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Dudding, Lindsay Rose

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THE EFFECT OF HUMAN CYTOMEGALOVIRUS INFECTION OF HUMAN MONOCYTES ON THE EXPRESSION OF MONOCYTE-ASSOCIATED MEDIATOR GENES WITH PARTICULAR REFERENCE TO INTERLEUKIN 1

Lindsay Rose Dudding

A Thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg for the Degree of Doctor of Philosophy

Johannesburg, 1989

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Lindsay Dudding

Lindsay Rose Dudding

30th day of November 1989

DEDICATION

TO MY PARENTS AND SISTER

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ABSTRACT

Monocytes and tissue macrophages play important roles in host defence against virus infections and can respond to infections by secretion of inflammatory mediators. The effect of HCMV infection on monocyte cytokine production was therefore studied at functional and molecular levels. This study primarily investigated the influence of HCMV infection on IL-1β production and expression in peripheral blood monocytes and the promyelocytic cell line, ML-3. The expression of several other inflammatory response genes, TNFα, CSF-1, MAD-2, MAD-6 and MAD-9 was investigated in ML-3 cells. LPS-stimulated and unstimulated monocytes infected with a clinical isolate of HCMV showed expression of HCMV IE antigens and were significantly more suppressive for lymphocyte proliferation than were strain AD169-infected monocytes, which rarely expressed detectable HCMV IE viral antigens. HCMV infection of LPS-stimulated and unstimulated monocytes resulted in abrogation of functional IL-1 bioactivity, with the effect being marked in LPS-stimulated monocytes infected with the clinical isolate of HCMV. Addition of IL-1 to infected, stimulated monocytes completely restored lymphoproliferative responses to PHA, whereas addition of this monokine to infected, unstimulated cells could not restore this response. At the level of IL-1β steady state mRNA expression, both RNA dot-blot

and in situ hybridization studies demonstrated that infection of monocytes with HCMV lead to sustained expression of IL-1β mRNA for up to 96 hr, which contrasted markedly with mock-infected or LPS-stimulated monocytes. Exposure of ML-3 cells to virus prior to induction of differentiation had little influence on mediator gene expression. However, induction of the macrophage phenotype by pretreatment of ML-3 cells with the phorbol ester, PMA, followed by HCMV challenge resulted in a greatly extended period of expression of IL-1β, TNFα, MAD-9 and CSF-1 but not MAD-6 and MAD-2. Constitutively expressed genes such as lysozyme and actin were not similarly modulated. Flow cytometric analysis of the intracellular levels of IL-1β protein in ML-3 cells indicated that more protein was produced in infected cells than in uninfected controls. Enhanced levels of the intracellular form of IL-1β in monocytes was confirmed by Western blot analysis. Cotransfection experiments were performed using IL-1β -CAT chimeric plasmids together with plasmids encoding HCMV IE gene region products. Transactivation of the IL-1β gene by region 2 of the IE gene was observed in ML-3 cells that had been induced to differentiate prior to transfection. No stimulation of IL-1β promoter activity was observed in ML-3 cells that were undifferentiated prior to transfection.

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1.0.0. INTRODUCTION

1.1.0. General characteristics of the cytomegalovirus (CMV) group

The family of Herpesviridae is divided into three subfamilies consisting of the herpes simplex viruses (Alphaherpesvirinae), the cytomegaloviruses (Betaherpesvirinae) and the lymphoproliferative virus groups (Gammaherpesvirinae). Each subfamily has been taxonomically named according to its biological properties (Roizman, 1982).

Human herpesvirus 6 (HHV-6) is a new herpesvirus, antigenically distinct from other human herpesviruses and has been isolated recently from peripheral blood lymphocytes of patients with the acquired immunodeficiency syndrome (AIDS) (Lopez et al., 1988; Tedder et al., 1987), from patients with lymphoproliferative disorders (Salahuddin et al., 1986) and from infants with exanthem subitum (Yamanishi et al., 1988).

The main characteristics of the cytomegalovirus (CMV) group are the following (Mathews, 1979):

1) The complete virion is 150 to 200 nm in diameter,

and consists of an inner core, a capsid and an envelope. The core contains the DNA genome which has a molecular weight of 150 x 10^6 with a G + C ratio of 50%. The virus nucleocapsid is an icosahedron (90 to 110nm) consisting of 162 capsomere subunits. The capsid is surrounded by the tegument which is enclosed by a lipid bilayer envelope.

2) The reproductive cycle is slow (>24 hr) and is characterised by slowly forming progressing foci in cell culture and enlargement of the infected cell in vivo and in vitro (cytomegalia). Inclusion bodies containing DNA may be present in the nuclei and cytoplasm late in infection.

3) Mouse cytomegalovirus (MCMV) persists in the salivary glands of mice and can also latently infect mouse macrophages (Brautigam et al., 1979). The site of human cytomegalovirus (HCMV) persistence is much less certain. Epithelial cells in salivary glands and renal tubules have been suggested as possible sites (Sissons et al., 1986).

4) CMV displays strong species and cell specificity. In cell culture, the virus usually grows best in fibroblasts of the species from which it was originally isolated, but exceptions do exist.

1.1.1. Medical Importance

The genus human cytomegalovirus (HCMV) is recognised as an important pathogen.The incidence of HCMV infection of humans is high, but most postnatal infections are acquired without clinical symptoms (Huang and Pagano, 1977). During their lifetime, 60 - 80% of individuals in developed countries and almost 100% in developing countries become infected with HCMV (Peckham et al., 1983 ; Griffiths et al., 1985). After primary HCMV infection in vivo, the virus often establishes a latent existence which is clinically important (Diosi et al., 1969). The severe diseases caused by HCMV frequently result from reactivation of latent virus, and are usually associated with immunosuppression due to a variety of conditions such as malignant disease, chemotherapy, organ transplantation (Ho, 1982) and the acquired immunodeficiency syndrome (AIDS) (Drew et al., 1985). HCMV is the causative agent of a wide variety of diseases ranging from the classical cytomegalic inclusion disease to intrauterine death, prematurity, congenital defects, infectious mononucleosis, postperfusion syndrome, and interstitial pneumonia in transplant patients (Rapp, 1980; Weller, 1971). In kidney, bone marrow or heart transplant pacients and patients with malignancies or connective tissue disease who are maintained on immunossupressive agents, HCMV

infection is commonly associated with organ rejection or loss. Infection also contributes to mortality and predisposes these patients to severe or fatal bacterial, fungal and parasitic infections by causing further immunosuppression.

Drug therapy of herpesviruses has recently advanced with the development of nucleoside analogs that are active only in herpesvirus infected cells (Biron et al., 1985). The most recent therapy has used ganciclovir, an acyclovir analogue, that is active against HCMV and all herpes viruses (Mar et al., 1983). The mechanism of action has not been fully elucidated but appears to be due to the selective phosphorylation of the drug to ganciclovir triphosphate in HCMV infected cells but not in uninfected cells (Biron et al., 1985). It is a more potent inhibitor of HCMV in vitro than acyclovir and has exerted an antiviral effect against HCMV in bone marrow and organ transplant recipients (Erice et al., 1987). It has also been used in clinical trials in patients with AIDS who have serious HCMV infection (Laskin et al., 1987). Clinical trials have also used vidaribine monohydrate (Meyers et al., 1982), acyclovir (Wade et al., 1982 ; Balfour et al., 1982), interferon (Meyers et al., 1980) or combinations of these various agents (Shepp et al., 1984). However, none of these agents has exerted the same antiviral effect as that shown by ganciclovir.

The prevention of primary infection by means of vaccinati[illegible] presumably depend on antibody recogniz[illegible] izing determinants on the virion (Sissons e[illegible] The most suitable vaccine would be a subunit v[illegible] e of live HCMV as a vaccine has been reported by [illegible] et al. (19[illegible]. There are however numerous draw[illegible]cks to this type of vaccine. The extent of attenuation of the strains is uncertain, as well as the biological pro[illegible]erties and ability to establish latency.

In addition to the clinical manifestations mentioned, HCMV has been implicated with malignant disease by virtue of its ability to transform cells in vitro and the finding of HCMV DNA, RNA and virus specific antigens associated with Kaposi's sarcoma, prostatic adenocarcinoma, cervical and colon cancers (Huang et al., 1984). These findings have more recently been substantiated by Fletcher et al. (1986) who have detected restriction endonuclease sites colinear with virion DNA in a small percentage of histologically abnormal cervices. Morphological transformation studies have failed to demonstrate a viral oncogene, a virus - coded transforming protein or any sequence of DNA that uniquely transforms cells according to "one - hit" kinetics. The transformed cells are however all oncogenic in the host animal and in immunocompromised

mice (Macnab, 1987). Transforming domains among the herpesviruses have been localised in small DNA fragments (Nelson et al., 1984; Galloway et al., 1984) and have stem/loop structures bounded by direct repeats. The morphological transforming region of HCMV DNA (mtr) consists of three regions mtrI, mtrII and mtrIII which have been mapped in the long unique region of the viral genome. Razzaque et al. (1988) have identified the mtrII of HCMV. In view of the transcriptional control signals and stem/loop structures that have been identified in mtr II, it is possible that mtr II transforms cells by increasing the transcription of critical cellular genes or may induce transformation by altering the structure of host DNA through fusion and gene rearrangement (Jariwalla et al., 1986 ; Bejeck and Conley, 1986). Molecular studies have also suggested a role for HCMV in carcinogenesis. The major enhancer of HCMV increases transcription of cellular genes (Boshart et al., 1985) which may be of great importance in cell behaviour during the progression of carcinogenesis.

1.1.2. Structure of HCMV

1.1.2.1. DNA of HCMV

The HCMV genome consists of double stranded DNA with a molecular weight of 150-155 x 10^6. It is a linear molecule consisting of a long unique (L) and a short unique (S) component which are covalently linked (Figure 1). Each component may be inverted which gives four different isomeric arrangements of the molecule. The L and S components are flanked by different repeat sequences that are inverted relative to each other (Stinski et al., 1981). The biological significance of this DNA structure is unknown. A number of strains of HCMV have been isolated including the Davis, Esp, Kerr Towne and AD169 strains. The commonly used laboratory strains of HCMV (AD169, Towne and C87) had approximately 80% genome homology when analysed by nucleic acid reassociation kinetics (Huang et al., 1976).

1.1.2.2. Structural Proteins of HCMV

Many of the structural proteins of HCMV have been determined using chemical denaturation of virions purified by physical methods (Gibson, 1983; Irmiere and Gibson 1983) (Table 1). More recently, Wright et al. (1988) have identified the proteins encoded by the 2.2 kb early transcript of AD169. The proteins were phosphorylated and had molecular weights of 84K, 50K, 43K and 34K. They were associated predominantly with the nuclei of infected cells.

