytokine PCR products were normalized against β-actin PCR products which were obtained from the same RT-reaction. The induction index values for each of the cytokines were determined using the calculation: (relative absorbance of the HHV-6 cytokine PCR product/relative absorbance of the HHV-6 β-actin PCR product)/(relative absorbance of the mock-treated cytokine PCR product/relative absorbance of the mock-treated β-actin PCR product).
ND, not determined

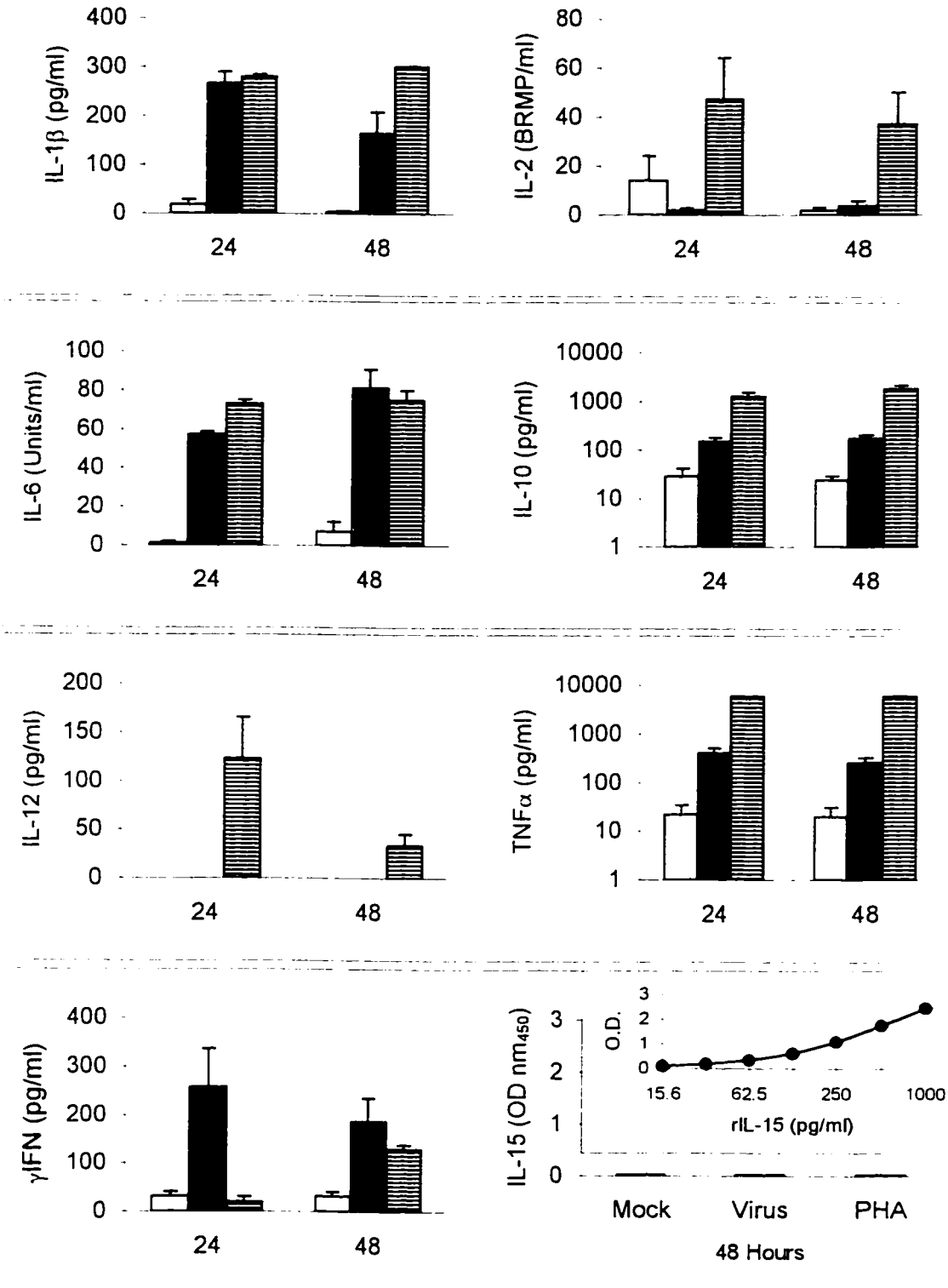
In summary, γIFN was induced in all donors tested, while IL-1β, IL-6, IL-10 and IL-15 were induced in greater than 50% of donors and IL-11, IL-12 and TNFα were induced in less than or equal to 50% of donors.

To confirm that the HHV-6 induced cytokine mRNAs were translated and the respective cytokine released into the culture medium, culture supernatants were assayed by ELISA (IL-1β, IL-2, IL-10, IL-12, IL-15, γIFN and TNFα) or by cytokine-specific

biological assay (IL-6) at 24 and 48 hours post-infection (Fig. 2). The protein values in Figure 2 represent all donors tested, even those that were negative for mRNA induction. Purified T cells ($2x10^6$/ml) were infected with mock or HHV-6 (added at $3x10^6$ infectious units/ml cells) following the infection protocol outlined in Materials and Methods. Uninfected, PHA-stimulated T cells were used as a positive control. Results indicate that HHV-6 infected T cells secreted a number of interleukins that were not secreted by mock infected cells. Analysis of the T-cell culture supernatants for IL-1β (n=4 donors) showed that HHV-6-infected T cells produced 266 ± 33 and 164 ± 43 pg/ml of IL-1β by 24 and 48 hours, respectively, versus 18 ± 10 and 3 ± 3 pg/ml of IL-1β for mock-infected T cells. This represents a 15- and 55-fold induction in the amount IL-1β at 24 and 48 hours post-infection. Analysis of IL-6 activity by biological assay revealed that culture supernatants from HHV-6-infected T cells (n=4 donors) contained 57 ± 1 and 81 ± 10 U of IL-6/ml at 24 and 48 hours post-infection, versus 1 ± 1 and 7 ± 5.2 U of IL-6/ml in the mock-infected cell culture supernatants. This represents a 57- and 12-fold induction in IL-6 activity. HHV-6 also induced IL-10 in T cells (n=6 donors). After 24 and 48 hours, HHV-6-infected T-cell culture supernatants contained 148 ± 31 and 171 ± 32 pg/ml of IL-10 versus less than 29 ± 12 and 24 ± 5.3 pg/ml of IL-10 for mock-infected cells, and thus represented a 5- to 7-fold increase in the levels of IL-10.

Due to the ability of IL-12 to activate NK and T cells during viral infections (Reiter et al., 1993), IL-12 production from T cells of two donors was also assayed. IL-12 concentrations were found to be less than 5 pg/ml in HHV-6 infected cells or in mock infected cells at 24 and 48 hours despite an increase in RNA in one donor (Fig. 1).

Figure 2. Analysis of cytokine levels in culture supernatants from T cells exposed to either mock or HHV-6 infection, or to PHA. Cytokine concentrations were measured from the culture supernatants of T cells which were incubated at $2x10^6$/ml for 24 and 48-hour intervals following exposure to HHV-6 (■), an equal volume of mock virus preparation (□), or PHA (1:200 v/v) (▤). The IL-15 inset represents the recombinant IL-15 standard with O.D. values. IL-15 levels were measured at 48 hours only. Values are expressed as the mean concentrations ± SEM from three or more experiments. Only PHA induced IL-12 into the culture supernatant and hence no bars are visible for mock and HHV-6 induced IL-12.


IL-1β (pg/ml)
400
300
200
100
0
24
48
IL-2 (BRMP/ml)
80
60
40
20
0
24
48
IL-6 (Units/ml)
100
80
60
40
20
0
24
48
IL-10 (pg/ml)
10000
1000
100
10
1
24
48
IL-12 (pg/ml)
200
150
100
50
0
24
48
TNFα (pg/ml)
10000
1000
100
10
1
24
48
γIFN (pg/ml)
400
300
200
100
0
24
48
IL-15 (OD nm450)
3
2
1
0
O.D.
3
2
1
0
15.6
62.5
250
1000
rIL-15 (pg/ml)
Mock
Virus
PHA
48 Hours

The inability of the HHV-6-infected T cells to express significant IL-12 protein in the culture supernatant was not due to T-cell unresponsiveness. Parallel cultures of PHA-activated T cells produced more than 123 ± 42 and 33 ± 12 pg/ ml of IL-12 after 24 and 48 hours, respectively. Analysis of IL-15 from two donors revealed no increase following HHV-6 infection as compared to mock-infected controls. IL-15 concentrations were less than 15 pg/ml in HHV-6 or mock infected T cell culture supernatants at 48 hours (Fig. 2). Even though the IL-15 ELISA assay was capable of detecting at least 15 pg/ml of rIL-15 protein, IL-15 was not detectable in the HHV-6 infected or PHA stimulated T-cell culture supernatants using two different ELISAs despite the presence of IL-15 transcripts (Fig. 1). In contrast to most other cytokines, the synthesis and secretion of IL-15 may be controlled predominantly by regulation at the level of translation or post-translation rather than at the transcriptional level.

In addition to inducing several interleukins, HHV-6 also strongly induced the cytokines IFN-γ and TNF-α in T cells. IFN-γ concentrations in T-cell culture supernatants (n=6 donors) following HHV-6 infection were found to be 257 ± 78 and 186 ± 48 pg/ml of IFN-γ at 24 and 48 hours, versus 33 ± 9 and 32 ± 8 pg/ml for mock-infected T cells. This represented a 8- and 6-fold induction at 24 and 48 hours, respectively. Finally, TNF-α levels were increased in HHV-6-infected T-cell culture supernatants (n=3 donors) as compared to mock-infected cells. TNF-α was found in the supernatants at 398 ± 104 and 256 ± 71 pg/ml at 24 and 48 hours, respectively, versus mock-infected cells which displayed no greater than 22 ± 12 pg/ml of TNF-α. This represented a 18- and 12-fold induction of TNF-α by HHV-6.

Since HHV-6 appeared not to alter IL-2 mRNA expression, it was decided to determine whether HHV-6 suppressed IL-2 protein expression. As shown in Figure 2, significant amounts of IL-2 were not detectable in the HHV-6-infected T-cell culture supernatants (n=4 donors) while in control cultures, T cells released less than 2 ± 1 and 4 ± 2 U/ml of IL-2, respectively, into the culture supernatant during the 24 and 48 hour period after infection. Parallel cultures of PHA-activated T cells produced more than 47 ± 17 and 37 ± 13 U/ml of IL-2. The observed presence of IL-2 mRNA in HHV-6-infected T cells versus mock-infected T cells, and absence of extracellular protein expression in these samples may be due to either an inhibition or attenuation of mRNA translation (Asson-Batres et al., 1994), or possible loss of crucial posttranslational processing (Rubartelli et al., 1993). It should be noted that the relationship between cytokine mRNA level and protein production is not always correlative (Mohler and Buttler, 1990; Gosselin et al., 1992). These data illustrate that results obtained by RT-PCR analyses do not always reflect the amounts of protein secreted into supernatants.

In summary, HHV-6 infected T cells secrete IL-1β, IL-6, IL-10, IFNγ and TNFα protein into the culture supernatant (Fig. 2) but IL-2, IL-12 and IL-15 were not expressed, which could compromise T cell and NK cytolytic functions during HHV-6 infection. Under such conditions, one could envisage HHV-6 promoting not only its own replication, but the replication of other viruses (e.g. HIV) through the induction of cytokines such as IL-1β, IL-6 and TNFα.

To determine if HHV-6 infection can inhibit mitogen driven cytokine gene transcription, RT-PCR analysis of cytokine RNA was performed after mock infection,

HHV-6 infection. PHA stimulation or PHA stimulation and HHV-6 infection at 24, 48 and 72 hour time intervals. T cells that were both PHA stimulated and HHV-6 infected were stimulated with PHA for five hours before addition of HHV-6. Figure 3 shows representative results for IL-2 and γIFN. For IL-2, similar levels of transcript can be seen following mock and HHV-6 infection (lanes 1 and 3 respectively) at all three timepoints. PHA stimulation markedly increased IL-2 transcript levels as expected (lane 2) particularly at 24 and 48 hours post stimulation. HHV-6 infection of PHA stimulated T cells (lane 4) did not affect the level of IL-2 transcript compared to PHA alone. For γIFN, HHV-6 infection did induce an increase in γIFN transcript at 24 and 48 hours post-infection (lane 3) compared to mock (lane 1) as shown previously (Fig. 1). Similar to IL-2, HHV-6 infection did not affect the ability of PHA to induce γIFN mRNA. HHV-6 infection also did not affect PHA stimulated transcription of IL-1β, IL-4, IL-6, IL-8, IL-9, IL-10, IL-13, IL-15, and TNFα mRNAs. HHV-6 infection also did not affect PHA stimulated cytokine protein expression for IL-1β, TNFα, IL-12, IL-10, IL-2 and γIFN. The protein levels induced by mitogen stimulated, infected T cells were comparable to cytokine protein levels from mitogen stimulated T cells. HHV-6 does not interfere with mitogen-driven cytokine mRNA or protein induction for the cytokines measured. The consequence of infection on the cell's ability to respond to mitogen as measured by a functional assay, is addressed in Part II.

To determine if the induction of cytokine required viral replication, RT-PCR analysis was done on HHV-6 infected T cells from one donor that were cultured in the presence of phosphonoacetic acid (PAA), an inhibitor of the HHV-6 DNA polymerase (Fig. 4B). PAA inhibits HHV-6 DNA replication (Di Luca et al., 1990) and should

Figure 3. RT-PCR analysis of cytokine (IL-2, γIFN) RNA from PHA stimulated and HHV-6 infected T cells. T cells were seeded at $2x10^6$/ml following exposure to HHV-6 ($3x10^6$ infective virus particles), an equal volume of mock virus preparation, PHA (1:200 v/v), or first PHA stimulated for five hours before addition of virus for 24, 48 and 72 hour intervals prior to collection of total cellular RNA. 2 μg of total RNA was reverse transcribed and PCR amplified as detailed under Materials and Methods. Cytokine primers for IL-2 and β-actin are listed in Table I and primers for γIFN were obtained from Stratagene (La Jolla, CA). Lanes 0, 1, 2, 3 and 4 shows RNA isolated from fresh T cells before culture, mock infected, PHA stimulated, HHV-6 infected and PHA and HHV-6 infected respectively at 24, 48 and 72 hours. Molecular weight markers (Gibco BRL) are indicated in lane m. PCR products were resolved in a 1.5% agarose gel.