Figure 1
Key

S	- Bam H I
Q	- Taq I
R	- Eco RI
IR_L	- inverted repeats of long and short
IR_S	portions of the genome
TR_L	- terminal repeats of long and short
TR_S	portions of genome
U_L	- unique long and short regions
U_S	

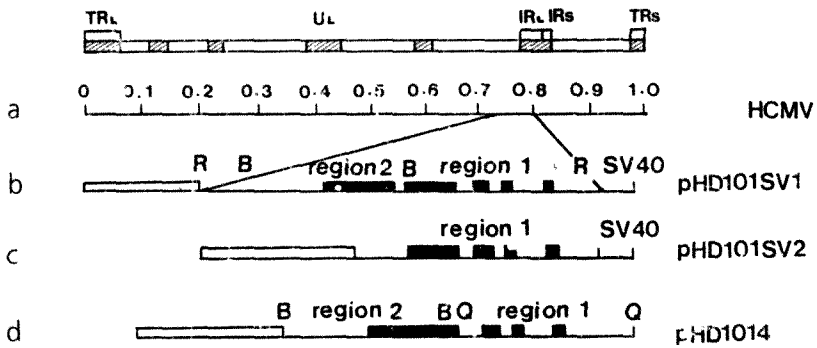

Figure 1 Schematic representation of the HCMV genome and 1E plasmid constructs (after Davis *et al.*, 1987).

(a) Fractional length of the HCMV genome

(b) Plasmid pHD101SV1. Plasmid sequences derived from pBR 322 (open box) and RNA transcripts corresponding to HCMV 1E regions 1 and 2 (closed boxes) are shown right to left.

(c) Plasmid pHD101SV2. The *Bam* HI fragment from region 2 of pHD101SV1 has been deleted to form pHD101SV2.

(d) Plasmid pHD1014. The *Bam* HI fragment from region 2 of pHD101SV1 was inserted downstream from the *Taq*I fragment containing the promoter and first three exons of 1E region 1.

M_r	Provisional name	Virion	NIEP	Dense Body
212 000	High molecular weight protein	+	+	
153 000	Major capsid protein	+	+	
149 000	Basic phosphoprotein	+	+	
115 000	-----	+	+	
74 000	Matrix protein	+	+	
39 000	Matrix protein	+	+	+
36 000	Assembly		+	
34 000	Minor capsid protein	+	+	
8 000	Minor capsid protein	+	+	

NIEP - Non infectious envelope particle

Data from Gibson (1983) and Irmiere and Gibson (1983)
(After Griffiths and Grundy (1987))

Table 1 Major Structural proteins of virus particles

Three distinct families of disulphide-linked glycoprotein complexes have been observed in the envelope of HCMV and have been designated gcI, gcII and gcIII (Gretch et al., 1988). The three complexes are almost certainly important targets of the human response to HCMV infection (Gretch et al., 1988). The gcI complex contains the gp55 gene product which has amino acid sequence homology with HSV gB, EBV gB, VZV (varicella zoster virus) gpII and pseudorabies virus gpII suggesting that the gcI family may play a role in the fusion of viral and cellular membranes. Functional roles for gcII and gcIII have not yet been assigned.

Each protein of HCMV will ultimately be identified as α, β or γ, being derived from α, β, or γ primary genomic transcripts respectively. Most of the structural proteins are classified as γ. Examples of β proteins are the virion DNA polymerase, the 50K phosphorylated DNA-binding protein (Gibson, 1983) and the 140K non-phosphorylated protein which binds single-stranded DNA (Anders et al., 1986). The most prominent α protein is phosphorylated and has a molecular weight of between 70 KDa and 79 KDa (Michelson-Fiske et al., 1977; Stinski et al., 1983).

The assembly of proteins to form progressively more mature A,B and C capsids is shown in Figure 2. Two morphological forms other than virions can be identified when HCMV is passaged in cell culture. One

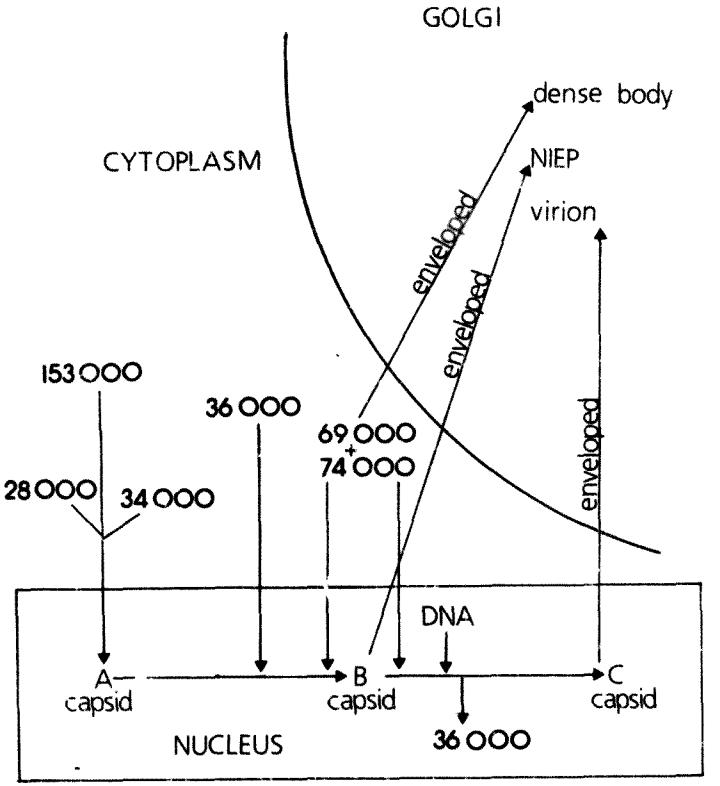

Figure 2
Schematic outline of assembly of CMV particles (after Griffiths and Grundy, 1987)
Numbers represent the Mr of identified proteins

is the dense body, which is much larger (300 nm) and more pleomorphic than the virion. More than 90% of its protein is the 69 KDa matrix protein together with envelope glycoprotein (Irmiere and Gibson, 1983). Dense bodies do not contain nucleic acid or a nucleocapsid. The second form is known as a non-infectious enveloped particle (NIEP) which resembles a virion without a central core containing DNA. The NIEP is thought to be an enveloped B capsid containing an excess of the 36K assembly protein.

1.1.3. Regulation of HCMV Gene Expression

HCMV undergoes a sequential order of gene expression in permissively infected cells (McDonough and Spector, 1983 , Wathen and Stinski, 1982). The first genes to be expressed are the α or immediate-early (IE) genes (Figure 1) which can be transcribed in the absence of de novo protein synthesis (McDonough and Spector, 1983 ; Stinski et al., 1983 ; Wilkinson et al., 1984). The products of the α genes are those the virus requires to take over control of host cell macromolecule synthesis (Griffiths and Grundy, 1987). The major site of IE transcription for AD169 and Towne is located in the long unique segment (Stinski et al., 1983), is mapped at coordinates 0.71 to 0.75 (Figure 1) and encodes at least three transcription units designated IE1, IE2 and IE3 (Wathen and Stinski, 1982 ; Stenberg et al., 1984). A fourth IE transcription unit

initiating in this region specifies a 5 kb RNA (Jahn et al., 1984).

IE1 encodes a 72 kDa nuclear phosphoprotein (Stinski et al., 1983 ; Stenberg et al., 1984) and is efficiently expressed following either infection or transfection of the isolated gene into tissue culture cells. The expression is mediated via a strong enhancer element consisting of different subsets of repeats that can independently substitute for enhancer activity (Boshart et al., 1985 ; Stinski and Roehr, 1985). An HCMV component further enhances IE gene expression in trans via these repeat sequences (Stinski and Roehr, 1985).

IE2 is immediately downstream of IE1 and consists of two subregions IE2a and IE2b (Hermiston et al., 1987). Transcription is highly complex with different transcripts being produced as a result of differential mRNA splicing. Predominant transcripts consist of all four exons of IE1 but there are also transcripts consisting of the first three exons of IE1 linked to IE2a (Hermiston et al., 1987).

An important phenomenon that has been observed in the infection of nonpermissive cells is the expression of α and β genes only. Transcription of the late genes is blocked. This has been shown in human monocytes and lymphocytes after in vitro infection (Rice et al., 1984; Einhorn and Ost, 1984).

Early or β genes (Figure 1) are transcribed before the onset of viral DNA replication and their transcription requires prior expression of one or more of the genes. Products of the β genes are required to control production of daughter virions (Griffiths and Grundy, 1987). The mechanism of this activation as well as the *cis*-acting elements of the HCMV early genes responsible for that temporal regulation have not yet been fully elucidated (Staprans *et al.*, 1988). These investigators have shown thus far that the HCMV major IE region can *trans*-activate the 2.2 kb early RNA promoter. Further studies will determine if additional viral or cellular factors are involved in the regulation of HCMV 2.2 kb early RNA transcription.

The γ or late genes (Figure 1) code for the structural components of the virion (Griffiths and Grundy, 1987). Geballe *et al.* (1986) have shown that expression of the late genes is controlled by post-transcriptional events. Two late genes encoding infected cell protein (ICP)27 and ICP36 are transcriptionally active at early times (4h post infection) but their expression is delayed. This may depend on post-transcriptional influences such as transcript transport to the cytoplasm, transcript accumulation, differential polysome association or differential stability of mRNA.

1.1.4. Trans-activation by the HCMV Immediate-Early Gene Products

The region of the HCMV genome which reportedly activates heterologous promoters in transient assays is the Hind III E fragment for strain AD169 and Xba I E fragment for the Towne strain (Everett, 1984). This region of the genome has at least four transcription units within approximately 20 kb (Stinski et al., 1983). Three of these units are positioned downstream of the major IE promoter regulatory region which contains cis-acting enhancer elements (Boshart et al., 1985; Stinski and Roehr, 1985). These HCMV genes have been designated IE1, IE2 and IE3 (Stinski et al.,1983). It has been postulated that one or more of these genes may function to regulate HCMV by stimulating transcription from viral and other promoters. Numerous studies have proposed that IE1 might be responsible for this function (Everett, 1984; Spector and Tevethia, 1986; Stinski et al., 1983). However, IE1 is expressed at a much higher level than is necessary for most trans-acting proteins, and reaches maximum levels 5-6 hrs after infection, long before extensive transcription of the genome begins. When tested in permissive cells, the IE1 gene product had little stimulating effect on the adenovirus E2-inducible promoter (Hermiston et al., 1987). IE2, however, is

made at levels more consistent with its role as a regulatory protein and reaches a maximum level of transcription 48 to 72 hr postinfection, when viral gene expression is at its highest (Wathen and Stinski, 1982). It has been found to express a regulatory factor that significantly stimulates the adenovirus E2 promoter as judged by expression of the chloramphenicol acetyltransferase (CAT) gene linked to this promoter (Hermiston et al., 1987). There are protein products of various sizes associated with IE2. Two of these of 27 and 30 kDa coded for by the IE2a gene can independently stimulate the adenovirus E2 promoter, as judged by CAT activity.

Pizzorno et al.(1988) have also shown that one of the phosphorylated nuclear proteins encoded by the major IE gene region acts as a powerful but nonspecific trans-activator of gene expression. In transient CAT assay experiments, the Hind III C fragment of HCMV Towne strain DNA, encompassing the IE1 and IE2 coding regions (exons 1 to 5), was able to stimulate expression from a variety of promoters. These included the human beta interferon gene. The IE1 coding region alone (exons 1 to 4) was inactive, but trans-activation was restored by insertion of the IE2 coding region (exon 5) in the correct orientation downstream from IE1.

Davis et al. (1987) showed that the HCMV IE gene region stimulated expression of the CAT gene linked to the human immunodeficiency virus (HIV) promoter. The HIV proviral genome has a promoter located within the 5' - long terminal repeat (LTR) that is trans-activated by the HIV-encoded gene tat (trans-activating transcriptional activator). Sequences located on the 3' side of the mRNA start site termed transcriptional activating region (TAR) are necessary for trans-activation by tat to occur (Chen, 1986). The HCMV IE gene region continued to trans-activate the HIV promoter even when the tat-responsive sequence had been deleted, which suggested that the HIV tat and HCMV IE region trans-activated the HIV promoter using different mechanisms. It was also found that the IE region 2 gene products could trans-activate the HIV promoter independently of the IE region 1 gene products.

1.1.5. Replication in Nonpermissive and Permissive Cells of Human Origin and Latency of HCMV

In the human host HCMV can infect a variety of cell types and presumably enters both nonproductive (nonpermissive) and productive (permissive) cells. Several studies have proposed that HCMV early gene expression but not late gene expression occurs in nonproductive cells (Mocarski and Stinski,1979; Wathen

et al., 1981) and that the nonproductive cell may favour latency of the viral genome.

HCMV frequently establishes a latent infection after an asymptomatic infection in a healthy individual. It can be reactivated, usually in association with immunosuppression to cause interstitial pneumonia and systemic disease (Ho, 1982). The site of latency and the molecular mechanisms of the establishment and maintenance of HCMV latency have yet to be determined. Seronegative individuals have been infected via blood transfusions from healthy seropositive donors (Stevens et al., 1970). This has led to speculation that peripheral blood mononuclear cells (PBMC) in asymptomatic individuals may be a resevoir for latent HCMV. Hybridization experiments using a cDNA probe specific for IE transcripts of HCMV have suggested that natural infection in healthy seropositive individuals may result in a low frequency of circulating PBMC that express HCMV RNA (Schrier et al., 1985). This finding has several implications. First, a population of cells capable of harbouring HCMV has been identified. Second, the presence of primarily HCMV IE RNA transcripts indicates that virus replication is restricted. Transfusion-mediated transmission suggests that infectious HCMV can be

reactivated from a lymphoid population. The biological mechanism remains unclear.

Smith (1986) attempted to define the characteristics which render a cell permissive for HCMV. A number of human cell lines differing in morphology, ploidy and extent of differentiation were screened. High titre HCMV replication occurred in a well-differentiated, diploid epithelial cell line derived from normal human colonic mucosa. Aneuploid cell types were all nonpermissive. Not all fibroblastic cell lines were permissive for HCMV. When permissive and nonpermissive cell types were cocultured prior to infection, neither cell type released soluble mediators capable of reversing the HCMV susceptibility of the other cell type.

Several investigators have attempted to determine an in vitro HCMV latency model in an attempt to establish which cells harbour HCMV in the latent state, the mechanisms involved in reactivation and the state of the latent genome in persistently infected cells. Tanaka et al. (1987) attempted to establish an in vitro model using a human epithelial thyroid papillary carcinoma cell line (TPC-1). HCMV was reactivated from latently infected TPC-1 cultures by decreasing the incubation temperature from 40.5 C to 37 C, with cultures subsequently entering into virus persistent

infection. Virus-specific DNA polymerase (an early viral protein) was not induced in latently infected TPC-1 cultures, suggesting that the block in replication occurred at the early stages. Latently infected cells were susceptible to superinfection by homologous and heterologous strains of HCMV. In persistently infected cultures, 38% of the cells could be lysed by reaction with HCMV immune serum and complement. Complement-mediated immune cytolysis could not be detected in latently infected cultures. Their data suggested that a temperature sensitive cellular function(s) that controls the expression of the HCMV early functions plays an important role in the maintenance of the HCMV genome in the latent state and its reactivation to a persistent infection as a result of decreasing the temperature. Subsequent studies by Tanaka et al. (1988) have shown that when the latently infected cells were treated with indomethacin or tetracaine immediately after being shifted to 37 C, reactivation of latent virus was not observed. Indomethacin and tetracaine inhibit replication of DNA viruses in cells, suggesting a role for arachidonic metabolites as regulatory agents for productive viral infection.

Gonczol et al. (1984, 1985) examined HCMV infected human teratocarcinoma cells as a possible latency model for HCMV. Undifferentiated cells produced neither

viral antigens or progeny virus. However, treatment with retinoic acid allowed for expression of viral antigens and release of progeny virus.

LaFemina and Hayward (1986) localised the block in the nonpermissive teratocarcinoma cells as occurring before IE protein synthesis and suggested that it occurred at the level of IE transcription. After differentiation, synthesis of the IE 68K protein was induced and viral DNA replication occurred.

1.1.6. HCMV Infection in Normal Hosts

Primary infection is generalised and portals of entry may include blood via blood transfusions, oral or orogenital contact or possibly genital contact alone. Viraemia is detectable for a few weeks to a few months (Klemola, 1973). The polymorphonuclear leukocyte is the main source of HCMV in the blood, but monocytes and occasionally T lymphocytes may harbour HCMV in a form as yet undefined (Carney and Hirsch, 1981; Fiala et al., 1975; Garnett, 1982; Rinaldo et al., 1977; Rinaldo et al., 1979).

Cell-mediated immunity is depressed with symptomatic primary infections, with T cell dysfunction being prominent (Rinaldo et al., 1977). Peripheral blood

monocytes in blastogenic assays respond poorly to the mitogens concanavalin A and pokeweed mitogen, HCMV-specific antigens and other herpesvirus antigens during the acute phase of HCMV-associated illness (Rinaldo et al., 1977). Responsiveness to mitogens and heterologous antigens returns before responsiveness to HCMV-specific antigens. Cytotoxic/suppressor lymphocytes (T8) increase strikingly while the helper/inducer subset (T4) cells are slightly diminished, resulting in a reversal of the normal ratio of these cell types (Carney et al., 1981).

1.1.7. Infection in Immunosuppressed Hosts

Following renal transplantation, reactivation of latent infection from the recipient or from the donated allograft accounts for most infections (Betts et al., 1975; Pass et al., 1973). Virus shedding posttransplant in individuals who were seropositive prior to transplant is referred to as nonprimary infection and shedding and seroconversion is referred to as primary infection. Approaches to the prevention of primary HCMV infections in transplant recipients have included use of seronegative blood products in seronegative patients who received marrow transplants from seronegative donors (Hersman et al., 1982)., the use of live attenuated vaccine in renal transplant recipients (Plotkin et al., 1984), use of HCMV immune plasma

(Winston et al., 1982) or HCMV immune globulin (Condie and O'Reilly, 1984).

Grundy et al. (1986) used restriction endonuclease analysis of HCMV isolates to show that HCMV infection of a seropositive renal allograft recipient was due to a virus from the donor kidney. This finding suggested that HCMV infection could behave as a primary infection when there was transmission of a viral strain from the donor that was different from that of the recipient. Rakela et al. (1987) have shown that serology of the donor is still the most important factor in predicting primary infections. Chou (1987) showed that antigenic relatedness among HCMV strains may determine if reactivation of endogenous strains or reinfection occurs in seropositive organ recipients receiving transplants from seropositive donors.

HCMV commonly infects patients with AIDS and may be an important co-factor for the full expression of AIDS (Drew et al., 1985). More than 95% of homosexual men are seropositive for HCMV and many actively shed virus (Huang et al., 1985). Recent observations have shown that an elevated titre of HCMV antibody is a significant predictor of the development of AIDS in HIV-seropositive individuals (Polk et al., 1987). Furthermore, HCMV viremia increases with progressive immune deficiency in patients infected with HIV-1 (Fiala et al., 1986).

HCMV has the potential for interacting with HIV at molecular and functional levels, thereby contributing to progression of the immunodeficiency syndrome (Nelson et al., 1988). HCMV-induced immunosuppression (Carney and Hirsch, 1981 ; Schrier et al., 1986) may lower the immunocompetence and allow for spread of HIV. At the in vivo level, Nelson et al. (1988) have shown direct interaction between HIV and HCMV. Double labelling techniques have shown that HIV and HCMV can coinfect central nervous system tissue in AIDS patients. This observation, together with the report of Davis et al. (1987), that HCMV can increase transcription of HIV in vitro, suggest a direct role for HCMV in the pathogenesis of AIDS.

Severe bacterial and fungal infections are increased in cardiac and renal transplant patients following HCMV infection, particularly when it is of the de novo primary type (Chatterjee et al., 1978; Braun and Nankervis, 1978;). These investigators have shown that since the early use of immunosuppression in transplant surgery, infections - in particular with Pseudomonas and other Gram-negative bacilli, Nocardia, opportunistic fungi and Pneumocystis carinii - were the major causes of death. Their study showed that opportunistic fungal infections occured much more frequently in renal allograft recipients who tested HCMV seronegative prior to transplantation.