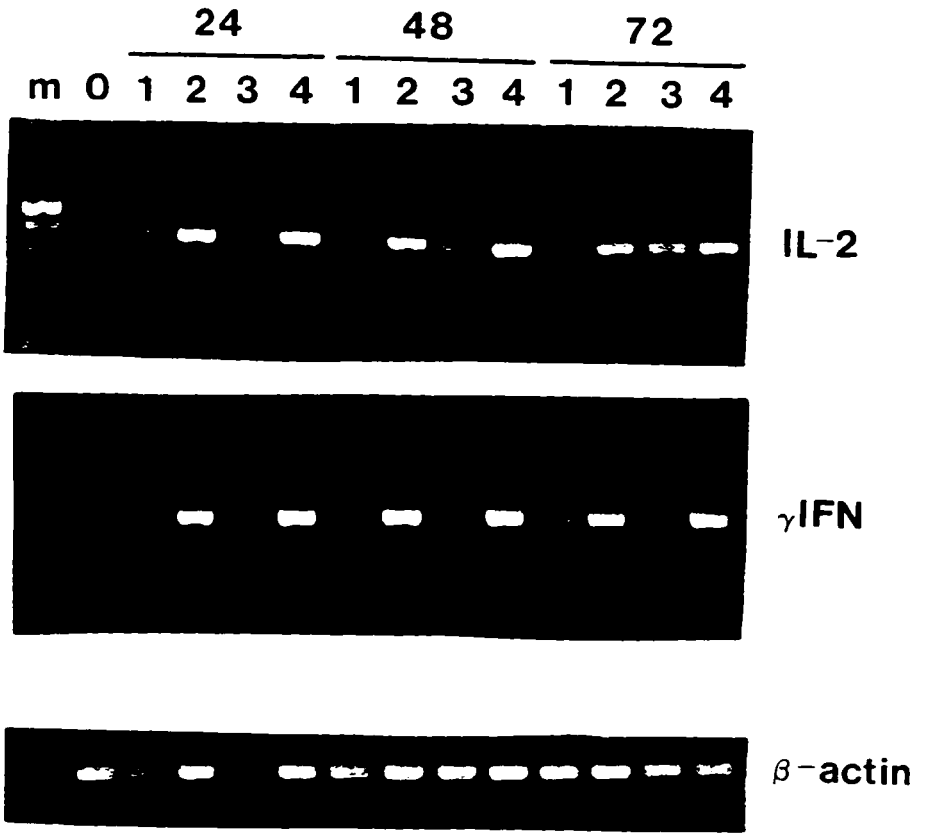
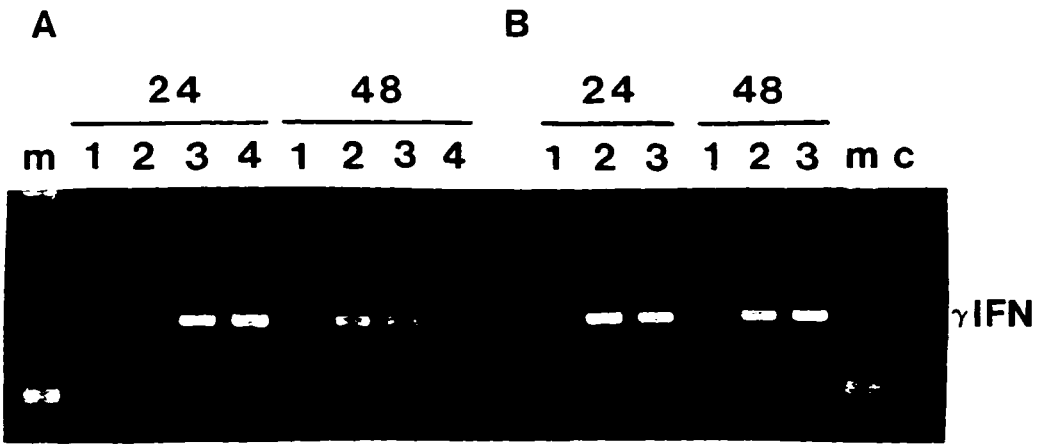

24
48
72
m 0 1 2 3 4 1 2 3 4 1 2 3 4
IL-2
γIFN
β-actin

Figure 4. RT-PCR analysis of γIFN cytokine RNA following infection with inactivated HHV-6. A) T cells ($2x10^6$/ml) were either mock infected (lane 1), infected with untreated HHV-6 (lane 2), infected with UV treated HHV-6 (lane 3) or infected with psoralen inactivated HHV-6 (lane 4) for 24 and 48 hour intervals prior to collection of total cellular RNA. B) T cells were either mock infected (lane 1), infected with untreated HHV-6 (lane 2) or infected with HHV-6 and after one hour of culture exposed to PAA (125μg/ml) (lane 3) for the rest of the culture period. For both Fig. 4A and 4B, total RNA was extracted at 24 and 48 hours, reverse transcribed and PCR amplified as detailed under Materials and Methods. Molecular weight markers (Gibco BRL) are indicated in lane m. PCR products were resolved in a 1.5% agarose gel. Lane c refers to PCR reagent control with no cDNA template.


A
B
24
48
24
48
m 1 2 3 4 1 2 3 4
1 2 3 1 2 3 m c
γIFN

therefore inhibit late viral gene transcription which is dependent on viral DNA replication while not abrogating adsorption, entry and early gene transcription. The concentration of drug used has previously been shown to inhibit HHV-6 replication (Di Luca et al., 1990). T cells were either mock infected (Fig. 4B, lane 1), infected with HHV-6 (Fig. 4B, lane 2) or infected with HHV-6 in the presence of PAA (Fig. 4B, lane 3). Lane 2 shows HHV-6 induced γIFN mRNA (lane 2) when compared to mock infected T cell RNA (lane 1). The presence of induced transcript in the presence of PAA at 24 and 48 hours (lane 3) shows that viral replication is not required for this stimulation effect. The cytokine inducing effect could be due to a virion component or the result of early gene expression. HHV-6 induced cytokine mRNA of IL-10, TNFα, IL-6 and IL-15 was also analysed in the presence of PAA with similar results. PAA treatment did not appear to affect the ability of HHV-6 to induce cytokine mRNA. This was extended to the protein level for γIFN and TNFα, which were the only two cytokine proteins measured from infected T cells in the presence of PAA. HHV-6 infected T cells treated with PAA induced γIFN protein (120 ± 6 pg/ml) to levels equal to untreated cells (128 ± 9 pg/ml). These results are derived from the same donor shown in Figure 4B. Similarly, PAA treatment of HHV-6 infected T cells did not affect the ability of HHV-6 to induce TNFα protein (491 ± 68 vs. 323 ± 21 pg/ml). Figure 5 below illustrates these results.

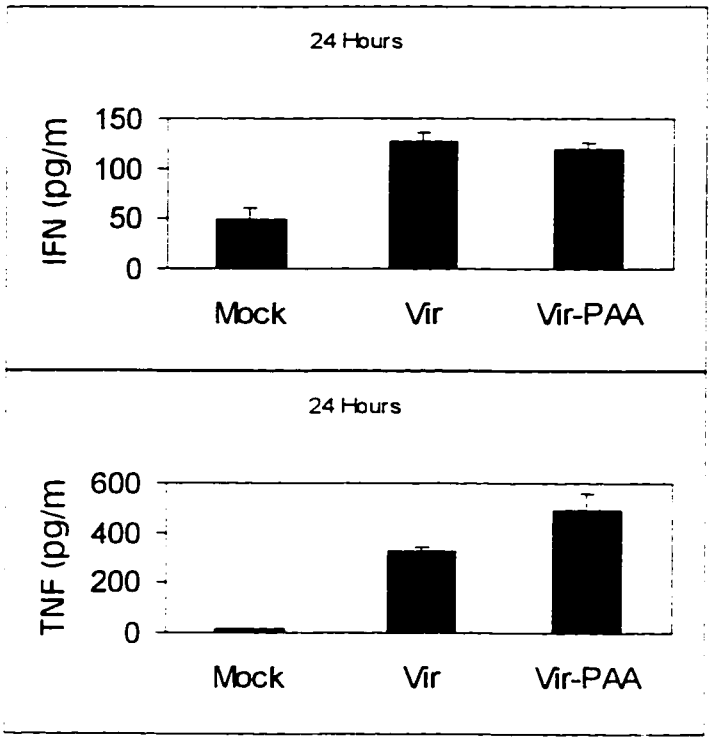


Figure 5: Analysis of γIFN and TNFα protein levels in culture supernatants from T cells exposed to either mock, HHV-6 or HHV-6 in the presence of PAA. T cells ($2x10^6$/ml) were either mock- (Mock), HHV-6-infected (Vir) or infected with HHV-6 and after one hour of culture exposed to PAA (125μg/ml) (Vir-PAA). After 24 hours, culture supernatants were tested for γIFN and TNFα protein levels by ELISA as outlined in Materials and Methods. Error bars represent standard deviation from duplicate measurements.

In an attempt to confirm if cytokine up-regulation requires the presence of infectious virus or is due to the effect of a virion's structural molecule, two methods of HHV-6 inactivation were used: UV and psoralen treatment. UV treatment is a standard method for blocking viral replication. UV radiation (260 nm) causes adjacent pyrimidines to become covalently linked by the formation of a 4-membered ring structure called a pyrimidine dimer. To further control for any possible transcriptional leakage of HHV-6

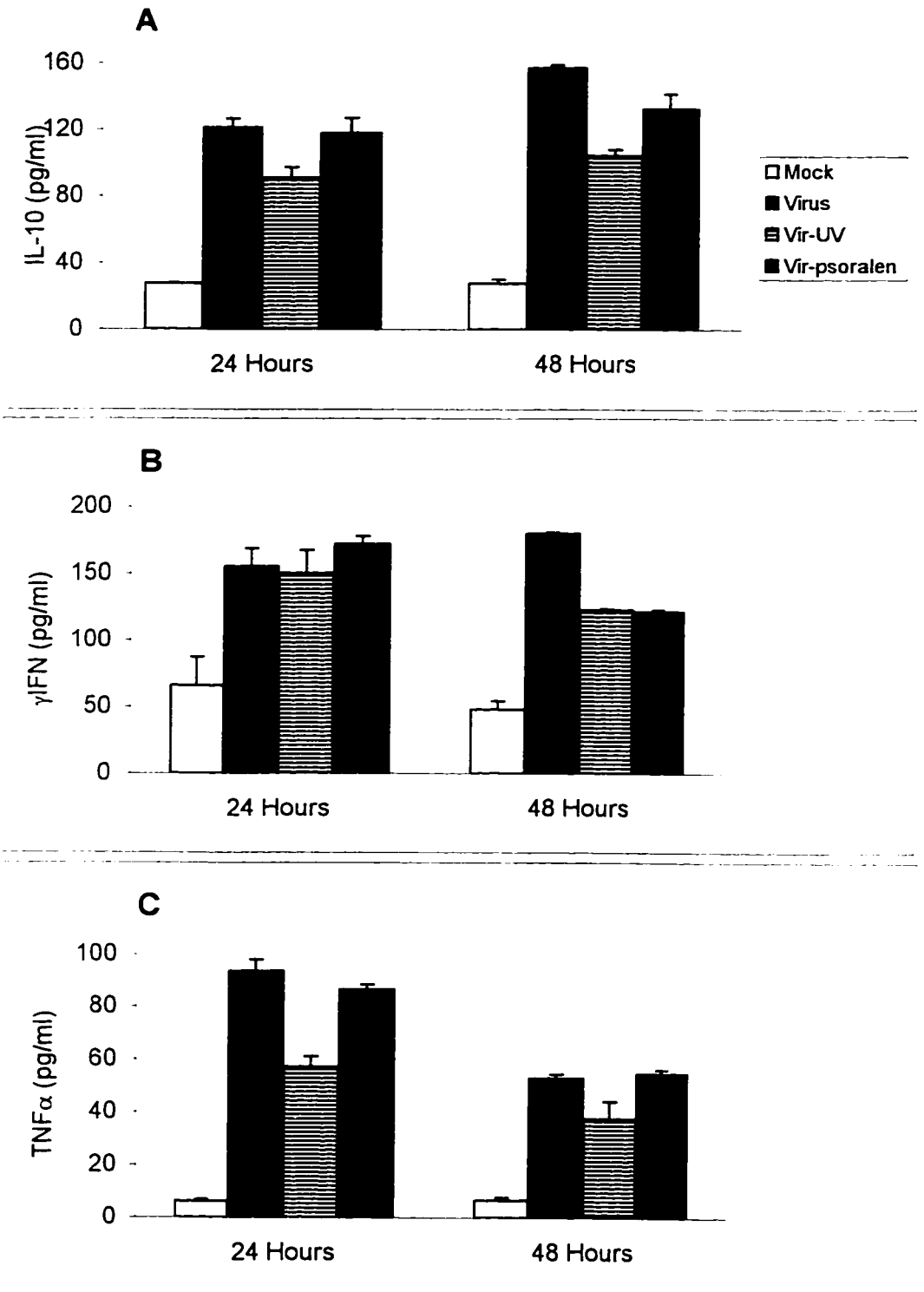
transcription following UV treatment, psoralen was also used. Psoralen, composed of three aromatic rings, can intercalate into DNA and with subsequent photoactivation by long-wavelength UV radiation, forms covalent adducts to pyrimidines. It can join a pyrimidine in one DNA strand to an appropriately situated pyrimidine below in the other strand thereby cross linking both strands. This interstrand cross linking prevents DNA strand separation and blocks DNA replication and transcription. Thus, psoralen irreversibly inhibits HHV-6 gene transcription without grossly affecting virion protein structures. To determine if virus binding alone was sufficient to induce cytokine mRNA and protein, T cells were infected with either HHV-6, UV- or psoralen-treated HHV-6 and supernatants were collected at 24 and 48 hours post-infection for cytokine protein measurement. Since γIFN, IL-10, IL-15, TNFα, IL-6 mRNA were found to be induced with HHV-6 infection they were analysed by RT-PCR after inactivated virus treatment. IL-2 was also included in this analysis. Figure 4A shows the effect of virus inactivation on γIFN mRNA at 24 and 48 hours post-infection. Mock infected γIFN mRNA levels are shown in lane 1 and HHV-6 induced levels are shown in lane 2. UV irradiation (lane 3) and psoralen inactivation (lane 4) of HHV-6 also induced γIFN transcript levels compared to mock. It appears that viral binding and/or internalization is sufficient to induce cytokine transcription. The induction of γIFN mRNA with UV and psoralen treated virus (Fig. 4A, lanes 3 and 4) at 24 hours, is greater than with untreated HHV-6 (lane 2). The reason for this is not clear and could be a donor specific effect. Unfortunately, this was not confirmed with another donor. In total, these experiments showed that viral binding is sufficient to induce cytokine mRNA in T cells.

Cytokine protein from two donors was measured to ensure the induced T cell RNA levels after inactivated virus exposure could be correlated to induced protein. IL-10, γIFN and TNFα were measured, as they were consistently induced (mRNA and protein) after HHV-6 infection. An induction of cytokine synthesis was observed with both UV and psoralen-treated HHV-6 that was comparable to cytokine synthesis by cells infected with untreated HHV-6. As shown in Figure 6, untreated, UV-irradiated and psoralen-treated HHV-6 induced significant and comparable levels of IL-10, γIFN and TNFα into the culture supernatant. This was in contrast to mock infected T cells which expressed low level cytokine synthesis. These results confirm that viral replication was not necessary to mediate cytokine induction and that virus particle-cell contact is sufficient to enhance cytokine release.

In summary, Figures 3-6 reveal that HHV-6 infection did not appear to affect the T cell's ability to respond to mitogen-driven cytokine induction at either the mRNA or protein level. Viral replication was not required for this effect as shown by the ability of cells to respond to HHV-6 by cytokine induction even in the presence of a viral polymerase inhibitor, PAA, or by exposure of T cells to UV-inactivated and psoralen-treated virus.

To ensure that psoralen inactivation of virus was complete, infected cultures were tested for HHV-6 DNA using a colorimetric microwell assay for detection of HHV-6-specific PCR products as outlined in Materials and Methods. HSB-2 cells were infected as per usual, and at 72 hours post-infection, DNA was extracted. Results indicate that mock antigen (derived from uninfected HSB-2 supernatant) that was either untreated, UV- or

Figure 6. Analysis of cytokine levels in culture supernatants from T cells exposed to either mock or untreated-, UV-inactivated- or psoralen-treated HHV-6. Cytokine concentrations were measured by ELISA from the culture supernatants of purified T cells from two donors which were incubated at $2x10^6$/ml for 24 and 48-hour intervals following exposure to mock (□), HHV-6 (■), UV-inactivated HHV-6 (▤) or psoralen-treated HHV-6 (▨). Error bars represent standard error of duplicate samples from two donors.


A
160
120
80
40
0
IL-10 (pg/ml)
24 Hours
48 Hours
Mock
Virus
Vir-UV
Vir-psoralen
B
200
150
100
50
0
γIFN (pg/ml)
24 Hours
48 Hours
C
100
80
60
40
20
0
TNFα (pg/ml)
24 Hours
48 Hours

psoralen-treated had no HHV-6 detectable sequences as expected (see Table III below). Untreated HHV-6 infection of HSB-2 cells gave a positive reading of 0.795 OD. This value represents input virus and DNA from subsequent rounds of HHV-6 infection. UV inactivation decreased the untreated value by 40%. This value likely represents input UV-inactivated virus. Psoralen crosslinks viral GC pairs and hence no HHV-6 primer annealment or extension should theoretically occur and this is supported by the negative OD reading. This confirms that the psoralen-treated virus inactivation was efficient and complete and no viral transcription or replication occurred.

Table III: HHV-6 DNA Measurement after Infection of HSB-2 Cells

Treatment	OD Reading
Mock	0.003
Mock-UV	0.001
Mock-psoralen	0.004
HHV6	0.795
HHV6-UV	0.487
HHV6-psoralen	0.002

HSB-2 cells (10^6/ml) were infected with either 100X mock or HHV-6 stock (at $2.3x10^8$ infectious units/ml) at 2 µl/ml HSB-2 cells. Cells were exposed to virus for 2 hours on ice, then washed twice to remove unadsorbed virus and resuspended to 10^6/ml and incubated for 72 hours in a 24-well plate in a 1 ml volume. DNA was extracted using Tri-Reagent following manufacturer's instructions and used as template in a HHV-6 specific PCR reaction. PCR products were assayed using a HHV-6 probe and detected using the Digene Sharp Signal System (Digene Diagnostics) as described in Materials and Methods.

III.2. Suppression of Cellular Proliferation by HHV-6 Infection

These results indicate that HHV-6 is a strong inducer of several proinflammatory cytokines, several of which are T or NK cell-activating, and thus would be predicted to stimulate immune cells to respond to virus infection (Fig. 1 and 2). However, HHV-6 is also noted for its strong immunosuppressive activity on PBMC and T cells (Horvat et al., 1993). The nature of this suppression or its mechanism of action is still unresolved. Studies by Flamand et al. (1995) have suggested that the immunosuppression is due to an inhibition of IL-2 synthesis. Since in my experiments the mRNAs for the T cell activating cytokines, IL-2 and IL-15, were present (Fig. 1), but their respective proteins were not significantly increased or detected in the T-cell supernatants (Fig. 2), the ability of infection to suppress T-cell proliferation through the inhibition of these cytokines was tested. In these experiments UV-irradiated HHV-6 was used to eliminate potential post-infection virus replication and thus prevent cytolysis of T cells. The ability of UV inactivated virus to inhibit T cell proliferation induced by exogenous cytokines along with the mitogen PHA was tested. UV-irradiated HHV-6 results in a 98% loss of the original virus infectivity as measured by reactivity of monoclonal antibodies with an infected indicator cell line as described in Materials and Methods. T cells were cultured with a suboptimal concentration of PHA plus either IL-2, IL-12 or IL-15, with or without UV-inactivated virus. After 4 days in culture, proliferation was assayed by thymidine incorporation (cpm). As shown in Table IV, UV-treated HHV-6 suppressed PHA or PHA and cytokine stimulated T cell proliferation. This was not due to insufficient interleukin,

Table IV: Proliferative responses of T cells, PBMC, Cord Blood to PHA and cytokines in the presence or absence of UV-treated HHV-6.

	CPM mock	Stimulation Index	CPM HHV6-UV	Stimulation Index
T Cells				
Medium	10295 ± 2745	-	2604 ± 657	-
IL-2 (4U/ml)	113324 ± 4262	11.0	4854 ± 238	1.9
IL-12 (0.1μg/ml)	15660 ± 1264	1.5	4586 ± 463	1.8
IL-15 (0.1μg/ml)	66127 ± 1372	6.4	3256 ± 339	1.3
PHA	103732 ± 4190	10.0	6775 ± 3229	2.6
PHA + IL-2	127448 ± 4738	12.0	3121 ± 1258	1.2
PHA+IL-12	165314 ± 1198	16.0	14667 ± 519	5.6
PHA + IL-15	186382 ± 1062	18.1	9509 ± 321	3.6
PBMC *				
Medium	2297 ± 1326	-	1780 ± 24	-
PHA	108671 ± 3756	47.3	10518 ± 1977	5.9
Cord Blood				
Medium	22229 ± 3676	-	5911 ± 21	-
PHA	53783 ± 2535	2.4	6149 ± 644	1.0

Triplicate 96-well cultures of T cells, PBMC, or cord blood ($2x10^6$/ml) were cultured for 4 days in complete medium in the presence or absence of PHA (1:200) and UV-treated HHV-6 ($2x10^8$ infectious particles/ml prior to UV-treatment) or an equal volume of mock-infected stock. Cells were pulsed during the final 18 hours of culture and harvested. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD for triplicate samples. Stimulation Indices were calculated by dividing the cpm of stimulated cells by the untreated (medium) control.

* Results are the mean of triplicate samples (SEM, n=3) from three different donors

PHA, Phytohaemagglutinin

since mock-infected T cells vigorously responded to PHA or PHA and cytokine. Basal T cell proliferation was reduced by 75% with HHV-6 infection in untreated T cells compared to mock infected controls. Results shown for T cells in Table IV are from one donor. Similar results were obtained when the experiment was repeated using T cells from two other donors. Mock infected T cells proliferated in response to exogenous interleukin IL-2, IL-12 and IL-15, as indicated by the high cpm, to 11-, 2- and 6-fold higher levels than basal proliferation (medium) respectively. Although the thymidine incorporation for UV-virus infected T cells was significantly lower, they were still able to respond to exogenous interleukin by a 2-fold increase in counts compared to unstimulated cells. UV-virus infected T cells displayed a loss of 93% in the thymidine incorporation as compared to that induced by PHA-stimulated mock controls. The UV-virus infected T cells minimally responded to PHA and cytokine compared to the response of mock treated cells. Therefore, it appears that adding exogenous cytokines that are not induced by HHV-6 infection (IL-2, IL-12 and IL-15) is not sufficient to overcome the suppression of HHV-6. The loss of T cell proliferative response was not simply due to a loss in cell viability, since HHV-6 infected T cells and mock-infected T cells had comparable numbers of viable cells when counted just prior to the [^{3}H]-thymidine pulse (on day three, ~90% viable for mock-infected and HHV-6 infected, as measured by trypan blue exclusion).

To investigate the role of non-T cells found in PBMCs in HHV-6 mediated suppression of proliferation, PHA-stimulated PBMC from three separate donors were exposed to UV-HHV-6 and cellular proliferation measured. Proliferation of PHA-stimulated PBMC infected with UV-treated HHV-6 was reduced by 90% compared to

PHA-stimulated controls (Table IV). It appears that the other cell types within the PBMC population did not contribute any factor(s) that could restore PHA responsiveness to infected cells. Similar results were obtained if cells were activated for 24 hours with PHA prior to HHV-6 infection implying that cells are susceptible to HHV-6 mediated suppression regardless of their activation state. Infection 24 hours after PHA stimulation resulted in 75 ± 5% suppression of proliferation (n=2 donors; data not shown). To determine whether naive T cells were similarly susceptible to the suppressive effects of HHV-6, PHA-stimulated cord blood was infected with UV-treated HHV-6 and PHA stimulated (Table IV). The PHA induced stimulation index of UV-virus treated cord blood cells was half that observed in PHA stimulated controls.

To further eliminate any potential proliferative background due to possible non-T cell contaminants in the T cell preparations, the effect of UV-virus infection on basal proliferation on three established human T cell lines which do not require IL-2 or PHA stimulation for growth was investigated (Table V). Three T cell lines (HSB2, Jurkat and H9) were infected with UV-HHV-6 which resulted in suppression of proliferation by 97, 92 and 88% respectively. This experiment was repeated five times with similar results; a representative experiment is shown.

Table V: Proliferative responses of T cell lines to UV-irradiated HHV-6.

	CPM mock	CPM HHV6-UV	% Suppression HHV6-UV
T Cell Lines (untreated)			
HSB2	119807 ± 2252	3677 ± 333	97
Jurkat	145971 ± 1417	11652 ± 475	92
H9	84234 ± 1002	10349 ± 122	88

Triplicate 96-well cultures of T cell lines ($1x10^5$/ml) were cultured for 4 days in complete medium in the presence of UV-irradiated HHV-6 ($2x10^8$ infectious particles/ml prior to UV-treatment) or an equal volume of mock-infected stock. Cells were pulsed during the final 18 hours of culture and harvested. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD for triplicate samples.

PHA stimulation offers a crude estimate of T cell function, because it may use both antigen-specific (TCR/CD3) and non-antigen specific (e.g. CD2) activation pathways. Thus various T cell activation pathways (CD2, CD3, CD28) were tested to try to map HHV-6-associated T cell anergy by analyzing surface and transmembrane activation pathways. To achieve this, T cells were activated polyclonally with PHA or via surface activation pathways by antibodies directed against different T cell mitogenic surface receptors (CD2, CD3, CD28) or via transmembrane activation pathways using reagents that bypass membrane signals, phorbol ester and calcium ionophore. UV-treated HHV-6 (at $3.8x10^6$ infectious units/ml of cells) or mock infected T cells were resuspended to $2x10^6$/ml and stimulated with various agents. Response of T cells to activating stimuli was measured by the standard proliferation assay as described in Materials and Methods. As shown in Table VI, exposure of unstimulated T cells to UV-treated HHV-6 reduces basal

Table VI: T-Cell proliferation after various mitogen treatments by exposure to UV treated HHV-6

	CPM Mock	**S.I.**	**CPM HHV-6-uv**	**S.I.**
Medium	8665 ± 597	-	2044 ± 225	-
PHA	85561 ± 2336	9.9	4394 ± 317	2.1
anti-CD2	3850 ± 451	NS	1316 ± 38	NS
anti-CD2 + anti-CD28	2772 ± 491	NS	1241 ± 88	NS
anti-CD3	15497 ± 1785	1.8	2145 ± 243	NS
anti-CD3+ anti-CD28	28361 ± 616	3.3	2559 ± 84	1.3
PDB+Ionomycin	127895 ± 1867	14.8	6739 ± 245	3.3
Medium +IL-2	16922 ± 882	-	1901 ± 166	-
PHA +IL-2	94213 ± 1359	5.6	4633 ± 417	2.4
anti-CD2 + IL-2	15451 + 1741	NS	1143 + 73	NS
anti-CD2+anti-CD28 + IL-2	16301 + 1296	NS	1467 + 33	NS
anti-CD3+ IL-2	29666 ± 698	1.8	2227 ± 183	1.2
anti-CD3+ anti-CD28 + IL-2	36491 ± 4432	2.2	2313 ± 86	1.2

T cells which were seeded at $2x10^6$/ml with PHA (1:200 v/v), PDB (5ng/ml), ionomycin (0.5μg/ml), IL-2 (50U/ml), anti-CD2 (5 μg/ml), anti-CD3 (1:200), or anti-CD2, anti-CD3 plus anti-CD28 (1:400) in the presence or absence of UV-treated HHV-6 were cultured for 3 days followed by an 18 hour pulse with [^{3}H]-thymidine. Results are listed as the mean cell proliferation (cpm) ± SEM from triplicate samples. Stimulation Indices were calculated by dividing the cpm of stimulated cells by the untreated (medium) control cpm.
PHA, Phytohaemagglutinin
PDB, Phorbol 12, 13-dibutyrate
S.I., Stimulation Index
NS, No stimulation

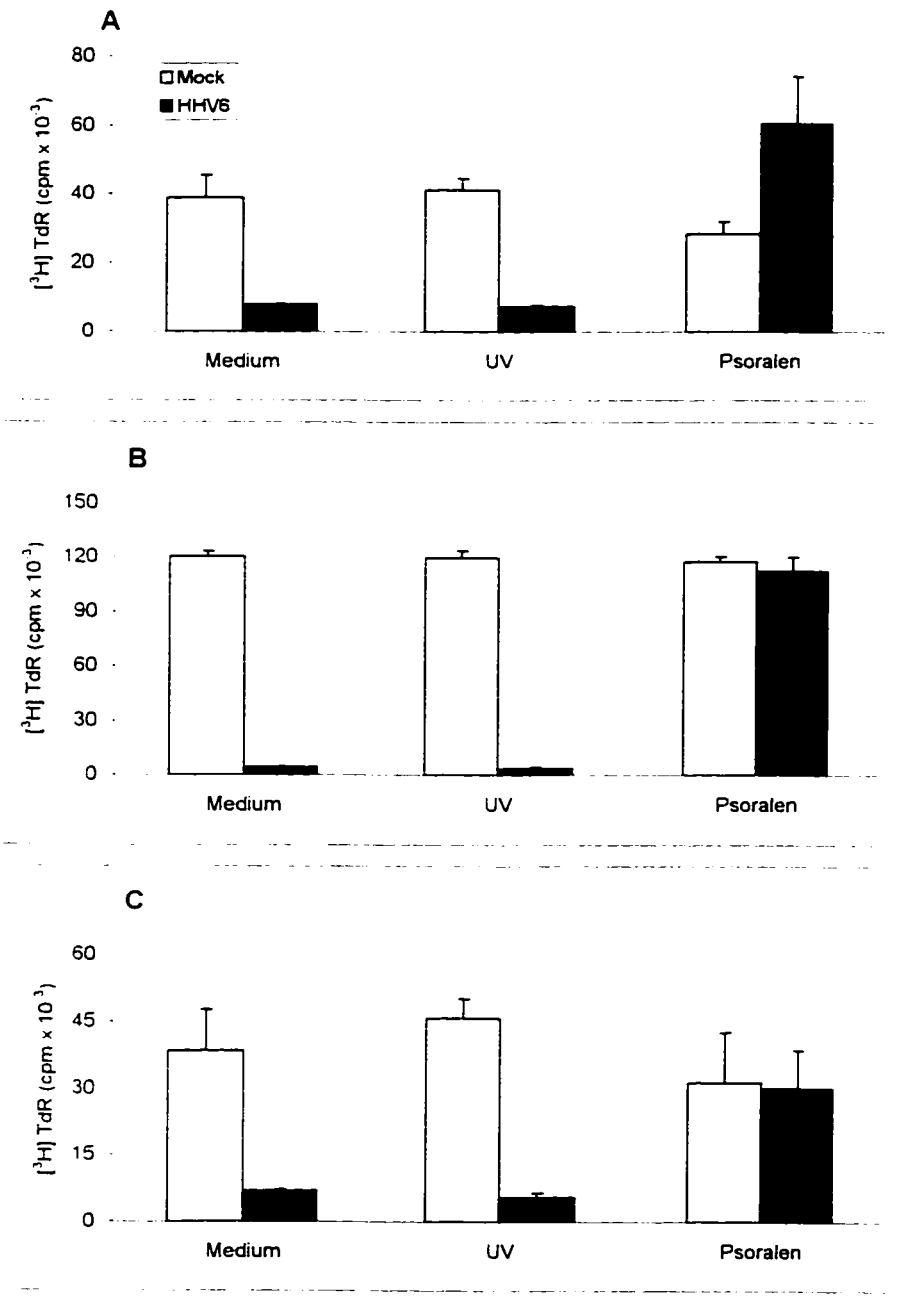
proliferation (Medium) by 76%. Upon stimulation with PHA, UV-treated HHV-6 exposed T cells proliferated less than mock infected control cells (SI of 2.1 compared to 9.9). This response was not corrected by addition of exogenous IL-2. Control cells did not respond to anti-CD2 stimulation, so the effects of HHV-6 exposure could not be determined. The lack of response to anti-CD2 stimulation may be due to the quality and suboptimal concentrations of reagent used. The CD3-mediated response was impaired in HHV-6 exposed T cells compared to controls in response to CD3- and CD28-mediated activation. Stimulation of T cells with the protein kinase C activator, PDB, and the calcium ionophore, ionomycin, also failed to induce the HHV-6 treated T cells to proliferate to levels comparable to that of uninfected cells. Other herpesviruses (EBV, CMV) have been shown to impair CD3-induced proliferative responses, but not to alter other T-cell activation pathways (Perez-Blas et al., 1992, Timon et al., 1993). The anergic response to PHA or anti-CD3 stimulation in EBV infection can be restored to normal levels by addition of exogenous IL-2 to cultures (Perez-Blas et al., 1992). Similarly, loss of responsiveness to anti-CD3 in acute CMV infection can also be corrected with exogenous IL-2 (Timon et al., 1993). Addition of IL-2 to HHV-6 infected T cells with either PHA, anti-CD3 or anti-CD3 and anti-CD28 did not restore normal proliferative responses to these stimuli. It appears that HHV-6 infection results in a generalized hypo-responsiveness of exposed T cells to various mitogenic stimuli and may be the cause of the clinically observed transient immunosuppression associated with acute HHV-6 infection.

The results in Figures 5 and 6 showed that virus transcription/replication was not required for cytokine protein induction. To determine if HHV-6 transcription/replication

were required for suppression of cellular proliferation. UV and psoralen treated HHV-6 was used to infect PBMC, cord blood cells or HSB-2 T cell line (Figure 7). Figure 7A shows that infection of PBMC with untreated virus (Medium) or UV-treated virus suppressed proliferation after PHA stimulation by 80 and 83% respectively when compared to proliferation of mock infected cells. Psoralen treated HHV-6 did not suppress proliferation of PBMC by PHA and in this particular donor only, enhanced proliferation (Fig. 7A). When infected with either untreated or UV-irradiated HHV-6 basal proliferation of HSB-2 cells was suppressed by 97% compared to mock infected cells (Fig. 7B). When infected with psoralen treated virus, there was no effect on basal proliferation. Similarly, PHA stimulated cord blood cells were suppressed by 82 and 88% when infected with either untreated or UV-irradiated HHV-6 respectively, but were unaffected in their proliferation to PHA stimulation when infected with psoralen treated virus (Fig. 7C). Thus, it appears that viral transcription is required for suppression of cellular proliferation and simple virus binding and/or internalization does not mediate the suppression. Results from Part I indicate that cytokine production requires only virus binding and/or internalization, therefore two separate mechanisms appear to be mediating cytokine secretion and inhibition of cellular proliferation, with one requiring virus binding and/or internalization and the other requiring some virus transcription independent of virus binding.