1.2.0. Comparison of HCMV Laboratory Strains and Clinical Isolates of HCMV

Rice et al. (1984) showed that monocytes, natural killer cells and T and B lymphocytes can be abortively infected with HCMV in vitro, as demonstrated by immunoflourescent staining using monoclonal antibodies to HCMV IE antigens. This abortive infection was most easily demonstrable with fresh, low-passage clinical isolates. The frequency of appearance of IE antigens was much lower when the laboratory adapted strain AD169 was used, which suggested an increased lymphotropic effect of fresh isolates of HCMV compared with strains of virus which were extensively adapted to growth in [illegible]sts. Among PBMC, monocytes comprise the cell [illegible] with the greatest proportion of cells [illegible]ing the immediate-early gene product. All 15 passage isolates of HCMV used in the study completely abrogated the mitogenic response of peripheral blood mononuclear cells to phytohemagglutinin (PHA). The dichotomy in the behaviour of freshly isolated and laboratory adapted strains of HCMV has been corroborated by Einhorn and Ost (1984), who also found that mononuclear cells had a differential tropism for the different strains of virus. The highest percentage of IE antigen positive cells in this study was monocytes. Infected cells remained IE-antigen positive in culture for 7-13 days.

Restriction site polymorphisms among clinical isolates and laboratory strains of HCMV have been compared (Chandler and McDougall, 1986). These authors compared Hind III and Eco RI restriction sites in the long and short unique regions of the HCMV genome among 20 low passage clinical isolates and four laboratory strains. Southern blot analysis with a series of subgenomic cloned fragments of AD169 was performed. Although no two strains were identical throughout the genome, identical patterns of variation in a given region frequently occurred in multiple strains. Polymorphisms occurred throughout the entire genome, including the region coding for immediate-early functions. All strains studied showed an identical fragment which hybridized to the cellular transforming region of AD169. No specific difference which could be related to length of time in tissue culture passage was found between the laboratory strains and the clinical isolates in their study.

The differential tropism that has been observed may be an important phenomenon among the herpesviruses. Sixbey et al. (1983) showed that recently isolated strains, but not laboratory adapted strains of Epstein-Barr virus could infect human epithelial cells and that viral genomic expression was incomplete in cells infected with recently isolated strains.

1.3.0 MONONUCLEAR PHAGOCYTES

All mononuclear phagocytes constitute a cell lineage that originates from a pluripotent stem cell in the bone marrow. The differentiation pathway proceeds through the stages of monoblasts, promonocytes, monocytes and macrophages (van Furth, 1980). Monoblasts and promonocytes are located in the bone marrow and are actively dividing cells that give rise to monocytes. Monocytes are transported via peripheral blood to the tissues where they become macrophages. Depending on the destination tissue site they become histiocytes of connective tissue, osteoclasts in bone tissue, Kupffer cells in the liver, alveolar macrophages in the lung, macrophages of the spleen, lymph nodes and bone marrow and free macrophages of the pleural and peritoneal cavity. It is becoming more evident that in addition to being effectors of non-specific host defenses, monocytes also play an important role in the induction and modulation of specific immune responses.

Monocytes measure 12 to 15 um and make up 3 to 8 percent of peripheral blood leukocytes. The nucleus is indented and has a characteristic kidney shape. The cytoplasm contains many fine azurophilic granules (Nichols et al., 1971). Electron microscopy shows the Golgi apparatus of monocytes to be well developed;

lysosomal granules are numerous and mitochondria are evenly distributed. Nucleoli are seen in about half the cells. Cytochemical studies help identify promonocytes, monocytes and macrophages, the latter being positive for non-specific esterases (Lam et al., 1971). Macrophages represent a later stage in the development and maturation of monocytes. In the transition from monocyte to macrophage, there are increases in the number of lysosomes, the number of mitochondria, the activity of mitochondrial enzymes and the rate of cellular respiration.

1.3.1. Monocyte Cell Lines

Studies of macrophage differentiation using primary cultured cells are difficult due to the heterogeneity between and within macrophage populations. The highly variable state of differentiation that cells of the mononuclear phagocyte series may exhibit and the influence that this may have on virus exposure and latency are issues that are presently being addressed. For these reasons, monocyte or macrophage cell lines are used in many areas of macrophage research. Of special interest are cell lines such as ML-3 and HL-60 which can be induced to change differentiation states in response to stimulation with a variety of chemical and biological agents (Harris and Ralph, 1985).

The human promyelocytic cell line, ML-3, used in this study is essentially identical to the HL-60 cell line established by Collins et al.(1977).

1.3.1.1. Phenotype of Uninduced Promyelocytic Cells

The phenotypic character of HL-60 reflects its leukemic origins. Phenotypically HL-60 resembles blast cells of their lineage and are believed to be the neoplastic derivatives of commited progenitors of granulocytes and monocytes. Histochemistry and morphology of these cells typify them as immature cells of the myelomonocyte lineage. They have a population doubling time of 24-48 hr and stain as myeloblasts and promyelocytes, though approximately 10% appear to be more mature cells (Gallagher et al., 1979). Cells are characterised by a lack of lymphoid markers, growth as compact colonies in semisolid culture and nonadherence to substrata. Cells possess few Fc-IgG and C3b receptors and weakly phagocytize latex or yeast particles. HL-60 cells also possess receptors for the chemotactic tripeptide formyl-methionyl-leucyl-phenylalanine and insulin.

1.3.1.2. Differentiation of Human Promyelocytic Cells along the Monocyte/Macrophage Pathway

Treatment of HL-60 cells with 12-O-tetradecanoylphorbol 13-acetate (TPA) results in terminally differentiated cells with several characteristics of macrophages (Rovera et al., 1979). Addition of TPA to suspension cultures of HL-60 results in more than 80% of the cells adhering to the plastic substrate within 24 hr. Within the same time period immature azurophilic granulations typical of HL-60 promyelocytic cells disappear, nuclear chromatin becomes more condensed, but the nucleolus is retained. Attached cells stop dividing and DNA synthesis stops. The phenomenon is irreversible and independent of the continuous presence of TPA. There is no change in the percentage of cells bearing surface Fc receptors for IgG. Cellular levels of NADase, acid phosphatase and non-specific esterase are markedly increased after TPA treatment, these enzymes being characteristic of macrophages. More lysozyme is found in the medium of TPA-treated cells than in the medium of untreated cells which is also characteristic of the macrophage phenotype (Rovera et al., 1979).

1.3.1.3. Differentiation of Human Promyelocytic Cells along the Granulocyte Pathway

Treatment of HL-60 cells with dimethyl sulfoxide (DMSO) results in terminally differentiated cells with several characteristics of mature granulocytes (Collins et al., 1978). The addition of DMSO to cultures resulted in the

following morphological changes: smaller size, decreased nuclear/cytoplasmic ratio, less prominent cytoplasmic granules, marked reduction or complete dissapearance of nucleoli and marked indentation, convolution and segmentation of the nuclei. Functional changes include increased phagocytic ability. Proliferation of HL-60 cells ceases after addition of DMSO and is irreversable.

1.4.0. VIRUS INTERACTIONS WITH MONOCYTES / MACROPHAGES

Macrophages are susceptible to infection by a number of viruses and their function may be altered as a result despite their role as phagocytic [illegible] several viruses preferentially replicate in macrophages [illegible] lactic dehydrogenase virus (LDH) of mice only replicates in macrophages (Porter et al., 1969) LDH is [illegible] example of a virus which appears to replicate in cells of the infected host without causing any cytopathic damage. The virus impairs the clearance from the blood of the endogenous enzyme, lactic dehydrogenase, by mouse macrophages. Antigen presentation is also affected (Isakov et al., 1982) in infected cells, but all other functions appear to be normal.

Macrophage resistance to virus infection may be of two types: extrinsic or intrinsic resistance (Stohlman et al., 1982). Extrinsic resistance of macrophages is

defined as their ability to inactivate extracellular virus or reduce production in other surrounding cells that are normally permissive. Intrinsic resistance is defined as the permissiveness/non-permissiveness of the macrophage itself for growth of a virus. The interaction may be completely non-specific or may be modified immunologically in either direction. For intrinsic interactions, there may be a multitude of possible outcomes. These include abortive, cytolytic, persistent, latent infections and transformations.

Interferons produced by macrophages appear to play a complex role in intrinsic resistance. Interferon, induced by a high multiplicity of infection with herpes simplex virus (HSV) was correlated with inhibition of productive virus replication in human monocytes in vitro (Linnavuori and Hovi, 1983).

Increasing evidence suggests that monocytes and macrophages also play a major role in the propagation and pathogenesis of HIV infection. Monocytes can be infected in vitro with HIV and virus can be isolated from blood and various organs of the HIV-infected individual (Nicholson et al., 1986). In the brain monocytes/macrophages are the major cell type infected with HIV (Koenig et al., 1986). A number of monocyte

functional abnormalities have been reported in monocytes infected with HIV in vitro. These include defective chemotaxis and killing of microorganisms. Although these abnormalities may be solely due to direct infection of monocytes by HIV, it seems unlikely, as the frequency of infection of circulating blood monocytes is low. The deficiency of inductive signals from T4 cells is likely to contribute to functional defects in monocytes (Fauci, 1988). The most important implication of HIV- infected monocytes is the possibility that they serve as the major resevoir for HIV infection in the body, in view of the extended time frame (more than 5 yrs) from initial infection with HIV to clinically detectable disease manifestation. Monocytes are relatively refractory to the cytopathic effects of HIV. The virus can therefore survive in these cells and be transported to various organs in the body such as the lungs and brain. Cells may survive that are either latently infected (integrated provirus without virus expression) or chronically infected (low-level virus expression). Activation signals are required for the establishment of a productive infection in vitro (Folks et al., 1986). In view of this, it is likely that various activation signals in vivo contribute to conversion from latent or chronic infection to productive infection, such as antigenic stimulation. Physiologic cellular inductive signals that might be encountered as part of the normal immune

response might also play a role. Folks et al. (1987) showed that cytokine-containing supernatants from PHA-stimulated human mononuclear cells as well as granulocyte/macrophage-colony stimulating factor (GM-CSF) were capable of inducing virus expression in a latently infected, cloned, promonocyte cell line (U1) which did not constitutively express virus. In addition, HIV infection of the U1 clone resulted in upregulation of IL-1β expression, an indication that HIV infection may have important influences on the expression of certain cellular genes.

1.4.1. Effects of Activation and Differentiation Signals on Virus Interaction with Monocytes / Macrophages

Numerous studies have indicated that macrophage resistance to virus infection (intrinsic resistance) changes with the state of differentiation of the macrophage. It is well established that macrophages that differ in the state of activation, anatomical location or in vitro treatment show differences in resistance to infection by the same virus (Morahan et al., 1985). Infection of human macrophages in vitro with HSV-1 varies with the differentiation state of the macrophage (Daniels et al., 1978).

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Studies of visna virus infection of sheep have suggested a crucial role for macrophage differentiation in the persistent nature of this disease (Gendelman et al., 1986). Immature macrophages in the bone marrow show a restricted, noncytolytic pattern of visna virus infection. These cells give rise to infected monocytes, which upon infiltrating an inflammatory site undergo differentiation to an inflammatory macrophage that triggers a productive, lytic infection thereby serving to disseminate the virus.

HIV may also rely on persistent, noncytolytically infected macrophages to disseminate virus to tissue sites of inflammation such as the brain and lung (Streicher and Joynt, 1986). Recent reports have shown that treatment of the monocyte-like cell line, U937, with agents that induce differentiation increased resistance to HIV (Hammer et al., 1986; Clapham et al., 1987).IFN- and GM-CSF increased resistance only when in culture both before and during the infection (Hammer et al., 1986). PMA (13-phorbol-12-myristate acetate) treatment has been reported to decrease HIV replication in U937 cells by decreasing the surface expression of the HIV receptor, the CD4 molecule.

Murine CMV can be activated to produce infection in teratocarcinoma lines only after differentiation is chemically induced by dimethylacetamide (Dutko and

Oldstone, 1981). Gonczol et al. (1984) found that only human embryonal carcinoma cells induced to differentiate with retinoic acid expressed CMV antigens and produced infectious virus. Phorbol esters have repeatedly been shown to activate EBV replication in lymphoblastoid cell lines that are low level producers of infectious EBV particles (Yamamoto and Zur Hausen, 1979).

Tenney and Morahan (1987) have characterised the effect of differentiation on the resistance of the mononuclear phagocyte to HSV-1 using U937 cells. Replication of HSV-1 was compared in undifferentiated U937 cells and in cells induced to undergo differentiation by PMA treatment. Undifferentiated cells were highly resistant to infection while PMA-differentiated U937 cells acquired full susceptibility to HSV-1-induced cytopathic effects. U937 cells were also induced to differentiate by treatment with retinoic acid, DMSO and lymphokine. The change in permissiveness, however, was uniquely associated with PMA treatment. Undifferentiated U937 cells adsorbed as much virus as PMA-differentiated cells, but immunoflouresence assays as well as DNA hybridization analysis demonstrated an early block in replication in undifferentiated cells, before synthesis of viral protein and DNA. It was also established that PMA must be added before infection, suggesting that replication is enhanced as a result of

the differentiated phenotype.

Weinshenker et al. (1988) have made a similar study of HCMV infection of human monocyte cell lines. They studied the susceptibility of a number of different cell lines to infection by HCMV in vitro. These were HUT 102, THP-1, MOLT-4 and HL-60. THP-1, a monocytic cell line, could be infected by HCMV with a full cycle of replication after induction with TPA which results in differentiation of the cell line into cells with characteristics of mature macrophages. HL-60, a promyelocytic cell line, was not susceptible to CMV infection after induction with TPA, suggesting that cell lineage was important. They were not, however, able to establish whether the enhanced susceptibility of THP-1 to CMV was due to an increase in viral attachment and penetration (i.e an increase in receptors) or to enhanced transcription of the intracellular viral genome. Failure to induce expression of the virus by treatment with TPA after infection suggested that the enhancement was due to an increase in viral receptors.

1.4.2. Interaction of HCMV with Monocytes

Virus-monocyte interactions were evaluated in patients with mononucleosis due to HCMV (Carney and Hirsch 1981). The study showed that the suppressor cell during acute HCMV mononucleosis had the characteristics of cells of the monocyte-macrophage series and was infected with HCMV. In addition, monocytes from uninfected control donors were infected in vitro with HCMV and evaluated for the induction of suppressor activity. HCMV-infected monocytes were significantly more suppressive for autologous lymphocyte responses to con A than were uninfected monocytes.

Einhorn and Ost (1984) showed that clinical isolates of HCMV, passaged one to ten times in vitro, regularly induced HCMV early antigens in human leukocytes. The population of leukocytes that were most positive for HCMV early antigens (EAs) were monocytes. No evidence of viral production was found. In contrast, EA-positive leukocytes were only rarely found after exposure to the HCMV laboratory adapted strain, AD169. These findings were corroborated by Rice et al. (1984) who demonstrated that HCMV could infect T and B lymphocytes, natural killer cells and monocytes. Virus expression was limited to the synthesis of immediate-early (IE) HCMV polypeptides and this abortive infection was also

most convincingly shown by strains of HCMV recently isolated from infected patients. In this study, monocytes were also found to comprise the cell population with the greatest proportion of cells expressing the IF gene product. In addition, infection of peripheral blood mononuclear cells with low passage isolates completely abrogated their response to PHA.

Kapasi and Rice (1986) have confirmed that the immunosuppressive effect of HCMV depends on the presence of monocytes in the cultures. They found that monocyte viability was unaltered by HCMV infection, but that phagocytic activity and respiratory burst were markedly suppressed. Kinetic studies revealed that the suppressive effect required at least 24hr to develop after infection.

1.5.0. INTERLEUKIN 1

1.5.1. Cell Sources of Interleukin 1

Interleukin 1-like factors are still identified by their ability to activate thymocytes. IL-1 is distinguished from IL-2, the only other cytokine known to have thymocyte activating activity, by its inability to support the growth of IL-2-dependent lymphocyte cell lines. Many cell types have been documented to produce IL-1-like activities (Dinarello, 1984; Oppenheim et

al., 1986), although the biochemical properties of the IL-1 activities produced by these diverse cell types have only been partly defined. Many non-monocytic cells produce IL-1-like activity with the same molecular weight and major pI's as monocyte derived IL-1. IL-1 mediates several diverse biological activities including induction of fever, stimulation of secretion of acute phase proteins from hepatocytes, PGE_2 from fibroblasts and synovial cells and a maturational influence on T and B lymphocytes (Appendix A).

1.5.2. Production and Release of IL-1

Most normal cell types and a number of cell lines produce IL-1 only in response to a variety of stimulants, although some transformed B and T cell lines spontaneously produce soluble IL-1-like factors. Reports of constitutive production of IL-1 by normal cells can probably be attributed to a continuation *in vitro* of a response to prior stimulation *in vivo* or as a result of a contaminating stimulant such as the ubiquitous endotoxin (LPS). Stimulants that induce IL-1 synthesis by monocytes act on the plasma membrane. For monocytes and macrophage cell lines, stimulants include LPS, PMA, CSF and IFN γ, with LPS being the most potent soluble inducer *in vitro*. Following LPS stimulation, low levels of IL-1 appear very rapidly (intracellular IL-1 activity appears within 30 min and extracellular

IL-1 within 60 min). High levels of intracellul extracellular IL-1 are reached 3h after stimu..ion and this LPS-induced increase is both actinomycin D and cycloheximide sensitive, thereby excluding a major contribution to IL-1 production by either preformed mRNA or protein (Oppenheim e. al., 1986). Further studies by Fenton et al. (1987) have shown that cycloheximide inhibition of de novo protein synthesis results in superinduced levels of IL-1βmRNA (400-600 fold over background) with accumulation rates and decay kinetics being identical. IL-1β is thus a transiently expressed, tightly regulated gene which implies a novel role for proIL-1β in monocyte differentiation particularly when considering that most of the IL-1β synthesized remains inside the cell (Matsushima et al., 1986). A puzzling feature of the IL-1 protein is its lack of a signal sequence which is usually characteristic of secreted proteins that are transported from an intracellular to an extracellular environment. This feature raises questions about whether IL-1 is truly a secretory protein and by what mechanism it is released from cells (Oppenheim et al., 1986)

Several investigators have shown that essentially all IL-1 is the product of two genes, designated as IL-1α and IL-1β that each encode distinct polypeptide products (Auron et al., 1984; March et al., 1985). The products are clearly defined on the basis of distinct

isoelectric points (namely pI 5 for IL-1α and pI 7 for IL-1β), but have indistinguishable biological profiles. Common structural genes essential to function are found in both IL-1α and IL-1β , which accounts for their similar biological activities.

Comparison of the gene sequences has revealed extensive similarity of genomic organization. Both the IL-1α and the IL-1 βgenes are split into seven exons, but the primary transcription products (7 kb for the beta gene and 10.2 kb for the alpha gene) are consistent with the relative mRNA sizes (1.6 kb for beta and 2.2 kb for alpha). Despite the poor overall amino acid homology (25%), there is a high conservation of organization for both exon size and position of splice junctions. Furthermore, the evolutionary constraints on this genomic pattern of organization have been maintained despite a sequence divergence of 45% (March et al., 1985). The paradox of the existence and functioning of two genes with indistinguishable biological activities is further complicated by differential expression of these two genes. Human monocytes transcribe the IL-1β gene 10-50 times more efficiently than the IL-1α sequence (March et al., 1985) and consequently produce about 10 times more pI 7 than pI 5 IL-1. This may be due to a more efficient set of typical eukaryotic promoter elements found in association with the IL-1β gene. The IL-1 βprecursor may be active without

processing (van Damme et al., 1985) whereas the IL-1α precursor may require cleavage. Considerable IL-1 activity can be extracted from cells with detergent and much of this cell- associated IL-1 activity is located in the cytosolic fraction of the cell. However, IL-1 activity that is exposed on the macrophage plasma membrane has been detected by Kurt-Jones et al. (1985).

Gel filtration or high pressure liquid chromatography reveals that a low level of the cell-associated IL-1β activity is associated with a 30-kDa precursor moiety, whereas most of the activity extracted with detergent from both cytosol and membrane exhibits a mw of 23kDa. In view of the absence of a nucleotide sequence in the cDNA for IL-1α or β that codes for such a signal peptide, it has been postulated that the IL-1 precursor undergoes sequential enzymatic cleavage into progressively smaller peptides from the biologically inactive 31 kDa precursor form to an intermediate 23 kDa intracellular and membrane form and subsequently to a 17 kDa extracellular peptide. Conlon et al. (1987) have performed studies on the localisation of the α and β forms of IL-1 in human mononuclear cells. They have demonstrated by two distinct criteria, flow cytometry and inhibition of biological activity of cell bound IL-1, that the α form is associated with the cell membrane, whereas the β form was not detected. If IL-1β was present on the surface, it might be there in a

biologically inactive form. Indeed, Mosley et al. (1987) have shown that only the processed 17 kDa form of IL-1β is active. Their conclusions were that IL-1α is preferentially associated with surfaces of cells, particularly those involved in antigen presentation, while the IL-1β form remains predominantly inside the cytoplasm and is only secreted upon stimulation.