HSV virion has been shown to contain an "inducing" factor that acts as a transactivator of α genes (Batterson et al., 1983). This factor is known as α-TIF (α-Trans-Inducing Factor) or VP-16. The HIV Tat protein must also be internalized in order to "deactivate" the T cell (Li et al, 1995). To determine if HHV-6 or a HHV-6 associated

Figure 7. Inhibition of lymphocyte proliferation following infection with HHV-6, UV-irradiated HHV-6 or psoralen-treated HHV-6. PBMC (A), HSB-2 (B) or cord blood (C) cells were either mock infected (□) or infected with either untreated (represented by Medium), UV-inactivated or psoralen-treated HHV-6 (■). Cells were infected at 10^6/ml for two hours and washed. PBMC, HSB-2 and cord blood were put into culture at $5x10^5$/ml, $2.5x10^5$/ml and $5x10^5$/ml respectively. PBMC and cord blood were stimulated with PHA (1:200 v/v). Cells were cultured for 4 days after which cells were pulsed with 1μCi of ^{3}H-thymidine for the final 18 hours of culture. ^{3}H-thymidine incorporation into DNA was counted and is represented on the *y*-axis. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD based on triplicate samples.


A
80
60
40
20
0
[3H] TdR (cpm x 10-3)
Mock
HHV6
Medium
UV
Psoralen
B
150
120
90
60
30
0
[3H] TdR (cpm x 10-3)
Medium
UV
Psoralen
C
60
45
30
15
0
[3H] TdR (cpm x 10-3)
Medium
UV
Psoralen

suppressor factor must also be internalized or whether this factor could act at the cell surface. HHV-6 or mock stock was UV- or psoralen-treated and coated onto ELISA plates, and tested for its ability to induce suppression of PHA stimulated lymphocyte proliferation. If the HHV-6 suppressor-factor needed to be internalized, then plastic-bound HHV-6 protein should not result in significant cell suppression. If, however, the HHV-6 protein recognized a surface molecule, then binding of plastic-bound HHV-6 protein could result in cell suppression. As shown in Figure 8, UV treated HHV6 coated onto plates was capable of suppressing PBMC proliferation by over 96% even in the presence of PHA and IL-2, IL-12, or IL-15.

T cell activation is accompanied by the induction of various cell surface molecules. Some of these molecules include cytokine receptors. To assess whether the observed diminished T cell proliferation induced by HHV-6 is attributable to the failure to express such molecules, the expression of two cytokine receptor chains, namely the IL-2 receptor α-chain, CD25 (Leonard et al., 1984) and the IL-15 receptor β-chain, CD122 (Giri et al., 1994) were measured by flow cytometry. Receptor levels were tested at 48 hours post-infection. As shown in Figure 9, mock and HHV-6-UV exposed T cells had the same level of CD25 and CD122 expression. PHA stimulation increased mock infected T cell expression of CD25 and CD122 to 62% and 49% respectively. Flamand et al. (1995) have previously shown that HHV-6 infected PBMC stimulated with PHA had an equal percentage and intensity of expession of CD25 as control PHA stimulated cells suggesting that reduced proliferation observed in infected PHA stimulated cells is maybe due to defective receptor signaling after binding of IL-2.

Figure 8. Inhibition of cellular proliferation by tissue culture plate-bound HHV-6. Concentrated UV-treated (30 minutes under UV light in a laminar flow hood) mock (□) or HHV-6 (■) stocks were coated onto sterilized ELISA plates as described in Materials and Methods. Following several washes with PBS and blocking of unbound plastic sites with complete medium, $1x10^5$ PBMC in 200µl were plated in the presence or absence of PHA (1:500 v/v) and IL-2 (50U/ml), IL-12 (100ng/ml) or IL-15 (100 ng/ml). Cells were cultured for 4 days and pulsed during the final 18 hours with 1 µCi/well [^{3}H]-thymidine. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD based on triplicate. samples.

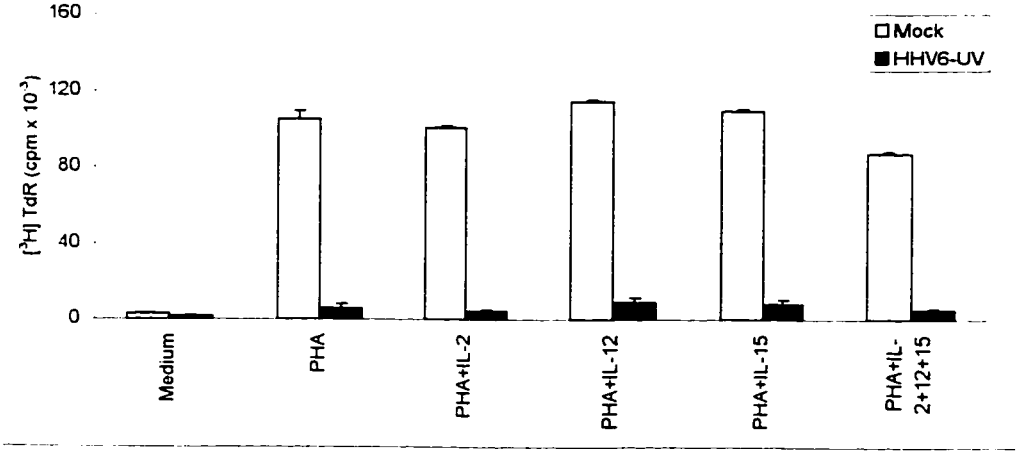
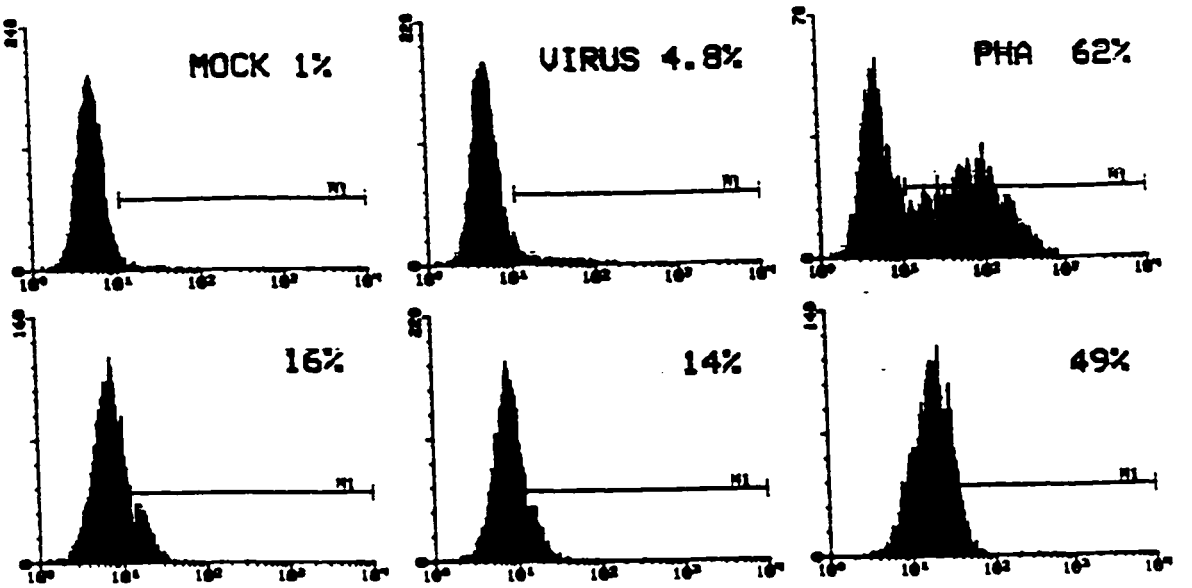

160
120
80
40
0
[3H] TdR (cpm x 10-3)
Mock
HHV6-UV
Medium
PHA
PHA+IL-2
PHA+IL-12
PHA+IL-15
PHA+IL-2+12+15

Figure 9. Expression of IL-2 receptor α-chain (CD25) or IL-15 receptor β-chain (CD122) on T cells following HHV-6 infection. T cells were incubated at $2x10^6$/ml for 48 hours following exposure to UV-treated HHV-6 ($3x10^6$ infective virus particles), an equal volume of mock virus preparation, or PHA (1:200 v/v). After 48 hours, cells were incubated at 4°C for 30 minutes with PE-conjugated anti-human CD25 (upper panel) monoclonal antibody (Becton Dickinson), or anti-human CD122 (lower panel) monoclonal antibody (Becton-Dickinson). Samples incubated with anti-human CD122 were detected with FITC-conjugated goat anti-mouse Ig (Becton-Dickinson). Cells were paraformaldehyde-fixed and analyzed by flow cytometry using a FACScan Flow Cytometer (Becton Dickinson). Results are expressed as the percentage of positively labeled cells versus that for isotype-matched, FITC or PE-conjugated control monoclonal antibodies. The *x* and *y* axes represent the log of fluorescence intensity and cell number respectively.


MOCK 1%
VIRUS 4.8%
PHA 62%
16%
14%
49%

Although the receptor for HHV-6 is not presently known, HHV-6 has previously been shown to down-regulate CD3 of the TCR complex late in virus infection (Lusso et al., 1991b) and to affect CD4 expression either positively (Lusso et al., 1991a, 1995) or negatively (Furukawa et al., 1994). The loss of this or other T-cell mitogenic receptor signals following HHV-6 infection could be a potential explanation for the reduced T cell responsiveness to PHA, or PHA and cytokine after virus exposure. To test whether this could be a possible mechanism for T cell anergy, flow cytometry was used to monitor expression of various molecules on the surface of T cells (Table VII). Results indicated that T-cell surface molecules, namely CD2, CD3, CD4, CD8, CD28, CD44, CD95/Apo-1, HLA class I and HLA class II, which are either associated with the TCR or involved in T-cell activation, apoptosis and anergy, and in cell adhesion, were tested and found to remain stably expressed at 24 hours. It has been suggested that loss of CD3 expression occurs late in infection and requires live virus, therefore PBMC were infected with untreated virus and CD3 expression measured at 96 hours post-infection. Again, CD3 expression remained stable (data not shown).

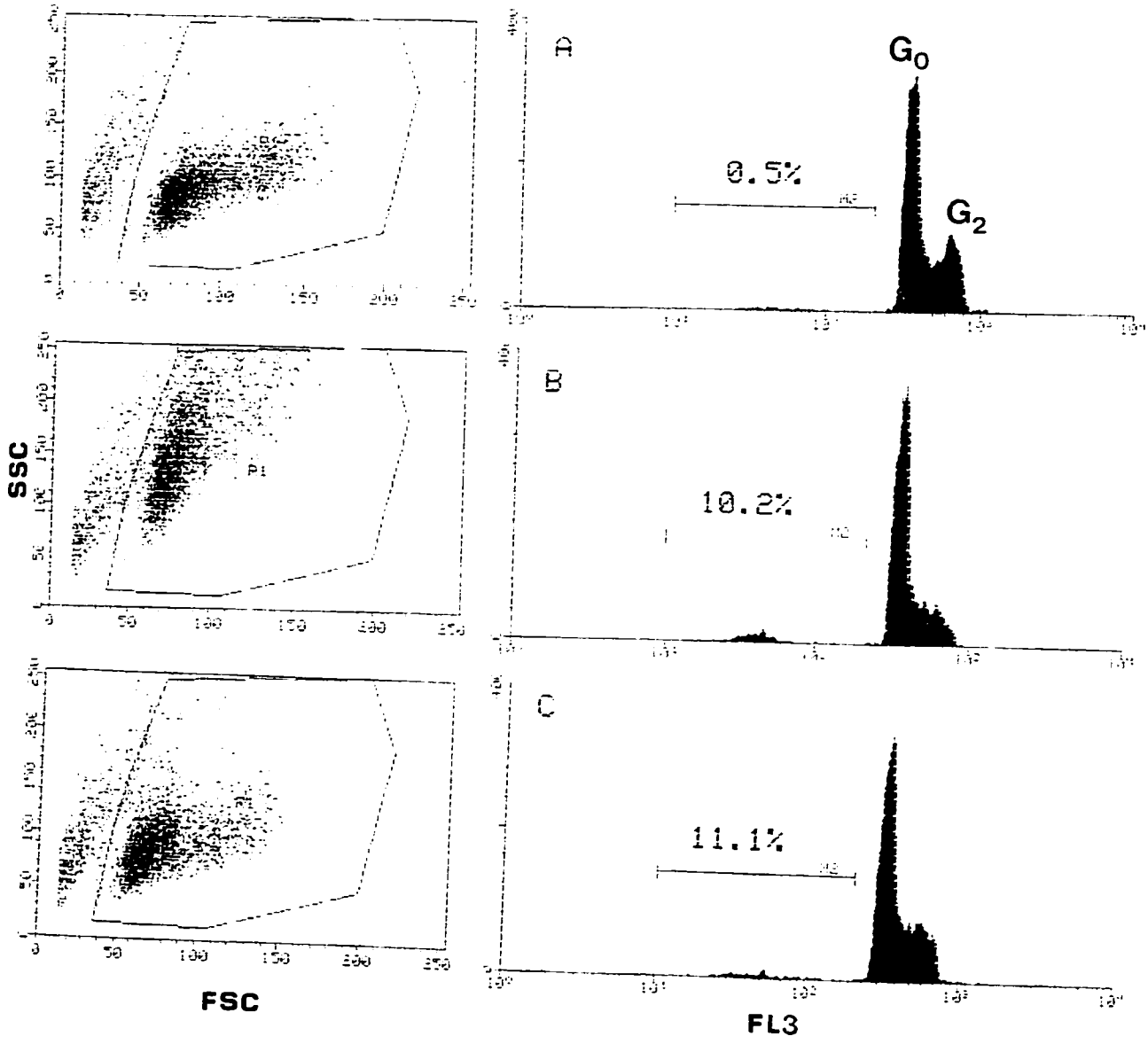
Table VII: Flow cytometric analysis of T-cell surface antigens following HHV-6 or PHA treatment

	CD2	CD3	CD4	CD8	CD28	CD44	CD95	HLA-I	HLA-II
Mock	88	88	54	27	70	70	20	87	6
HHV-6	89	85	56	25	76	80	28	93	7
PHA	ND	91	54	ND	ND	ND	86	ND	59

T cells ($2x10^6$/ml) which were either mock-infected, HHV-6-infected or PHA-stimulated (1:200 v/v) were seeded at 2 ml/well in a 24-well flat-bottom Falcon plate and cultured for 24 hours. For HHV-6 or mock treatments, T cells were initially inoculated with UV-treated HHV-6 ($3x10^6$ infective virus particles prior to UV-treatment) or an equal volume of mock preparation, and incubated for 2 hours on ice. Cells were subsequently washed in medium and seeded. After 24 hours, cell aliquots were incubated with antibodies and prepared for flow cytometric analysis as described in Materials and Methods. Results are expressed as the percentage of positively labeled cells versus that for isotype-matched, FITC or PE-conjugated control monoclonal antibodies. All fluorochrome-conjugated monoclonal antibodies, with the exception of CD95, were obtained from Becton-Dickinson (San Jose, CA). FITC-coupled anti-human CD95 monoclonal was obtained from MBL International (Watertown, MA).
ND, not determined

It was speculated that HHV-6 infection of T cells could result in the induction of apoptosis, thus impairing their ability to respond to mitogens and other stimuli. Apoptosis was investigated by measuring propidium iodide staining of DNA by either mock (Fig. 10A), HHV-6 (Fig. 10B) or UV treated HHV-6 (Fig. 10C) infected cells as outlined in Materials and Methods. Apoptotic nuclei appear as a broad hypodiploid DNA peak (shown under M2) which is easily discriminable from the narrow peak of normal (diploid) DNA. Operator-defined markers delineate the area where hypodiploid DNA would fall and the percentage of events found within this area are calculated. The data are shown as histograms of the fluorescence distribution. Using flow cytometry to measure fluorescence, only 10% of cells analyzed were apoptotic, which could not account for the global suppression on cellular proliferation observed. Propidium iodide staining of DNA

Figure 10. Cytofluorometric detection of apoptosis. 10^6/ml HSB2 cells were infected with either mock (A), HHV-6 (B) or UV-inactivated HHV-6 (C) and put into culture for 4 days after which cells were collected, resuspended in hypotonic PI buffer for 6 hours at 4°C then fixed with 1% paraformaldehyde before flow cytometric analysis as described in Materials and Methods. PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson) using the red (FL3) fluorescence channel. 10^4 cells of each sample were analyzed and all measurements were done under the same instrument settings. Cells falling within the 'M2' marker represent apoptotic cells.


A
G_0
G_2
0.5%
B
10.2%
C
11.1%
SSC
FSC
FL3

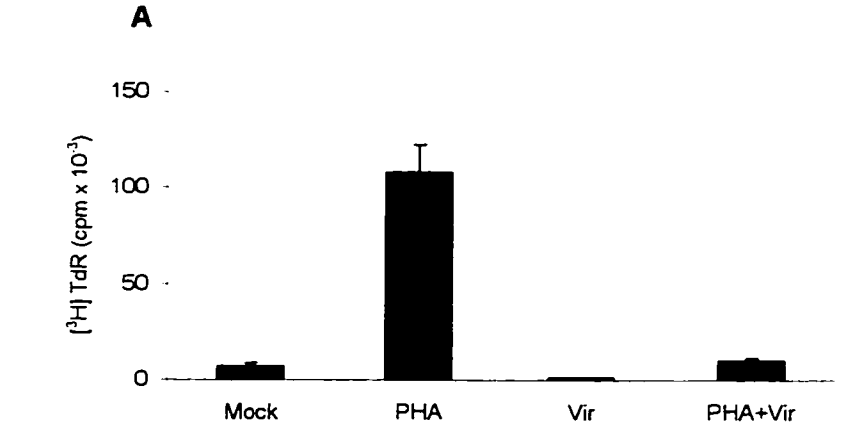
also allows cell cycle analysis. The $G_{0/1}$ phase is represented by the first (large) peak seen on the FL3 axis and the G_2 phase is the second (smaller) peak. The intermediate fluorescence represents cells in the S phase of the cell cycle. In infected cells (Fig. 10B), the G_2 peak is reduced compared to mock infected (Fig. 10A). This preliminary DNA analysis of HHV-6 infected cells suggests that transition from G_1 to S phase does not occur efficiently and HHV-6 exposed cells accumulate in the G_1 phase.

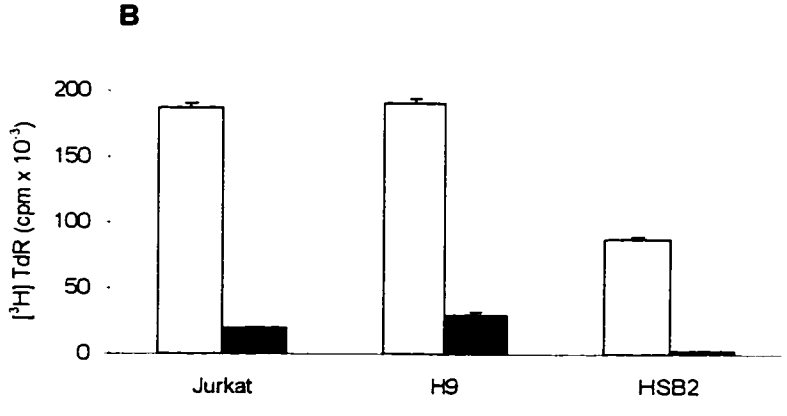
III.3. Suppressor Factor Characterization

To investigate the nature of the factor(s) mediating inhibition of T cell proliferation, virus-infected T-cell culture supernatants were harvested, filtered through a 0.2 micron filter to remove virus particles (Josephs et al., 1988a) and added to fresh autologous PHA-stimulated T cells at a final concentration of 1:10. Cells were cultured for three days in the presence of the supernatant before standard proliferation assays were performed. Filtered supernatant demonstrated no detectable virus when added to HSB-2 indicator cells as revealed by immunofluorescence staining with antibody (less than one positive cell/5000 cells). Purified T cells were exposed to supernatant from mock infected T cells (1:10 final dilution) in the presence or absence of PHA or exposed to supernatant from HHV-6 infected T cells (1:10 final dilution). The mock supernatant treated cells responded vigorously to PHA stimulation while the PHA-induced proliferation of T cells that were exposed to the HHV-6 infected T cell culture supernatant was suppressed by 96% (Fig. 11A). Comparable results were seen with several T cell lines that were similarly treated with virus infected supernatant. As shown in Figure 11B, the mean proliferative response for unstimulated, continuous T cell lines (Jurkat, H9 and HSB-2) exposed to virus infected supernatants was less than 10% of the proliferation of T cells exposed to mock infected cell supernatant.

To determine if different cell types could secrete this "factor" which had a similar suppressive effect on proliferation after infection, supernatants were harvested from either

Figure 11. Inhibition of T cell proliferation by HHV-6 suppressor factor containing culture supernatant. (A) Triplicate samples of purified T cells ($1x10^6$ cells/ml) were cultured with 1/10 volume mock supernant (Mock), PHA (1:200 v/v), 1/10 volume of filtered HHV-6 conditioned supernatant from autologous T cells (Vir), or PHA and filtered HHV-6-conditioned supernatant (PHA+Vir). (B) The T cell lines Jurkat, H9 and HSB-2 ($1x10^5$ cells/ml) were treated with either 1/100 volume of filtered mock (□) or HHV-6-infected (■) HSB-2 conditioned cell culture supernatant. Cells were cultured for 3 days and at the end of the third day were pulsed for an additional 18 hours with 1 μCi/well [^{3}H]-thymidine. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD based on triplicate samples.


A
150
100
50
0
[3H] TdR (cpm x 10-3)
Mock
PHA
Vir
PHA+Vir


B
200
150
100
50
0
[3H] TdR (cpm x 10-3)
Jurkat
H9
HSB2

PBMC or HSB-2 infected cells and tested for suppressive activity on the proliferation of T cell lines. Supernatants derived from PBMC or HSB-2 cells infected with UV irradiated- or psoralen-treated virus were also harvested, filtered and added to T cell lines at a 1:20 final dilution. Figure 12 shows that virus conditioned supernatant derived from PBMC (Fig. 12A) or from HSB-2 (Fig. 12B) after 4 days of culture with untreated or UV-irradiated HHV-6 caused comparable inhibition of proliferation of three T cell lines (Jurkat, H9 and HSB-2). Thus, virus conditioned supernatant derived from infected T cells (Fig. 11), PBMC or a T cell line (Fig. 12) inhibited mitogen driven cellular proliferation (T cells and PBMC) or basal proliferation of T cell lines. Supernatant derived from PBMC or HSB-2 cells infected with psoralen treated virus did not inhibit cellular proliferation when added to fresh T cell lines as compared to supernatants from cells infected with untreated and UV-irradiated HHV-6 (Fig. 12A, B). Thus it appears that some virus transcription is involved in mediating suppression of cellular proliferation.

In an attempt to determine whether co-culturing infected, irradiated cells with untreated cells would mediate inhibition of proliferation, an experiment was performed in which HHV-6 stock was UV-irradiated and used to infect PBMC as per the usual infection protocol. Two hours post-infection the cells were washed twice and then γ-irradiated (3,000 rads) to prevent their proliferation and incorporation of significant ^{3}H-Thymidine. Uninfected, autologous PBMC were similarly irradiated. These cells were then added to an equal number of autologous uninfected and non-irradiated PHA or cytokine stimulated PBMC. PHA and cytokine were added concurrently. The response of these cells was measured by a proliferation assay. Figure 13 indicates that "bystander" cells

Figure 12. Suppression of cellular proliferation by PBMC- or HSB-2-derived suppressor supernatant. Jurkat, H9 or HSB-2 at $2x10^5$/ml were exposed to PBMC (A) or HSB-2 (B) derived supernatant at a 1/20 final dilution. Supernatants were collected 3 days post infection of either UV-inactivated mock (□), HHV-6 [Vir (■)], UV-inactivated HHV-6 [Vir-uv(▤)] or psoralen-inactivated HHV-6 [Vir-ps (▦)] exposed PBMC or HSB2. Supernatants were filter sterilized and added to T cell lines. Cells were cultured for 4 days then pulsed with 1μCi ^{3}H-thymidine for the final 18 hours of culture. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD based on triplicate samples.

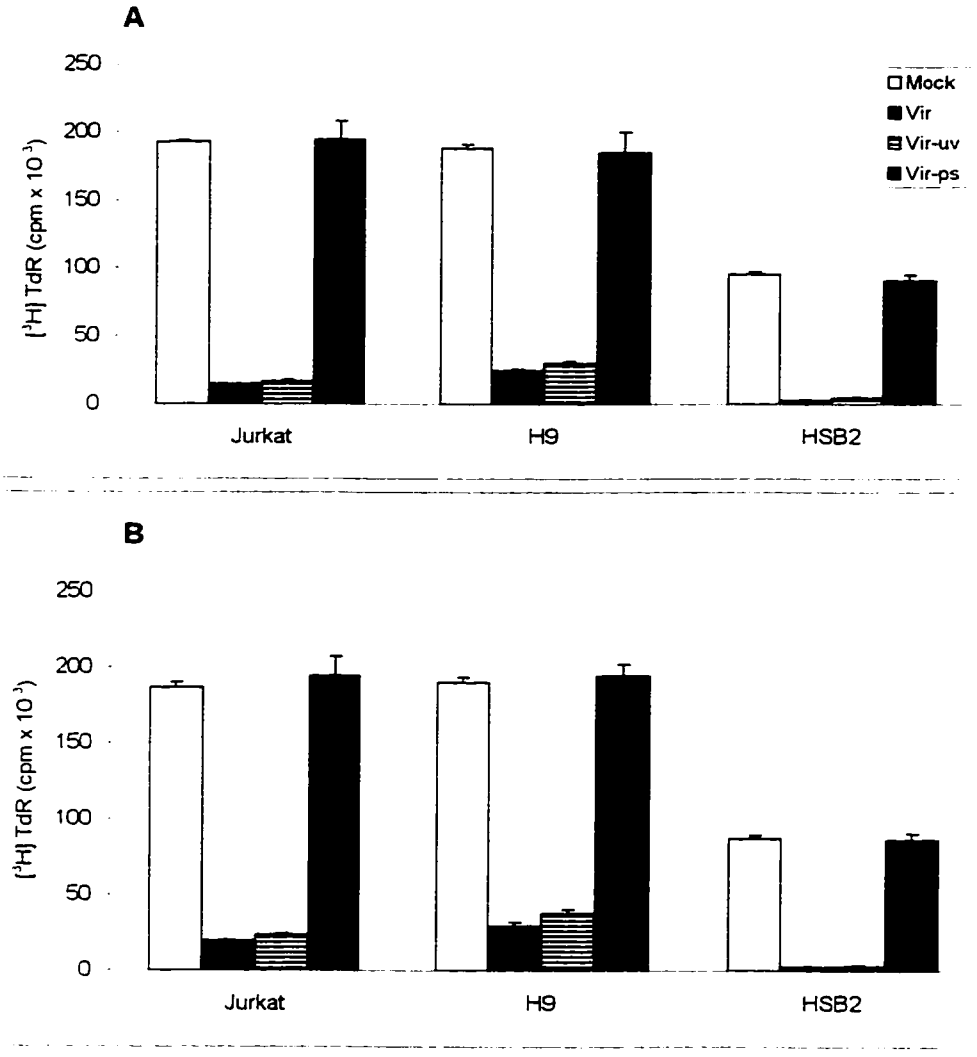
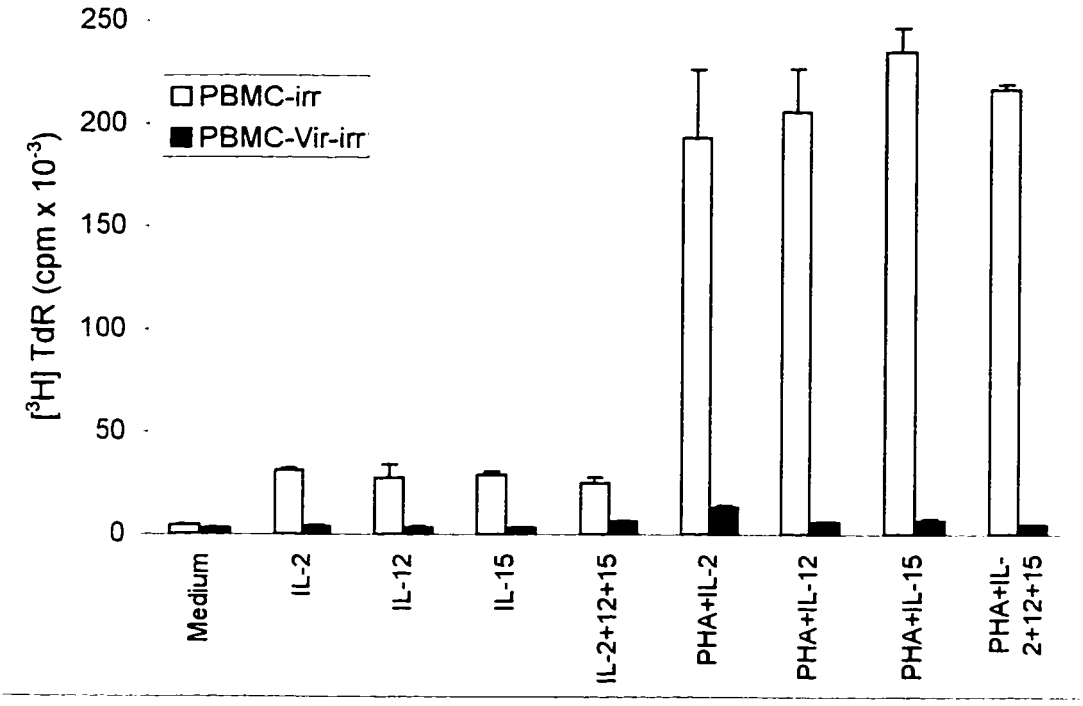

A
250
200
150
100
50
0
[³H] TdR (cpm x 10⁻³)
Mock
Vir
Vir-uv
Vir-ps
Jurkat
H9
HSB2
B
250
200
150
100
50
0
[³H] TdR (cpm x 10⁻³)
Jurkat
H9
HSB2

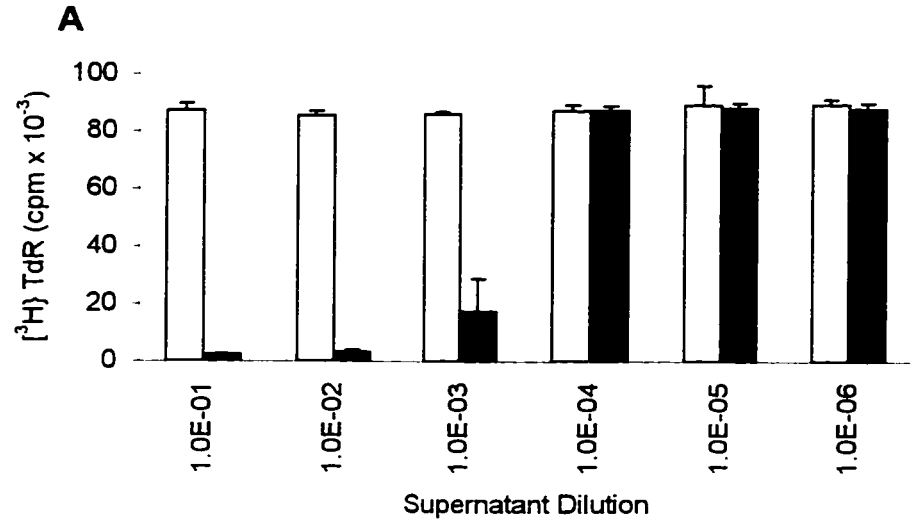
Figure 13. Proliferative response of cytokine- or mitogen-stimulated PBMC following culture with γ-irradiated PBMC or HHV-6 infected γ-irradiated PBMC. Uninfected PBMC (PBMC-irr) or UV-HHV-6 (PBMC-Vir-irr) infected PBMC ($5x10^5$/ml) that had been γ-irradiated (3,000 rads) were incubated with an equal number of uninfected PBMC ($5x10^5$/ml). Cells were either unstimulated (Medium), stimulated with combinations of cytokine [IL-2 (50U/ml), IL-12 (100 ng/ml), IL-15 (100 ng/ml)] and/or PHA (1:200 v/v). Cells were cocultured for 4 days then pulsed for an additional 18 hours with 1 μCi/well [^{3}H]-thymidine. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD based on triplicate samples.