1.5.3. Receptors for IL-1

The availability of purified recombinant IL-1 has made it possible to label IL-1 with ^{125}I to detect receptors on cell surfaces (Dower et al., 1985). In this study, it was found that a wide variety of cell types have a low number of binding sites. Fibroblasts had the highest number of sites (1500-5000 sites/cell). The murine T lymphoma LBRM-33-1A5 IL-1 dependent cell line bound 500 ^{125}I - IL-1 molecules / cell at saturation and of 12 polypeptide hormones, only IL-1 competed with labelled IL-1 in a dose dependent manner. A membrane polypeptide of mw 79 500 was found to be crosslinked to IL-1. Dower et al. (1985) also observed that unlabelled IL-1α competed equally with IL-1β for binding sites, suggesting that receptors for the two molecules may be identical. A cDNA clone encoding a molecule of 576 amino acids corresponding to the IL-1 receptor has been isolated (Sims et al., 1988). This transmembrane molecule appears to be a member of the

gene family. Mutations of a unique histidine residue (position 30) in IL-1β resulted in a 100 fold reduction in receptor-binding affinity (MacDonald et al., 1986).

Paradoxically, only 50 IL-1 binding sites / cell have been observed for peripheral T lymphocytes (Dower et al., 1985) - the reason for such a low detection is unknown. Little is known concerning biochemical events following interaction of IL-1 with its receptor. The calcium ionophore, A23187, has been shown to synergistically enhance the effects of IL-1 in the thymocyte activation assay (Matsushima and Oppenheim, 1985). This suggests that IL-1 depends on calcium channel formation for its effects, but attempts to ascertain whether IL-1 rapidly influences the intracellular activity of protein kinase C have been unsuccesful.

1.5.4. Genomic Structure of IL-1

1.5.4.1. Homology Regions within IL-1 Molecules

Since both IL-1α and IL-1β cDNAs have been shown to code for biologically active IL-1 , regions of homology between the two DNA sequences could identify the important regulatory functions of these proteins. The regions of strongest homology between the

human IL-1 α and human IL-1ßgenes respectively, are known as the R_1 and R_2 regions (Figure 3) .

March et al. (1985) have shown that biological activity is conveyed by 159 and 153 amino acids from the C - terminal end of the IL-1 α and IL-1 ß molecules respectively. They suggest that the primary difference between the IL-1α and IL-1ß species is that the primary translation product of the IL-1ß is biologically active, while that of IL-1α is not, as determined in a reticulocyte lysate system.

1.5.4.2. Regulation of IL-1 Gene Expression

Oppenheim et al. (1986) have analysed the rate of IL-1 mRNA accumulation in peripheral blood monocytes and also observed differential expression of the IL-1α and IL-1ß genes. Dot blot analysis revealed that the levels of IL-1 ß mRNA began rising 1 hour after stimulation of cells with LPS, reaching a maximum 6 hours after treatment and was 40 fold greater than the level measured in unstimulated cells. Spontaneous expression of low levels of both IL-1α and IL-1ß mRNA by the monocyte population was also observed. Following LPS stimulation, the amount of IL-1α and ß mRNA produced by human monocytes determined

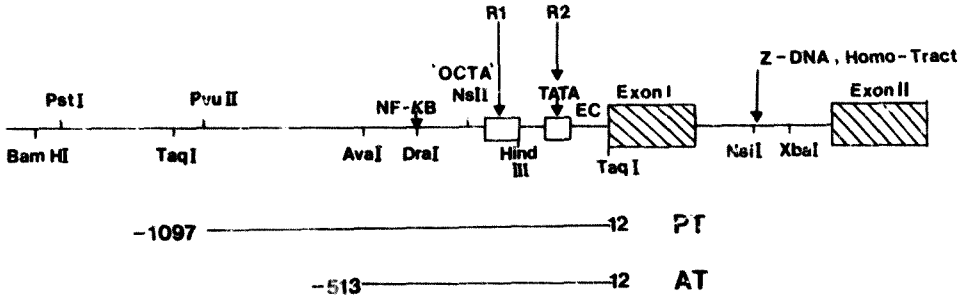

Figure 3
Schematic representation of IL-1ß putative regulatory regions (after Clark et al. 1988) and sequences tested in cotransfection experiments together with HCMV IE gene region constructs

the relative amount of IL-1α and β protein produced by the cells and 10 fold greater concentrations of both the IL-1β mRNA and protein were measured compared with the levels of IL-1α .

1.5.4.3. Role of cis and trans acting elements involved in expression of the proIL-1β gene.

Fenton et al. (1987) showed that the proIL-1β gene was differentially expressed in human monocytic cells following induction with LPS or PMA and that the kinetics of IL-1β expression is stimulant specific. LPS stimulation results in rapidly and transiently expressed IL-1β message whereas PMA leads to a much slower appearance and decay of IL-1β mRNA,suggesting separate membrane receptor/transduction systems for LPS and PMA. These data also suggested a model for regulation involving a transcriptional activator during the induction of the IL-1β gene followed by the action of a repressor to partially down-regulate transcription. Clark et al. (1988) therefore investigated whether cis and trans-acting elements may be involved in the regulation of the IL-1β gene.

Two approaches were used to analyze DNA sequences upstream of the transcriptional initiation site of the proIL-1β gene. The first used chimeric plasmids containing proIL-1β DNA sequences fused to the

bacterial CAT gene. The insertion of the proIL-1β promoter in front of the promoter-less CAT gene allows for promoter testing by the expression of CAT in a transfected host cell. Using this technique, they have identified cis-acting sequences of the proIL-1β gene necessary for expression of the gene. In order to define the cis-acting sequences necessary for expression, they tested constructs containing successively smaller fragments of the proIL-1β upstream sequence generated by progressive cleavage at specific endonuclease sites. Clones designated PT, AT, DT, NT and HT contained 1097, 513, 314, 235 and 132 bp of upstream IL-1β sequence respectively. Secondly electrophoretic gel mobility shift assays were used to identify sequences to which trans-acting factors could bind. This method provided additional information on the regions identified by the CAT functional analysis by determining if nuclear factors bound to cis-acting sequences.

Four different human cell lines, THP-1, U937, Colo/16 and HeLa were transfected with the various IL-1β - CAT plasmids. The data showed that the proIL-1β upstream sequences were only able to direct CAT expression in monocytic THP-1 and U937 cells and not in non-monocytic HeLa and Colo/16 cells. IL-1β - CAT constructs containing the SV40 enhancer could direct CAT expression in all cell types tested but, without the enhancer, only monocytic cell lines could express CAT.

In THP-1 cells, the DNA fragment containing the R_2 homology region was required to direct CAT expression. This expression was increased by additional sequences extending into and beyond the R_1 region. Clones AT and PT with 513 and 1097 bp of upstream IL-1β sequence and containing the R_2 fragment, directed CAT expression with nearly equal efficiency with or without the SV40 enhancer. Clone DT with 314 bp did not, suggesting that DNA sequences between -513 and -314 possessed enhancer-like activity.

The two upstream regions which were used as radiolabelled probes in the band shift assays contained the R_1 and R_2 regions respectively. These fragments were incubated with nuclear extracts from unstimulated or LPS-stimulated (for 1 or 5hr) THP-1 cells. Specific regions of the proIL-1β upstream sequence that bound nuclear factors were identified. A factor present maximally in 5hr extracts and specific for THP-1 cells, bound to the fragment containing the R_2 region with a kinetic binding profile reflecting that previously proposed for a transcriptional repressor that down-regulated IL-1β transcription.

1.5.5. Effect of IL-1 on T Cells

A recent model attempting to explain the function of IL-1 in T cell activation is that of Mizel (1987). In the presence of a specific antigen or T cell mitogen, T cells and accessory cells are mutually stimulatory. The accessory cell is induced to begin IL-1 synthesis and secretion. In return, T cells initiate IL-2 and IL-2 receptor synthesis and expression. In addition T cells are stimulated to produce IL-1 receptors and thus become fully responsive to IL-1. IL-1 responsiveness confers upon the T cell a heightened ability to produce IL-2 and IL-2 receptors in response to processed antigen / class II MHC antigen or mitogen. Interaction between IL-2 and its receptor then results in T cell proliferation. Activated T cells may also produce a lymphokine that augments the ability of accessory cells to produce IL-1 thus providing for a secondary amplification loop in the interactions of T cells and accessory cells.

IL-2 is the sole requirement for stimulation of the entire series of events known as G_1 progression in cells (Stern and Smith, 1986). IL-2 receptor density and the absolute number of receptor interactions determines the time required to transit the G_1 phase. At the level of the single cell, progression to DNA replication is determined precisely by a finite number of IL-2 receptor interactions. The actual molecular mechanism underlying competence, or G_0 to G_1

transition, is the expression of IL-2 receptors (Smith, 1988). In contrast, IL-2 promotes a gradual and sustained increase in cell size, known as the lymphocyte blastic transformation, and prepares the cell metabolism for DNA replication.

In addition to its effects on IL-2 production and action, IL-1 also induces other T cell effects including changes in membrane viscosity (Puri et al.,1980) and increased chemotaxis (Miossec et al.,1984). The production of several other T cell lymphokines in addition to IL-2, increases following IL-1 exposure. These include IFN-γ and CSF (Kasahara et al.,1985).

1.6.0. EFFECTS OF VIRUS INFECTION ON INTERLEUKIN 1 AND 2 PRODUCTION

Rodgers et al. (1985) showed that infection of human monocytes with the AD169 strain of HCMV abrogates their production of functional IL-1 bioactivity. This was associated with the release from infected monocytes of an inhibitor of IL-1 activity, which was also released after HCMV infection of the U937 macrophage-like cell line. It appeared that the inhibitor decreased the proliferation of the IL-1 responsive cells in the IL-1 assay (ymocytes). The action of HCMV strain AD169 was virus specific and required infectious virus but

occurred without virus replication or detectable expression of viral proteins. Fractionation studies did not reveal whether both inhibitor and IL-1 are present in supernatants of HCMV-infected monocytes at the same time.

In a different study by Smith et al. (1985) it was also shown that HCMV infection depressed monocyte production of functional IL-1 bioactivity. In both LPS-stimulated and unstimulated monocytes, IL-1 production by HCMV-infected monocytes was significantly depressed as determined by the thymocyte proliferation assay. However, in this system, CMV did not induce monocytes to release an inhibitor of thymocyte proliferation. The reduced proliferation was also not due to an increase in prostaglandin E (PGE) production.