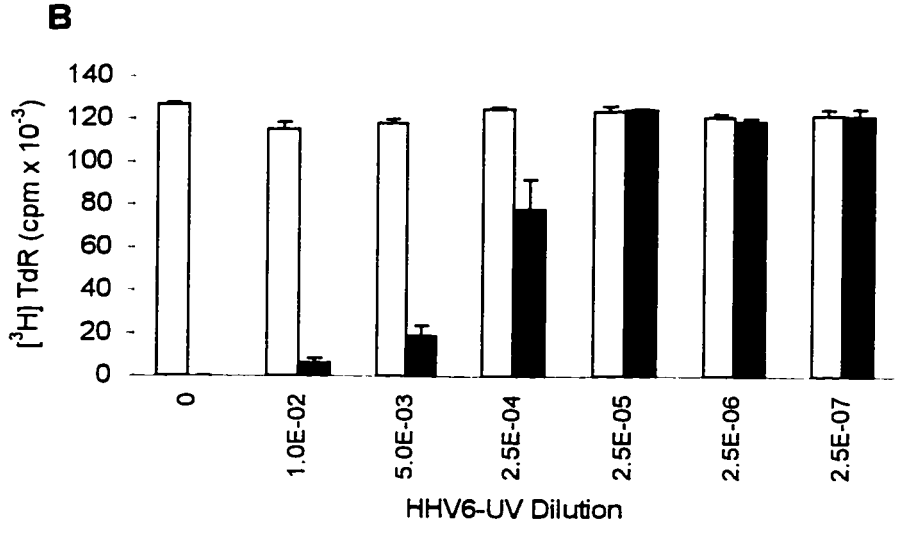

250
200
150
100
50
0
[3H] TdR (cpm x 10-3)
PBMC-irr
PBMC-Vir-irr
Medium
IL-2
IL-12
IL-15
IL-2+12+15
PHA+IL-2
PHA+IL-12
PHA+IL-15
PHA+IL-2+12+15

retain their ability to proliferate in response to PHA and cytokine (IL-2, IL-12 and IL-15) in the presence of irradiated uninfected PBMC ruling out any inhibitory effect from the irradiated cells. PBMC responded to mitogen and cytokine stimulation when cocultured with mock-infected, irradiated autologous PBMC. PHA and cytokine stimulated PBMC were suppressed in their proliferative response when cultured in the presence of HHV-6-infected, irradiated autologous PBMC. UV-irradiation of the virus blocks viral replication and hence subsequent rounds of infection but some viral transcription can occur. This experiment supports the idea that a viral-induced soluble factor is capable of inhibiting mitogen and cytokine driven proliferation but it cannot be distinguished whether it is a virus-derived product or a cellular factor induced following exposure to HHV-6.

If the suppression on cellular proliferation was due to a "factor(s)", the effect should be able to be titrated out. To investigate this, serial dilutions of virus-conditioned supernatant or UV-treated HHV-6 stock were added to an indicator T cell line (HSB-2) and a proliferation assay was performed. The suppressor factor(s) from virus conditioned PBMC supernatant could be titrated out at a 10^{-4} dilution of supernatant (Fig. 14A). Suppression of HSB-2 proliferation after infection with UV-irradiated HHV-6 was titrated out at a dilution of 2.5×10^{-5} (Fig. 14B).

Figure 14. Proliferative response of the T cell line, HSB-2, following treatment with serial dilutions of either suppressor-factor containing culture supernatant or UV-irradiated HHV-6 stock. (A) Titration of suppressive effect of HHV-6 conditioned supernatant on HSB-2 cells. HSB-2 cells were exposed to serial dilutions (10^{-1} - 10^{-6}) of suppressor supernatant. Suppressor supernatant was derived from either UV-inactivated mock infected PBMC (□) or UV-irradiated HHV-6 (■) infected PBMC three days post infection. Supernatants were filtered sterilized with a 0.2μm filter and added to HSB-2 T cell line ($2x10^5$/ml) and incubated for 3 days then pulsed with 1μCi ^{3}H-thymidine for an additional 18 hours of culture. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SEM based on triplicate samples. (B) Titration of UV-inactivated HHV-6 infection on T cell line proliferation. HSB-2 cells ($2x10^5$/ml) were exposed to serial dilutions (0-1/4x10^6) of either UV-inactivated mock (□) or UV-inactivated HHV-6 (■) stock. Cells were cultured for 3 days then pulsed with 1μCi ^{3}H-thymidine for an additional 18 hours of culture. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD based on triplicate samples.

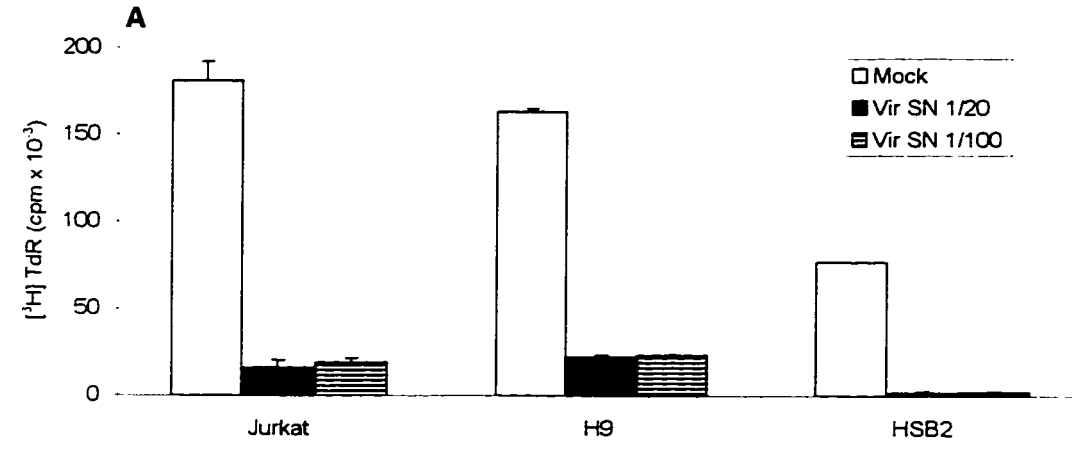

A
100
80
60
40
20
0
[3H] TdR (cpm x 10-3)
1.0E-01
1.0E-02
1.0E-03
1.0E-04
1.0E-05
1.0E-06
Supernatant Dilution

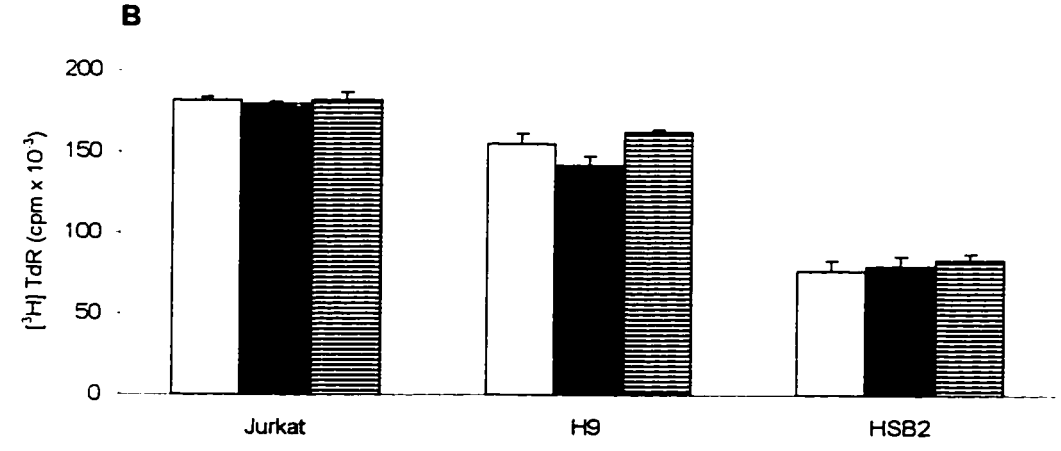

B
140
120
100
80
60
40
20
0
[3H] TdR (cpm x 10-3)
0
1.0E-02
5.0E-03
2.5E-04
2.5E-05
2.5E-06
2.5E-07
HHV6-UV Dilution

To determine if the suppressor factor present in supernatants from infected cells was heat-labile, supernatant from PBMC 72 hours post infection was filter sterilized and heated at 56°C for 1 hour prior to addition to T cell line cultures. Control supernatant from mock infected PBMC was similarly treated. The ability of the supernatant to mediate suppression of cellular proliferation was measured by a proliferation assay. As shown in Figure 15A, untreated supernatant was capable of suppressing Jurkat, H9 or HSB-2 proliferation by 90%, 86% and 97% respectively at 1/100 dilution as seen previously. After heat-inactivation, no suppression of proliferation was observed with the T cell lines (Fig. 15B) indicating that the suppressor factor(s) within the infected cell supernatant was heat labile and suggests it is a protein which requires its native conformation for action.

Since HHV-6 infected T cells expressed several potential immunosuppressive cytokines including IL-6, IL-10, TNF-α, IFN-γ (Fig. 2), corresponding anti-cytokine neutralizing antibodies were added to the culture supernatants to determine whether inhibition of cell proliferation was due to presence of cytokines in the supernatant. In addition, anti-human TGF-β neutralizing antibody was added, since TGF-β has also been shown to be a strong immunosuppressive agent (Whal, 1994). These blocking studies employed concentrations of anti-cytokine antibodies in excess of the neutralizing concentration specified on product sheets. Antibodies were added together with virus conditioned supernatant to PHA-stimulated T cells and cellular response measured by a proliferation assay after 4 days in culture. As shown in Figure 16, IL-6, IL-10, TNF-α, IFN-γ or TGF-β neutralizing antibodies failed to restore PHA responses, indicating that

Figure 15. Proliferative responses of T cell lines in the presence of heat-treated suppressor-factor containing supernatant. Jurkat, H9 and HSB-2 ($2x10^5$/ml) cells were exposed to supernatants derived from mock infected (□) PBMC at a 1/20 final dilution or from HHV-6 infected PBMC at 1/20 (■) or 1/100 (≡) final dilution. Supernatants harvested at three days post-infection were sterile filtered through a 0.2μm filter and an aliquot was heat-inactivated by heating at 56°C for one hour after which it was added to T cell line cultures (B) as were untreated sterile filtered supernatants (A). Cultures were incubated for 3 days then pulsed with 1μCi ^{3}H-thymidine for an additional 18 hours of culture. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SEM based on triplicate samples.


A
200
150
100
50
0
[3H] TdR (cpm x 10-3)
Mock
Vir SN 1/20
Vir SN 1/100
Jurkat
H9
HSB2


B
200
150
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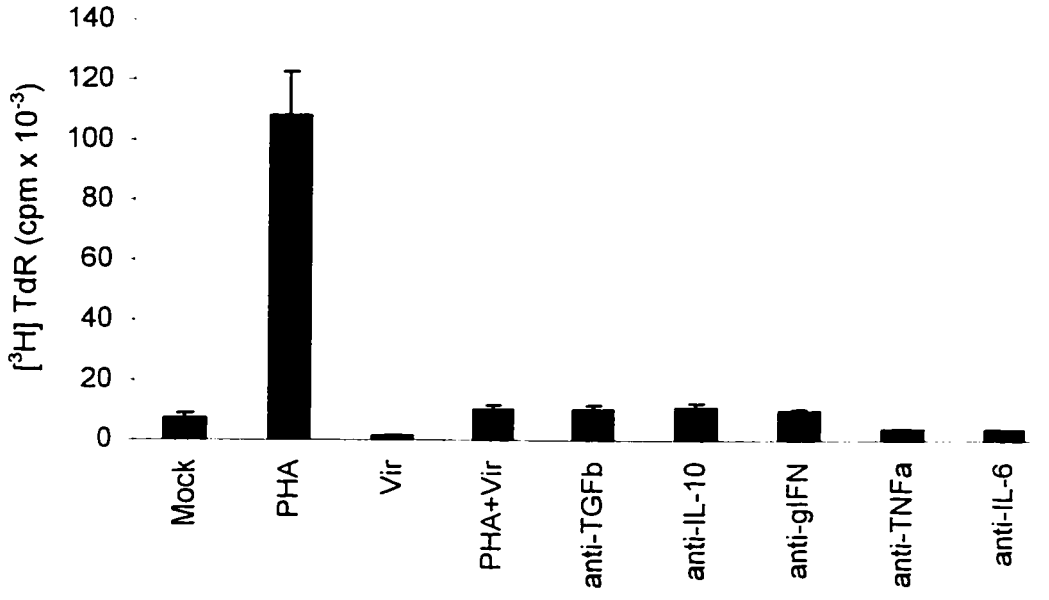
50
0
[3H] TdR (cpm x 10-3)
Jurkat
H9
HSB2

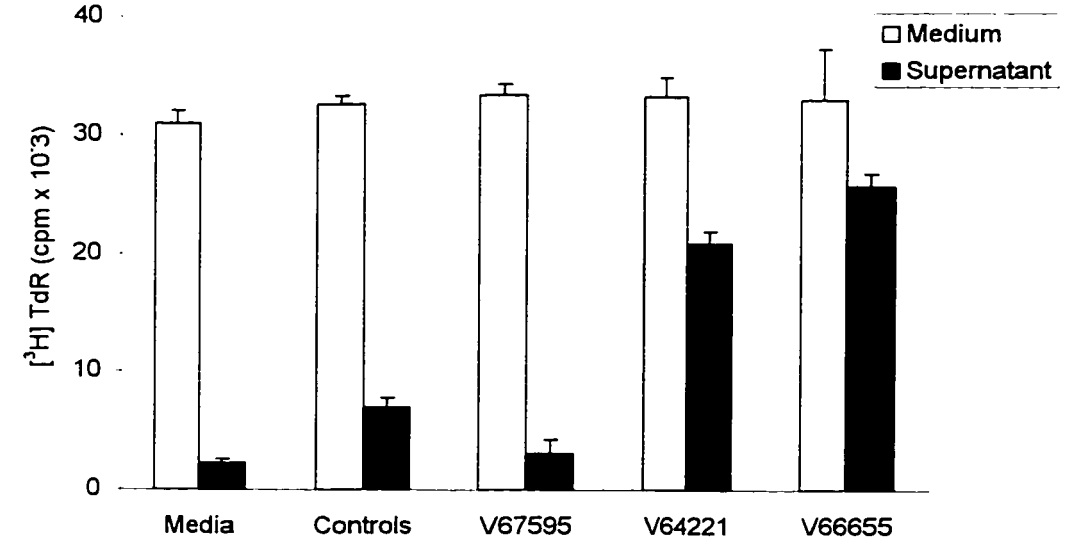
Figure 16. Contribution of endogenous cytokines to HHV-6-induced T cell anergy. Triplicate samples of purified T cells ($1x10^6$ cells/ml) were cultured with either 1/10 volume mock virus supernatant (Mock), PHA (1:200 v/v), 1/10 volume of HHV-6 conditioned autologous T cell supernatant (Vir), or PHA plus virus-conditioned supernatant (PHA+Vir), or PHA plus virus-conditioned supernatant (PHA+Vir) and 5 μg/ml of anti-cytokine neutralizing antibody specific for either human IL-6, IL-10, TNF-α, IFN-γ or TGF-β. Cells were cultured for 3 days and pulsed for an additional 18 hours with 1 μCi/well [^{3}H]-thymidine. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) ± SD based on triplicate samples.


140
120
100
80
60
40
20
0
[3H] TdR (cpm x 10-3)
Mock
PHA
Vir
PHA+Vir
anti-TGFb
anti-IL-10
anti-gIFN
anti-TNFa
anti-IL-6

HHV-6 suppression of T cell proliferation was not mediated by any of these five cytokines.

The lymphoproliferative inhibitory activity in infected cell supernatants is likely due to viral products or possibly virus-induced host products. To determine whether inhibition of lymphoproliferative response was due to viral antigens, anti-HHV-6 antibody-containing human sera was used to absorb viral proteins from virus conditioned supernatant which was then tested for its inhibitory effect on HSB-2 proliferation (Fig. 17). A virus conditioned HSB-2-derived supernatant that had previously been shown to suppress HSB-2 proliferation by over 90% was used as a source of "suppressor" viral proteins. Anti-HHV-6 human serum was obtained form pediatric patients with clinical symptoms consistent with HHV-6 infection. Their sera were tested for anti-HHV-6 IgG and IgM. Results of antibody screening and patient information are outlined in Materials and Methods. The antibody in the serum from patient or control samples was first coupled to Protein G-Sepharose and then added to "suppressor" factor containing supernatant or medium (control). The antibody-coupled beads with absorbed viral factors were pelleted and the depleted supernatant was added to HSB-2 cells at a 1:20 final dilution and proliferation measured by a proliferation assay. Maximum suppression of HSB-2 proliferation was 92.6% compared to medium exposed cells and this was considered to be 100% suppression. Values from supernatants treated with anti-HHV-6 serum were compared to this value. Control values in Figure 17 are the group mean from four healthy donors' sera. Their anti-HHV-6 antibody status was not tested. Results indicate that V64221 and V66655 serum samples (anti-HHV-6 antibody positive) were capable of

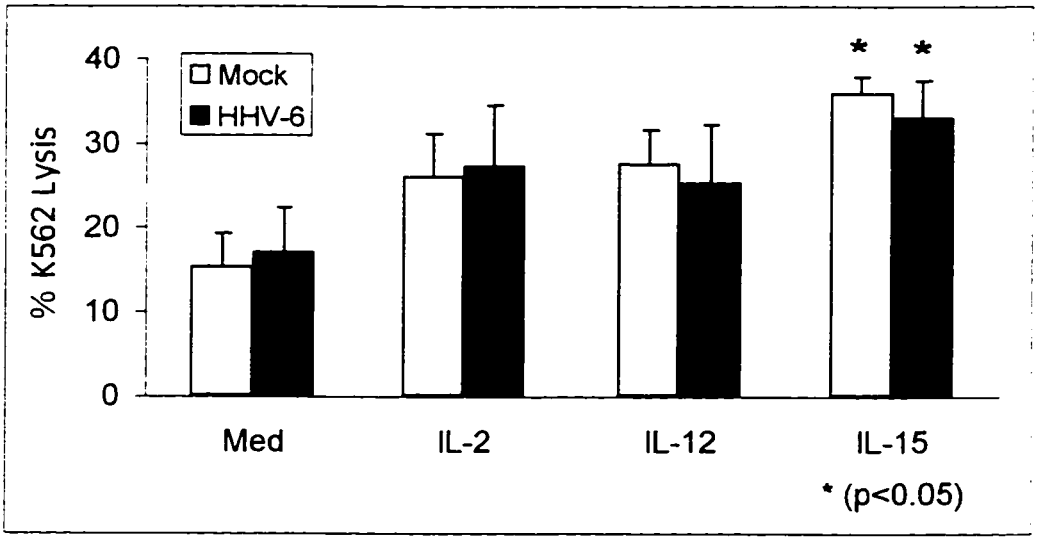
Figure 17. Proliferative response of HSB-2 cells to suppressor-factor containing culture supernatant following treatment with anti-HHV-6 antibody containing human serum. HSB-2 cells (10^5/ml) were exposed to suppressor supernatant that had been treated with either control or anti-HHV-6 human serum. The supernatant was derived from HSB-2 infected cells five days post-infection. Control or anti-HHV-6 human serum was first coupled to Protein-G Sepharose then incubated with supernatant to absorb out viral proteins. The treated supernatant was added to HSB-2 cells at a 1:10 final dilution and allowed to incubate for 4 days after which a proliferation assay was performed. Results are expressed as the mean [^{3}H]-thymidine incorporation (cpm) $\pm$ SD based on triplicate samples. Anti-HHV-6 antibodies IgG and IgM were measured by ELISA (Parker and Weber, 1993). Serum sample V64221 and V66655 are anti-HHV-6 antibody positive; V67595 is anti-HHV-6 antibody negative. Controls are the mean from four healthy donors whose anti-HHV-6 antibody status was unknown. Details of serum samples are described in Materials and Methods.


40
30
20
10
0
[3H] TdR (cpm x 10'3)
Medium
Supernatant
Media
Controls
V67595
V64221
V66655

reducing the suppressive activity on HSB2 proliferation by 60% and 76% respectively compared to media control. Serum sample V67595 with no anti-HHV-6 antibody did not affect the suppressive ability of the supernatant and suppression was equal to that seen with unabsorbed supernatant. Interestingly, the serum samples from the four healthy donors (control) reversed maximal suppression by 15%. This activity could be due to the presence of anti-HHV-6 antibody in the donor serum from an earlier primary infection or non specific blocking. These results support the idea that suppression is mediated by a soluble factor encoded by the virus.

HHV-6 suppressor factor strongly inhibits lymphocyte proliferation even when cells are stimulated with mitogens and cytokines. To determine whether this factor was also capable of inhibiting immune cell cytolytic function during acute infection, NK cytotoxicity was measured. NK cells provide a first-line of defence against initial and recurrent HSV infections (Lopez, 1975) by recognizing and killing virally-infected cells during acute infection (Trinchieri, 1989). NK cells found in PBMC from 3 different donors were cultured in complete medium for 72 hours in the presence or absence of autologous, donor-derived HHV-6 suppressor factor supernatant at a 1:10 final dilution. These HHV-6 suppressor factor supernatant treated lymphocytes (the "effectors") were harvested and tested for cytotoxicity against the NK-sensitive cell line K562 using a standard ^{51}-Chromium release assay. As shown in Figure 18 which represents the mean of triplicate samples from three donors, the HHV-6 suppressor factor had no effect (either inhibitory or stimulatory) on NK cell killing of K562 cells. Supplementation of the culture supernatant with IL-2, IL-12 or IL-15 resulted in slight enhancement of the NK cytolysis

Figure 18. Natural Killer/Lymphokine Activated Killer (NK/LAK) cytolytic activity of PBMC following exposure to HHV-6 suppressor factor containing supernatant and IL-2, IL-12 or IL-15. PBMC ($1x10^6$/ml) were cultured for 72 hours in the presence of 1/10 volume of mock supernatant (□) or HHV-6 suppressor factor-containing culture supernatant (■). Mock and suppressor factor supernatants were supplemented with either IL-2 (50 U/ml), IL-12 (10 ng/ml) or IL-15 (10 ng/ml). PBMC were harvested after 72 hours of incubation and triplicate samples were seeded in 200 μl of compete medium with $1x10^4$ ^{51}Cr-labeled NK-sensitive target cells, K562, at a target to effector cell ratio of 1:50. After 6 hours, 100 μl of culture supernatant was removed and cell-free ^{51}Cr measured. Results are expressed as the mean specific cell lysis ± SEM from triplicate samples for three separate donors. Statistical analysis of data was performed using a Student's *t*-test. Differences were considered significant when $p<0.05$.


40
30
20
10
0
% K562 Lysis
Mock
HHV-6
*
*
Med
IL-2
IL-12
IL-15
* (p<0.05)

with IL-2 and IL-12 treated cultures while only IL-15 treated cells reached significant increased lysis ($p<0.05$) compared to medium treated cultures. These results indicate that although IL-2, IL-12 or IL-15 did not restore the proliferative potential of peripheral T cells or lymphocytes, IL-15 was capable of eliciting immune killing function by generating LAK cells even in the presence of suppressor factor. These results suggest that IL-15 may, therefore, be an important element in the resolution of HHV-6 infection by activating NK cells.

IV. Discussion

IV.1. Summary

The results indicate that HHV-6 infection of T cells by either replication competent or incompetent virus induced secretion of several cytokines, namely, IL-1β, TNFα, IL-6, IL-10 and γIFN while IL-2, IL-12 and IL-15 were not detected in culture supernatants of infected cells, despite the presence of IL-2 and IL-15 mRNA. Virus infection did not inhibit mitogen-driven cytokine mRNA transcription or protein synthesis, but did inhibit mitogen-driven cellular proliferation. Specific alteration of cell surface molecules involved with the TCR, T cell activation, apoptosis and cell adhesion was not observed. HHV-6-infected cells were viable, non-apoptotic and when stimulated with PHA, morphologically appeared activated. In an effort to map out the defect in cell signaling, PHA, CD2, CD3, CD28 stimulation or post membrane activation pathways (phorbol ester and ionomycin) were tested and all were found to be impaired in infected cells in the presence or absence of exogenous IL-2. There appeared to be a general defective membrane function resulting in a T cell activation deficiency. Supernatants harvested from infected cells, filtered through a 0.2 μm filter to remove virions contained soluble suppressor factor(s) which rendered fresh, uninfected cells unable to proliferate in response to mitogen. The suppressor factor could be derived from infection of T cells, T cell lines, cord blood and PBMC and mediate suppression of lymphoproliferation. Coculturing HHV-6 exposed, γ-irradiated PBMC with untreated PBMCs inhibited mitogen and cytokine-induced proliferation suggesting the HHV-6 exposed cells secreted a factor(s) capable of

suppressing proliferation of bystander cells. The suppressor factor would begin to lose some of its suppressive effect on lymphocyte proliferation at a 1:2000 dilution and completely lose it at 1:10,000 dilution. Therefore, the factor was either produced in high amounts or was extremely efficient at anergizing the cells. The suppressor factor found in the supernatant was inhibited by heat treatment suggesting it required its native conformation for activity. The factor is not one of the known cytokines induced during infection as the addition of neutralizing anti-cytokine antibodies together with suppressor supernatant does not reverse suppression. The majority (up to 76%) of the suppression was relieved when suppressor supernatant was pretreated with anti-HHV-6 human serum from patients suffering from acute HHV-6 infection (or reactivation) suggesting a virally-derived or cellular induced factor. NK cell function was found not to be affected by the factor and cytotoxicity could be augmented with supplementation of the NK activating cytokine, IL-15. Infection with UV-treated HHV-6 suppressed lymphocyte proliferation as efficiently as competent HHV-6 but psoralen inactivated HHV-6 did not affect proliferation suggesting suppression of lymphocyte proliferation requires some virus transcription while cytokine induction can be mediated by virus binding and/or internalization only.

IV.2. Cytokine mRNA

HHV-6 infected T cells expressed IL-1β, IL-6, IL-10, IL-15, IFN-γ and TNF-α mRNAs. Virus infection of T cells did not affect mitogen-driven cytokine gene transcription or protein synthesis for the cytokines measured. Virus replication was not required for the induction of cytokine mRNA as demonstrated by infection with either UV or psoralen treated HHV-6. Furthermore, T cells infected with HHV-6 in the presence of PAA, an inhibitor of viral DNA polymerase, did not affect cytokine mRNA levels as compared to PAA-untreated HHV-6 infected T cells. The mechanism of virus-induced cytokine gene activation is unclear. Virus contact with cell surface molecules may lead to the activation of transductional events resulting in cytokine gene transcription.

Most cytokines have mRNAs with short half-lives and available evidence suggests that the IL-2 gene is regulated primarily at the level of transcription initiation, although some contribution from post-transcriptional mechanisms has been demonstrated. mRNA for IL-2 was detected in HHV-6 infected cells but was not translated and released into culture medium. This could be due to the instability of the message or impairment of some other post-transcriptional event. It has been determined that mRNAs encoding highly inducible proteins, including cytokines, adhesion molecules and protooncogenes share a conserved AU-rich sequence in their 3' untranslated regions that may play a role in regulating their stability (Asson-Batres et al., 1994). Lindsten et al (1989) demonstrated that stimulation through the CD28 pathway resulted in the stability of mRNAs for several lymphokines, including IL-2. It was determined that the AU-rich elements were shared by these mRNAs (IL-2, TNFα, γIFN and GM-CSF). AU-rich elements could mediate mRNA

instability by serving as a target for specific AU-binding factors and/or endonucleases. It should be noted that other mRNAs (e.g. *c-fos* and *c-myc*) with AU rich regions are not stabilized with anti-CD28 stimulation therefore this sequence element is not always sufficient to confer mRNA stability. Garcia-Sanz and Lenig (1996) showed that IL-2 expression is controlled at the translational level by differential ribosome loading. They show that T cells anergized by 12 hour incubation with calcium ionophore express IL-2 mRNA but do not synthesize the protein and are refractory to subsequent stimulation as shown by the lack of IL-2 production with TCR complex stimulation and lack of proliferation in response to allogeneic stimulation. They found IL-2 mRNA was not polysome-bound in anergized, stimulated cells as compared to non-anergized, stimulated T cells. Translational control of IL-2 mRNA may be a molecular mechanism by which anergy is attained. The lack of ribosome loading or the assembly of the initiation complex in HHV-6 exposed cells may contribute to HHV-6 induced T cell anergy. Translational regulation also plays a role in the expression of IL-15. Similar to IL-2 mRNA, IL-15 mRNA was detected in almost a constitutive manner, yet no protein could be detected. On analysis of the 5' region of IL-15 mRNA, Bamford et al (1996) demonstrated that the predominant IL-15 message produced by Hut-102 is a chimeric message joining a segment of the R region of the LTR of HTLV-1 with the 5' UTR of IL-15 placing the IL-15 gene under the regulatory control of the HTLV-I-R element. Analysis of the 5' UTR of normal human IL-15 demonstrated the presence of 10 upstream AUGs. The presence of such upstream AUGs in the 5' UTR of mRNAs has been shown to dramatically reduce the efficiency of translation. In the case of Hut-102, the HTLV-I-R/IL-15 fusion message

eliminated over 200 nucleotides of the IL-15 5' UTR including 8 of 10 upstream AUGs that normally attenuate translation. No upstream AUGs are present in the 5' UTR of IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, G-CSF, GM-CSF or γIFN (Kozak, 1991). Kozak (1992) suggests that upstream AUG codons represent a strategy by the cell to yield poorly translated mRNAs that encode critical regulatory proteins whose efficient expression might be hazardous to the cell.

IL-15 shares many biological activities with IL-2. In situations where IL-2 is lacking, such as in HHV-6 and HIV infections, IL-15 may be able to compensate by providing activating signals to cells via β and γ chains of the IL-2R. The presence of mRNA and lack of protein of either of these cytokines argues against differential regulation between these two genes.

IV.3. Cytokine Protein

HHV-6 infection of T cells by either replication competent or incompetent virus induces secretion of several cytokines. IL-1β, TNFα, IL-6, IL-10 and γIFN were consistently induced, while IL-2, IL-12 and IL-15 could not be detected in culture supernatants of infected cells. The high levels of γIFN produced with HHV-6 infection were not induced by IL-12 as no IL-12 was detectable in virus infected T cell cultures. This suggests an IL-12 independent mechanism for γIFN induction which could be based on specific viral antigenic stimulation. High levels of IL-10 have been shown to inhibit both the antigen-presenting and accessory function of monocytes and IL-2 production by T cells (Roncarolo, 1995). The inhibition of macrophage/monocyte APC function by IL-

10 would affect most aspects of T cell activation, including proliferation and cytokine synthesis suggesting IL-10 may be involved in the induction of anergy in T cells.

Induction of IL-1β, TNFα and IL-6 from T cells was consistently seen with HHV-6 infection. Flamand et al. (1991) reported an induction of IL-1β and TNFα but not IL-6 from PBMC infected with HHV-6. The discrepancy may be explained by the different target cells used (PBMC vs T cells) or the method of measuring IL-6 (ELISA vs biological assay).

HHV-6 has been implicated as an important cofactor in HIV progression, as well as a possible transactivating element for several other virally-associated diseases (Flamand et al., 1993; Chen et al., 1994). Several of the cytokines induced by HHV-6 have been shown to stimulate other viruses to replicate (Antoni and Rabson, 1994; Tanner and Menezes, 1994) and in the case of IL-6, TNF-α or IFN-γ may also prevent target cell lysis if present at high levels (Reiter and Rapport., 1993). In addition, IL-10 is known to block T-cell or NK-cell function through altered antigen presentation (Suzuki et al., 1995).

The observation that inactivated virus particles can up-regulate certain cytokines suggests that virus/cell contact is sufficient to activate second messengers, leading to the transcription and translation of the respective cytokine gene.

IV.4. Suppression of Cellular Proliferation by HHV-6 Infection

HHV-6 was consistently seen to have a suppressive effect on basal and mitogen-stimulated proliferation. This was repeatedly demonstrated with purified T cells, T cell lines, umbilical cord blood leukocytes or PBMC. UV-inactivated HHV-6 had similar

effects as wild type HHV-6 which rules out the need for complete virus replication to mediate this effect. When infected T cells are exposed to mitogen, morphologically they resemble their uninfected control counterparts, i.e. the cells are clumped together in a characteristic blast-like appearance. The cells are viable as shown by trypan blue exclusion and presumably host cell protein synthesis is not shut off as the mitogen-induced cellular clumping may require expression of adhesion molecules or some modification of existing molecules.

The T cell activating cytokines IL-2, IL-12 and IL-15 were not induced with HHV-6 infection. The suppression of lymphoproliferation after HHV-6 exposure was not due to a hole in the cytokine response repertoire of T cell activating cytokines, as exogenously added cytokines (IL-2, IL-12 and IL-15) did not restore cytokine or mitogen- responsiveness. This phenotype might be achieved by a downregulation of surface cytokine receptor or a functional uncoupling of the receptor from its intracellular signaling pathway. Alternatively, the inability to proliferate in the presence of sufficient cytokine may reflect a defect or absence of a second factor required by the cells to enter the cell cycle. Stimulation of infected T cells with monoclonal antibodies against CD3 and CD28 or using activators of PKC and calcium (PDB and calcium ionophore respectively) also failed to deliver necessary intracellular signals leading to lymphoproliferation. Supplementing these stimuli with IL-2 also did not result in T cell responsiveness. There appears to be a general surface impairment for the induction of T cell proliferation. It has previously been documented that TCR modulation leads to a transient unresponsiveness to both CD2- and CD28-mediated activation signals (Clevers et al., 1988). No modulation of

any important surface molecules mediating activation such as CD3, CD2 or CD28 was detected. Since the T cell activating agents used in this study bind to different specific receptors, it is unlikely that HHV-6 protein(s) block activation through binding to all of these structures.

IV.V. Suppressor Factor(s)

HHV-6-infected cells expressed a soluble suppressor factor which prevented T-cell proliferation and was capable of abrogating activation signals. To date, the nature or origin of the suppressor factor for HHV-6 is unknown. Based on the inability to reverse T cell proliferative dysfunction with specific anti-cytokine antibodies, it is unlikely that the suppressive factor is cellular IL-6, IL-10, TNF-α, IFN-γ or TGF-β. The experiments using UV-treated virus or filtered culture supernatants from autologous HHV-6 infected T cells and PHA or PHA plus cytokine, or the experiments involving plastic-bound protein derived from UV-treated HHV6 infected T cells suggest that the non-responsiveness of HHV-6 infected T cells to PHA or other cell mitogens (Flamand et al., 1995) is due to the presence of a suppressive factor induced upon T cell infection. The suppressive factor appears to act on a cell surface molecule, since plastic-bound protein was sufficient to induce lymphocyte suppression of proliferation.