Kapasi and Rice (1988) also investigated the role of IL-1 and IL-2 in HCMV-mediated immunosuppression in vitro and found that HCMV infection of monocytes, lymphocytes or PBMC resulted in reduction of IL-1 and IL-2 production in the cultures. Addition of exogenous IL-1 or IL -2 did not restore these responses. However, if reduced production of IL-1 or production of an IL-1 inhibitor had been the only flaw, PBMC should still have responded to IL-2 which they did not. There was also no evidence of an IL-2 inhibitor, suggesting that

CMV causes a metabolic derangement in lymphocytes and monocytes and impairs their ability both to produce and respond to physiological mediators of the immune response.

Roberts et al. (1986) examined IL-1 and IL-1 inhibitor production by human monocytes exposed to influenza virus or respiratory syncytial virus (RSV) and showed that both influenza virus and RSV induce concomitant production of IL-1 and IL-1 inhibitors by peripheral blood monocytes. The net IL-1 activity produced after exposure to influenza virus resulted in enhanced thymocyte proliferation to PHA. In contrast, lymphocyte proliferative responses to mitogens and antigens were significantly depressed in the presence of influenza-infected monocytes. This suggests that this phenomenon was not due to inability of the infected monocytes to produce IL-1, but rather the effect of concomitantly produced inhibitors to IL-1. The net IL-1 activity produced by RSV-infected monocytes resulted in marked inhibition of thymocyte proliferation, either in response to any IL-1 activity that was produced at the same time, or in response to exogenous IL-1 added to the cultures.

Berman et al. (1987) performed studies using peripheral blood mononuclear cells from AIDS patients. In this study, however, in addition to finding marked increases

in the amounts of 50 000-100 000 and 6 000-9 000 molecular weight factors which inhibited IL-1 activity, results showed that PBMCs from patients with AIDS produced increased amounts of the 30 000-40 000 mw form of IL-1 compared with those of controls. The action of the 6 000-9 000 mw inhibitor of functional IL-1 activity appeared to be directed specifically against the maturational effect of IL-1 on immature T lymphocytes (thymocytes) - the same phenomenon reported by Rodgers et al. (1985) for CMV-infected monocytes. The inhibitor of functional IL-1 activity produced by PBMCs from AIDS patients did not inhibit proliferative responses to mitogens by peripheral blood T lymphocytes or IL-2 action or synthesis. The increased activity of an IL-1 form with higher mw in AIDS suggests either a decrease in IL-1 processing to the 17 000 mw form (Auron et al.,1984) or preferential synthesis of a certain type of IL-1, since IL-1 appears to represent a family of related peptides (Billian et al.,1985). Monocytes / macrophages also comprise cells of different stages of differentiation or activation which are likely to exhibit different properties in IL-1 production (Khansari et al.,1985). Such subsets of cells could be altered in AIDS. A change in monocyte differentiation or activation could be a result of the continuous activation of monocytes by the various opportunistic infections that accompany AIDS, or directly through infection of monocytes / macrophages by HIV.

In a study by Gupta et al. (1987), IL-1 activity in the supernatants of cultured peripheral blood monocytes from patients with AIDS was examined. Spontaneous IL-1 production was significantly increased in patients with AIDS, whereas IL-1 production by LPS-stimulated monocytes was significantly decreased. They suggest that in the early stage of the disease, adherent cells are activated in vivo. However, with progression of immune deficiency, adherent cells are functionally deficient. They did not assay for the simultaneous production of an IL-1 inhibitor by the LPS-stimulated monocytes.

Under normal conditions, the release of IL-1 and IL-1 inhibitors plays a role in tissue homeostasis (Larrick, 1989). Several molecules with IL-1 inhibitory activity (as tested in a limited number of assays) have been identified and partially characterised. Factors with distinct molecular weights of 8, 20 - 25 and 95 kDa have been identified. Molecules that limit transcription of IL-1, bind directly to the IL-1 gene, bind to its receptor or act at other sites to limit the activity of the receptor have been described. The altered production of IL-1 and/or IL-1 inhibitors in AIDS and other viral diseases may play a major role in the immune disfunction associated with these diseases (Larrick, 1989). The isolation and cDNA cloning of the

mRNAs giving rise to these molecules will permit their evaluation as modulators of immune function.

Folks et al.(1987) established a model system for cytokine-induced up-regulation of HIV-1 expression in chronically infected promonocyte clones. The parent promonocyte cell line U937 was chronically infected with HIV-1 and from this line a clone, U1, was derived. U1 showed minimal constitutive expression of HIV-1, but virus expression was markedly up-regulated by a PHA-induced supernatant containing multiple cytokines and by recombinant GM-CSF alone. Recombinant IL-1, IL-2, interferon-γ and tumour necrosis factor (TNF), did not up-regulate virus expression. Concomitant with the cytokine-induced up-regulation of HIV-1 expression of membrane bound IL-1β was selectively induced in U1 in the absence of induction of other surface membrane proteins. This cytokine up-regulation of IL-1β was not seen in the uninfected parent U937 cell line. These studies have implications for the understanding of the mechanism of progression from a latent or low-level HIV-1 infection to a productive infection with resulting immunosuppression. In addition, this model can be used to delineate the potential mechanisms whereby HIV-1 infection regulates cellular gene expression. Additional studies were performed to determine whether HIV induction occurred via activation of the HIV promoter. Constructs made from the HIV long

terminal repeat (LTR) and the CAT gene were transfected into U937 cells. Transfected cells were exposed to cytokines and showed increased levels of CAT activity, suggesting that the site of cytokine-induced activation resided in the LTR (A. Rabson and E.Duh et al., unpublished observations, In Rosenberg and Fauci, 1989). To further identify specific sites on the HIV LTR that were involved in virus induction, gel retardation experiments were performed using labelled oligonucleotides in the presence of nuclear extracts from stimulated and unstimulated cells. Nuclear extracts from PHA supernatant-stimulated cells retarded mobility of oligonucleotides from the HIV LTR region. The retardation could be inhibited by probes to binding sites within the transcription initiation region. The induction of virus expression from latently or chronically HIV-infected cell lines therefore occurs as a result of the interaction of specific DNA-binding proteins with the HIV LTR.

1.7.0. AIMS OF THIS STUDY

Monocytes play an important part in host defence against virus infection and in the case of HCMV may also be the reservoir for latent disease. The highly variable state of stimulation and/or differentiation that cells of the mononuclear phagocyte series may exhibit and the influence that this may have on the outcome of virus infection at functional and molecular levels have been the aims of investigation in this study.

At a functional level, monocytes play an essential role in the initiation of immune responses through antigen presentation and production of IL-1° and other mediators. Impairment of these accessory cell functions could be a major factor contributing to the immunosuppression and pathology of HCMV infection.

At a molecular level, there has been considerable interest in the IE gene products of HCMV since they possess transactivating properties and have been shown to transactivate transcription from viral and cellular promoters. Alteration in transcription of host cell genes might therefore also contribute to the pathology of infection at a molecular level.

The aims of this study therefore can be listed as follows:-

1. To evaluate the effect of HCMV infection on monocyte accessory cell functions, notably production of IL-1 bioactivity which is required for T lymphocyte responses to mitogens.

2. To compare effects of HCMV infection on production of IL-1 bioactivity in stimulated and unstimulated peripheral blood monocytes, in an attempt to evaluate an in vivo situation in which HCMV infection is frequently accompanied by superinfection with other organisms.

3. To evaluate effects of HCMV infection on expression of IL-1β steady state mRNA in peripheral blood monocytes.

4. To investigate the influence of various differentiation states on the expression of mediator genes in HCMV-infected and uninfected monocytes. In order to investigate the effect of HCMV exposure in terms of an overall response to infection and inflammation, the expression of IL-1β and several other monocyte-associated genes were studied. This was done using a promyelocytic cell line which can be induced to differentiate along different cellular lineages.

5. To determine whether the IE gene products of HCMV are capable of activating transcription of the IL-1β promoter.

2.0.0. MATERIALS AND METHODS

2.1.0. Antibody Status of Donors

Peripheral blood monocytes and autologous T lymphocytes were obtained from ten healthy adults, all of whom were seronegative for HCMV as determined by enzyme-linked immunosorbent assay (ELISA).

2.2.0. Isolation and Separation of Mononuclear Cells

Heparinised (15U/ml) human peripheral blood was diluted 1:2 with endotoxin free phosphate buffered saline (PBS), and separated into mononuclear and polymorphonuclear cell fractions using lymphocyte separation medium (Flow Labs. Inc., C.A., USA). The mononuclear cells were washed twice in endotoxin free PBS and once with endotoxin free RPMI 1640 culture medium (Cellgro, Mediatech) containing penicillin (100U/ml), streptomycin (100ug/ml) and L-glutamine (2mM). They were then resuspended in RPMI 1640 and 10% heat inactivated (56°C for 30 min) AB serum (South African Blood Transfusion Service, Johannesburg, SA), free of antibodies to CMV. Monocytes were removed by adsorption onto the flat surfaces of 100mm tissue culture petri dishes (Falcon Plastics. Oxnard, C.A., USA) at 37°C for 60 min. After incubation, the nonadherent cells were removed by washing with RPMI

1640. This nonadherent fraction is referred to as the lymphocyte population. Monocytes were recovered by incubating the adherent cells on the surfaces of tissue culture petri dishes with Versene solution (Gibco Labs, Grand Island, N.Y., USA) for 20-25 min at 4°C with occasional gentle shaking. After removal, monocytes were washed three times with RPMI 1640 medium. The resulting cell population was >95% monocytes as determined by non-specific esterase staining, and the viability was >95% as determined by trypan blue dye exclusion.

2.2.1. Non-specific Esterase Staining of Monocytes

Esterase staining of monocytes was performed using the α-napthyl acetate esterase staining kit (Sigma Diagnostics, St. Louis, MO., USA) according to manufacturers specifications.

2.2.2. Separation of T Lymphocytes

T lymphocytes were separated from other non-adherent cells by differential centrifugation of lymphocytes rosetted with sheep red blood cells (SRBC) treated with 2-amincethyl-isothiouronium bromide (AET), using the method of Boylston and Anderson (1979) (appendix 1). AET-treated SRBC were prepared from packed SRBC washed three times in PBS using centrifugations of 1000 g

for 10 mins each. Two and a half ml AET solution was added to 0,5 ml packed SRBC and incubated at 37°C for 15 min with gentle agitation. The SRBC were then washed with PBS until there was no haemolysis and resupended to a 10% concentration in RPMI 1640 and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA), preabsorbed with SRBC (appendix 1). The final cell density of non-adherent cells was adjusted to 3-4 x 10^6 cells in 2,5 ml RPMI 1640 and incubated at 37 °C with 0,5 ml of AET-treated SRBC and 1 ml of FCS preabsorbed with SRBC. The suspension was incubated for 15 min with gentle agitation and subsequently pelleted by centrifugation. After overnight incubation at 4 °C the pellet was resuspended by gentle agitation of the tube. The suspension was underlayed with a 10 ml cushion of lymphocyte separation medium (LSM) (Flow Labs.Inc,. C.A., USA) and centrifuged at 1000 g for 30 min. SRBC-rosetted cells (T lymphocytes) pellet through the LSM cushion, while non-rosetted cells remain at the interface. T lymphocytes were recovered by lysis of SRBC using freshly prepared cold (4°C) ammonium chloride lysing buffer (appendix 1), after which the cells were washed three times in RPMI 1640. Viability was assessed by trypan blue dye exclusion and was >95%.