The suppressor factor(s) could be generated from every cell type used i.e. T cells, T cell lines, PBMC and cord blood. The suppressive effect on lymphoproliferation could be titrated out and the agent mediating the suppression was UV and heat sensitive. The majority (up to 76%) of the suppression was relieved when suppressor supernatant was

pretreated with anti-HHV-6 human serum from patients suffering from acute HHV-6 infection (or reactivation). These data imply a virus-derived factor. The inability to completely reverse suppression with the anti-serum could be explained by the fact that the virion component may not be a good immunogen and did not induce sufficient antibody or not all viral "anergizing" ligands induced antibody. The suppressive effect may also not be solely mediated by a virion component. Virus binding to the cell may induce a cellular factor that participates in anergizing the cell. Therefore the anti-HHV-6 antibodies alone could not completely reverse the suppression.

Since HHV-6 encodes a number of proteins which have similarity to members of the Ig superfamily, chemokines, and chemokine receptors, it is possible that, like EBV, HHV-6 may have captured an immunosuppressive factor in order to blunt or abrogate acute immune-cell responses (Gompels et al., 1995). Interestingly, HHV-6 encodes a gene, U83, with structural features typical of the C-C chemokine subfamily which has the potential to be secreted, as well as two genes, U12, U51, which have homology to G-protein coupled receptors (Gompels et al., 1995). HHV-6 (and HHV-7) has been shown to induce EB1, a putative lymphocyte-specific G protein-coupled receptor in CD4− T cells (Hasegawa et al., 1994) that was originally reported to be induced by EBV infection in B cells (Birkenbach et al., 1993). The ligand for EB1 is unknown. It could be envisioned that HHV-6 uses molecular mimicry to evade immune responses by affecting cellular activation. Like other herpesviruses, HHV-6 suppression also appeared to involve loss of IL-2 expression; however, unlike CMV and EBV, where supplementation of IL-2 or the activation via the CD2 pathway reversed T-cell immunosuppression, these signals did not

appear to reverse the immunosuppression in T cells by HHV-6 (Perez-Blas et al., 1992; Timon et al., 1993). Whether the difference in the ability of CMV- and EBV-exposed T cells to respond to exogenous IL-2 is due to expression of a functional IL-2 receptor, or to differences in the primary viral target (i.e. B cells for EBV and large granular lymphocytes for CMV versus T cells for HHV-6) such that the HHV-6 T cells are directly impaired, remains to be tested. There may be different degrees or levels of anergic unresponsiveness such that the proliferative response of EBV or HCMV infected cells can be rescued because there is a "partial" block of cellular activation, as opposed to the "complete" block induced by HHV-6 exposure.

The *in vivo* significance of suppressor factors remains to be demonstrated. Suppressor cells and suppressor factors likely act together to form a suppressor network affecting various populations of immune cells in an antigen specific and non-specific way. Suppressor factors could suppress protective aspects of immunity, thus permitting sufficient virus spread to cause pathology. Whether these factors are produced *in vivo* or whether *in vitro* generated factors can modulate immunity *in vivo* has not been determined. Suppressor supernatant exposed NK cells were not affected in their ability to lyse a NK-sensitive cell line (K562) and their cytotoxic potential could be augmented by supplementation with a NK stimulating cytokine (IL-15). However, this same supernatant completely rendered T cells unresponsive to various activating stimuli. The effect on antigen-specific CTL was not investigated.

IV.6. Anergy

T cell anergy is characterized by a lack of certain functional responses when T cells are optimally stimulated through receptors required for full activation. Normally, the binding of IL-2 to its high-affinity receptor drives the progression of activated T cells through G_1 into S phase and ultimately into mitosis. To achieve this, many protein tyrosine kinases (PTKs) are activated following IL-2 binding to its receptor. The IL-2R subunits possess no intrinsic PTK activity of their own but are linked to at least three classes of PTKs, the Jaks and Syk- and Src-family members. IL-2 stimulation induces the coapproximation of the signal-transducing receptor subunits IL-2Rβ and γ and their associated PTKs, Jak1 and Jak3, respectively. This recruitment leads to the phosphorylation of tyrosines in the cytoplasmic domain on one or more receptor subunits triggering several downstream signaling cascades. Jaks are required for the phosphorylation and activation of the DNA binding Signal transducers and activators of transcription (Stat) factors (see Darnell et al., 1994 for review) and Ras. The Ras and JAK-Stat pathways are known to make their way to the nucleus, where they regulate the expression of specific genes related to IL-2 dependent T cell growth, survival or differentiation. HHV-6 renders cells unresponsive to IL-2 which would negatively affect IL-2 stimulated protein tyrosine phosphorylation, Ras activation, Stat factor activation and c-myc mRNA accumulation. Exactly which step is inhibited (from receptor to nucleus) is not known.

A minimum amount of IL-2 is required to commit T cells to proliferate. The lack of it results in anergy and "incompetence". Cells in early G_1 of the cell cycle contain high

levels of Kinase Inhibitor Protein-1 (KIP-1), which inhibits the activities of G_1 cyclin-associated cdks (Polyak et al., 1994). Cyclin-cdk complex activity ensure the orderly progression from G_1 to S phase (Kirschner, 1992). IL-2 is required for induction of further accumulation of various cyclin-cdk complexes as the T cells progress through G_1 (Ajchenbaum et al., 1993; Firpo et al., 1994). Continued IL-2 exposure decreases KIP-1 expression, thereby allowing the assembly of active G_1 cyclin-cdk complexes, which in turn drive the cell toward S phase (Nourse et al., 1994). The activated T cells cannot commit to S-phase entry until they receive a progression signal for KIP-1 downregulation, which is delivered through the IL-2R. HHV-6 may prevent the IL-2-induced decrease in KIP-1 and the subsequent activation of G_1 cyclin-cdk complexes and arrest the T cell in mid to late G_1. Anergy induction by insufficient IL-2 production (or an IL-2R signalling defect) is not the sole mechanism of unresponsiveness as induction of anergy following HHV-6 infection of the HSB-2 T cell line was IL-2 independent.

The classic model of anergy is where the TCR is occupied in the absence of costimulation. This leads to a failure to proliferate when restimulated. If CD4+ T cells recognize processed antigens presented by APCs that lack costimulators (B7), the T cells survive but are rendered incapable of responding to the antigen even if it is later presented by competent APC (Harding et al., 1992). Anergy can be induced with either immunogenic peptide and chemically fixed APC or altered peptide ligand and live APC (Jenkins and Schwartz, 1987; Sloan-Lancaster et al., 1993). For CD4+ Th1 clones and CD8+ CTL clones, anergy is caused by a block in IL-2 production, leading to a failure to activate $p21^{ras}$ which leads to a decrease in the activities of two of the mitogen-activated

protein (MAP) kinase pathways (Li et al., 1996; Fields et al., 1996). These data suggest that anergy can be induced at different nonexclusive levels. At the subcellular level, changes of phosphorylation patterns and lack of ZAP-70 recruitment have also been shown to play a role in the induction of anergy (Sloan-Lancaster et al., 1994; Cho et al., 1993). In some models of anergy, a failure to express transcription factors (e.g. NF-AT, AP-1, NF-κB) has been described (Becker et al., 1995; Wotton et al., 1995; Kang et al., 1992; Sundstedt et al., 1996). The lack of these factors may fail to allow for the assembly of a complete transcriptional complex on promoters regulating genes involved in T cell activation (e.g. IL-2).

The HHV-6-induced anergy rendered cells unable to respond to exogenous IL-2, despite expression of the receptor. This suggests the receptor is functionally aberrant and unable to transmit a proliferative signal when exposed to IL-2. IL-2 induces proliferative competence by binding to the p75 protein shown to be constitutively expressed on the surface of resting T cells. This interaction results in the rapid induction of various genes involved in T cell activation, including the gene coding for the α subunit. Inhibition of expression of the α subunit would prevent the assembly of the high affinity trimer capable of transmitting a proliferative signal by IL-2.

IV.7. NK Activity

The importance of natural immunity via NK cells in herpesvirus infections can be seen clearly in murine experimental models. Results re-emphasize the importance of NK cells in fighting herpesvirus infection, since HHV-6-infected or HHV-6 suppressor factor-

treated T cells were anergic, but NK cells treated with the same HHV-6 suppressive factor lysed the NK target cell K562. HHV-6 suppressor-treated NK cells were responsive to IL-15 as displayed by their enhanced cytolytic activity. This cytokine has been shown to enhance and induce cytolytic function, activate NK cells, as well as increase mitogenic effects on NK cells (Giri et al., 1995a; Howard et al., 1993; Trinchieri et al., 1992; Valiante et al., 1993). These results indicate that although IL-15 was unable to generate a mitogenic effect on infected T cells, addition of it was capable of promoting NK cytotoxic potential, even in an environment of suppressed T cell proliferation. Since IL-2, IL-12 or IL-15 were not detected following HHV-6 infection of its primary target (i.e T cells), an alternate source could be from other virally-infected or HHV-6-stimulated target cells. One possible cell type which may be an important target or source is the peripheral blood dendritic cell. Work with other herpesviruses has shown that virally-stimulated dendritic cells are a rich source of IFN-α and are crucial for the recognition and destruction of infected cells by NK cells (Feldman and Fitzgerald-Bocarsky, 1990). Interestingly, HHV-6 infected individuals showed increased levels of IFN-α in their serum during the acute febrile phase of the illness (Takahashi et al., 1992), leading one to speculate that, although HHV-6's primary target may be unable to properly alert immune cell to react to HHV-6, a circulating or resident dendritic cell stimulated by virus or viral antigens may act as a surrogate NK activator by producing IL-2, IL-12, IL-15 or IFN-α. These cells would then prompt the destruction of other virally-infected targets, including T cells.

IV.8. Model of HHV-6 Pathogenesis

HHV-6 induces a number of proinflammatory and immunoregulatory cytokines. Under such conditions, one can envisage HHV-6 promoting replication of endogenous viruses or itself, through the stimulation of viral-lytic inducing cytokines. HHV-6 could further promote viral spread by preventing the lytic cell from being lysed due to decreased target-cell sensitivity or alteration of viral antigen presentation. In addition the induced cytokines could contribute to the systemic effects of acute HHV-6 infection with IL-1β mediating fever and TNFα and IL-6 mediating acute phase responses. Thus, certain HHV-6 induced cytokines together with the induction of T cell anergy, could create an environment for the progression of virus infection. Exposure of T cells to HHV-6 allows cytokine production in the absence of proliferation. Whether anergic T cells retain cytolytic function remains to be tested. The inhibition of proliferation is a key control mechanism used for anergy. By preventing clonal expansion, HHV-6 controls amplification of the immune response against itself. This emphasizes the relevance of functional anergy in an *in vivo* setting as an immune response depends on clonal expansion for amplification of responses. In a non-viral setting, the ability of T cells to be anergized is an efficient way of controlling autoimmune responses and self-reactive lymphocytes *in vivo*. The viral ligand(s) and/or secreted factor(s) responsible for T cell anergy are not presently known. It is not clear if anergy is mediated by aberrant cytokine receptors, a failure of protein tyrosine phosphorylation, Ras or Stat factor activation or nuclear cyclin-cdk complex activation. From titration experiments, the suppressor factor(s) appear to be effective at very low concentrations and causes immunosuppression in the absence of

direct viral infection. If the factor is identified, neutralization of it could be beneficial in an acute HHV-6 infection, or it could be used where suppressing the immune response is desired (e.g. organ transplantation, autoimmune diseases). The lack of IL-15 (and IL-2) expression by T cells, which in turn stimulated NK cells, indicates a potentially critical role for this cytokine and NK cells in primary HHV-6 infection. In light of these *in vitro* results, it is tempting to propose that NK function-enhancing cytokines could serve as immunotherapeutic candidates for the intervention of severe HHV-6 infection. Whatever the mechanism by which HHV-6 induces anergy, further studies are required to better understand the immunopathogenesis triggered by HHV-6 infection and the consequence of T cell anergy.

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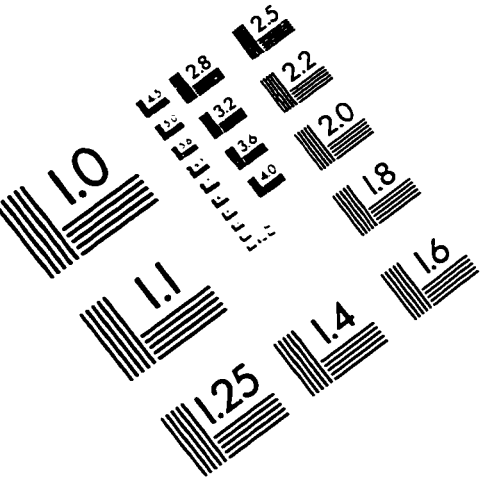
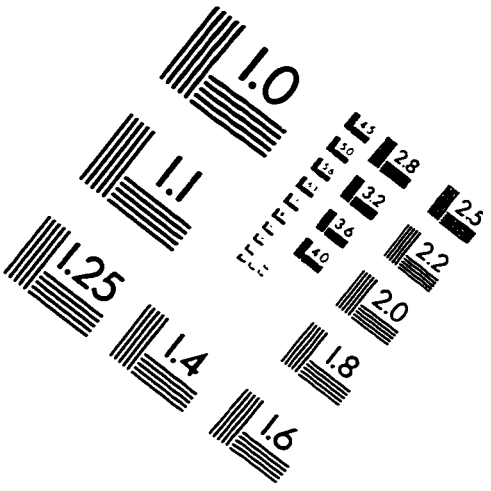
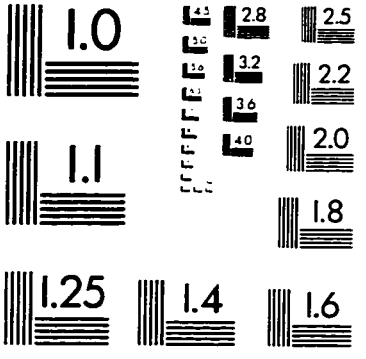
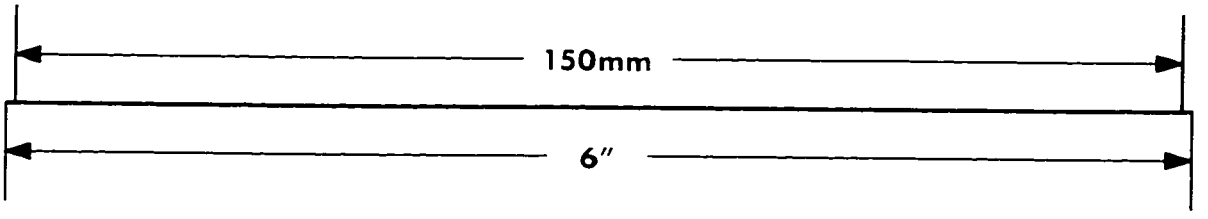
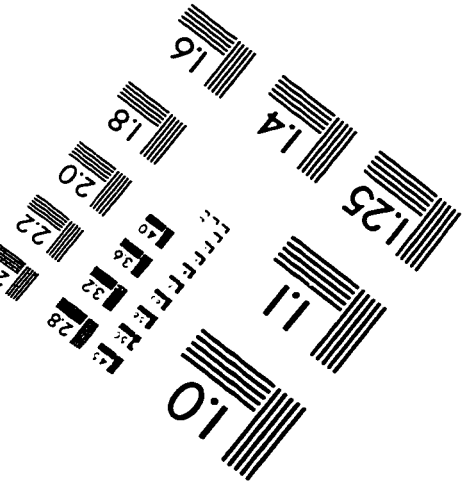
IMAGE EVALUATION TEST TARGET (QA-3)

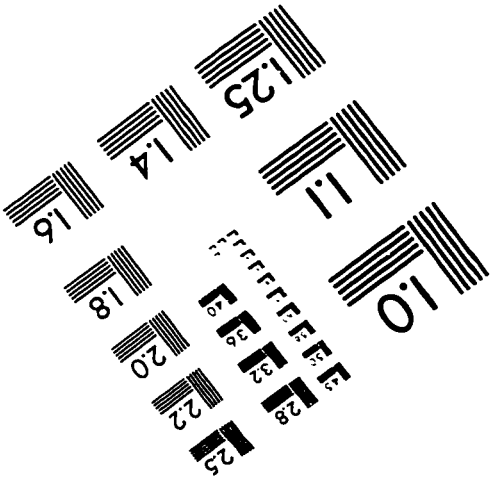












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989