2.3.0. Culture of ML-3 Cells and Induction of Cell Differentiation

ML-3 cells were grown in endotoxin free RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT, USA) and were subcultured every four days by resuspending at $2x10^5$ cells/ml in fresh media. For induction of cell differentiation, ML-3 cells were cultured in complete RPMI 1640 containing 10 ng/ml 13-phorbol-12-myristate acetate (PMA) or 10 ng/ml dimethylsulphoxide (DMSO) for 24 hr and then washed twice with RPMI 1640. Trypan blue dye exclusion showed that viability at the time of HCMV infection was >90% for control and PMA treated cells.

2.4.0. VIRUS STRAINS

2.4.1. CMV Strain AD169 and Towne Strain

Human embryonic lung fibroblasts (HEL) (Flow Labs. Inc., C.A., USA) were grown as monolayers in 25 cm^2 plastic tissue culture flasks (Falcon Plastics, Oxnard, CA, USA) in Eagle's minimal essential medium (MEM) (Gibco Labs,. Grand Island, NY, USA) supplemented with 10% FCS (Flow Labs. Inc., C.A. USA). Once a confluent monolayer had been obtained, cells were trypsinised using 0,25% trypsin (w/v), and the cells seeded into two new flasks of the same size. Stock virus was obtained by infecting a 90% confluent HEL monolayer with virus at a low multiplicity of infection (0.1 pfu/cell). After 90 min adsorption, the inoculum was

removed and replaced with Eagle's MEM supplemented with 6% (v/v) heat inactivated FCS. Culture supernatant containing virus was collected 7 days after inoculation and the quantity of virus determined by plaque assay (appendix 2). Virus was used for infection when the titer was 10^7 pfu/ml in HEL.

2.4.2. Clinical Isolate Strain

A fresh isolate strain of human CMV, V3474 - 84, (obtained from the virology laboratory, Medical University of South Africa) was isolated from the urine of an adult with CMV mononucleosis and passaged three times in mycoplasma-free HEL. Infected fibroblasts showing characteristic CPE were harvested and virus containing medium from passage three was used for infection of cells. The titre of the fresh isolate was $1x10^4$ pfu/ml in HEL.

2.5.0. Stimulation of Monocytes and Production of IL-1 Containing Supernatants

Monocytes were stimulated with lipopolysaccharide (LPS) from E.coli (Difco, Detroit, Mich., USA) for the generation of IL-1 containing supernatants (Lachman et al. (1977). Monocytes were resuspended to a concentration of $1x10^6$ per ml in RPMI 1640 and 5% heat inactivated AB serum. Aliquots (2 ml), were plated into 60 mm tissue culture dishes (Costar), with

or without 10ug/ml LPS. Dishes were incubated for 20 hr at 37°C in a humidified atmosphere containing 5% CO_2 in air. Supernatants were collected by centrifugation at 800g for 10 min and frozen at -20°C until used.

2.6.0. Virus Infection of Monocytes and ML-3 Cells

Virus was added to cell pellets in polypropylene tubes at varying multiplicities of infection (MOI) and incubated for 2 hrs at 37°C with gentle agitation every 20 min. After infection, the cells were washed twice in RPMI 1640. Monocytes were resuspended to a concentration of $1x10^6$ cells/ml in RPMI 1640 and 10% heat inactivated AB serum and ML-3 cells resuspended to the same concentration in RPMI 1640 and 10% FCS. Stimulated and unstimulated monocytes and ML-3 cells were mock infected with growth medium from uninfected fibroblasts.

2.7.0. Assessment of T Lymphocyte Transformation

Autologous T lymphocytes were prepared by differential centrifugation of lymphocytes rosetted with sheep red blood cells treated with AET, as described above.

T lymphocytes were washed and resuspended to a concentration of $1x10^6$ cells/ml in RPMI 1640 and 10% heat inactivated AB serum with or without a previously determined optimum dose of 2µg/ml of phytohemagglutinin (PHA) (Wellcome Reagents, Beckenham, England). T lymphocytes, $2x10^5$/well, were incubated alone or with uninfected or HCMV-infected, LPS-stimulated or unstimulated monocytes that had been cultured for 3 days. Maximum stimulation of T lymphocytes by PHA occurred after a further 72 hrs in culture. Lymphocyte stimulation was measured by the addition of 0.5µCi of [^{3}H]thymidine (specific activity, 19.3 Ci/mmol; New England Nuclear Corp., Boston, MA, USA) to each well 16 hr before harvest. Cells were harvested with a multiple automatic harvester (MASH II, Microbiological Associate Bioproducts) on glass fibre filters (Whatman, Clifton, N.J.), air dried and immersed in toluene scintillation fluid. [^{3}H]thymidine incorporation was assessed by liquid scintillation counting for 10 min in a liquid scintillation counter (Packard Instrument Co., Downers Grove, III) and expressed in dpm. All experiments were performed in triplicate and results averaged.

2.8.0. Immunoflouresence for detection of HCMV Immediate-Early Antigen Expression

Immunoflouresence for detection of HCMV immediate - early (IE) antigen expression in infected peripheral blood monocytes and ML-3 cells was assessed on cytocentrifuge preparations. Slides were air dried and fixed sequentially in the following solutions: 3.7% formaldehyde for 5 min at room temperature, methanol for 2-4 min at -20°C and acetone for 2 min at -20°C. Fixed monocytes were then blocked with 10% human AB serum for 30 min at 37°C to prevent non-specific binding of monoclonal antibody to monocyte Fc receptors. Slides were then rinsed in PBS and incubated for 1 hr at 37°C with a monoclonal antibody recognising the major 72-kilodalton (kDa) immediate-early protein of HCMV (a gift from L.Goldstein, Fred Hutchinson Cancer Research Centre, Seattle, Washington, USA). Control slides were incubated with PBS + 5% BSA instead of monoclonal antibody. Slides were then rinsed thoroughly in PBS and incubated with FITC labelled rabbit antimouse immunoglobulin (Cappel Labs.,Cochranville, PA.,USA) at 37°C for 1 hr. Slides were rinsed thoroughly in PBS and stained cells observed with a Leitz Weitzlar Dialux 20 microscope (West Germany).

2.9.0. Assay for Functional Interleukin 1 Activity

The assay for detection of functional IL-1 activity is based upon the enhancement of murine thymocyte

proliferation by IL-1 in response to suboptimal concentrations of the mitogens PHA or Con A (Gery et al., 1972). Single cell thymocyte suspensions were prepared by aseptically teasing the thymus glands of 6 - 8 week old Balb-c mice. The larger clumps of cells were allowed to settle, and the single cell suspension pelleted by centrifugation at 1000 g for 10 min.

Cells were washed twice in PBS and resuspended in RPMI 1640 containing 5% heat inactivated FCS, 2.5×10^{-2} M 2-mercaptoethanol, 2 mM glutamine and 2 µg/ml PHA. Viability was assessed by trypan blue dye exclusion. Aliquots, 100 µl, of the cell suspension containing 10^6 cells were plated into the wells of 96 well tissue culture plates (Falcon, Oxnard, CA, USA). Culture supernatants (100 µl) from infected or uninfected, stimulated or unstimulated monocytes that had been cultured for 3 days post infection were added to the wells. Thymocyte proliferation was maximal after 96 hr. Cultures were pulsed with 0.5 µCi of [^{3}H]thymidine per well for the final 16 hr of incubation. Cells were harvested with a multiple automatic harvester, and [^{3}H]thymidine incorporation assessed by liquid scintillation counting. Experiments were performed in triplicate and the mean disintegrations per minute calculated.

2.9.1. Detection of IL-1 β using ELISA

LPS-stimulated or unstimulated monocytes were infected or mock-infected as described above and then cultured for 1d after infection. After 24 hr, supernatants were removed, replaced with fresh medium and this supernatant collected after a further 24 hr (representing 1d-2d mediator production). Fresh supernatant was added and removed after an additional 24 hr (representing 2d-3d mediator production).

Quantitative measurement of secreted IL-1β in culture supernatants was performed using an IL-1β ELISA kit (Cistron Biotechnology, Pine Brook, NJ, USA) following instructions given by the manufacturers.

2.10.0. Addition of Pure IL-1 or IL-1 Containing Supernatant to HCMV-infected Monocytes

Pure IL-1 (a gift from L. Traub of the Immunology Department, South African Institute for Medical Research) was serially diluted from an initial concentration of 4U/ml using 0.1% bovine serum albumin (BSA) in PBS. IL-1 conditioned supernatant obtained from LPS-stimulated monocytes was diluted 1:2, 1:4 and 1:16.

Stimulated or unstimulated, HCMV-infected or uninfected monocytes were cultured in vitro for 3 days. After 3 days, culture medium was removed and cells washed with RPMI 1640.

T lymphocytes, 100 µl, (10^6 cells/ml) in RPMI 1640 supplemented with 10% heat inactivated AB serum and 2 µg/ml PHA, were added to wells containing monocytes (10^5 lymphocytes/well). Pure IL-1 or IL-1 conditioned medium serially diluted in RPMI 1640, 100µl, was added to the T lymphocytes - a total of 200µl added per well. Control wells consisted of monocytes, T lymphocytes and PHA, with no exogenous IL-1 added.

Infected and uninfected monocytes were incubated for a further 3 days in the presence of autologous T cells and PHA, with or without various concentrations of IL-1. Lymphocyte stimulation was assessed after 72h by the addition of 0.5 uCi of [^{3}H]thymidine (specific activity, 19.3 Ci/mmol) per well for 16 hr. Cells were harvested using an automatic cell harvester and incorporated radioactivity measured by liquid scintillation counting and expressed as mean dpm for triplicate samples.

2.11.0. Detection of Intracellular IL-1β in Monocytes and ML-3 Cells by Immunoblotting

2.11.1. Protein Isolation

HCMV-infected or mock infected ML-3 cells and peripheral blood monocytes were harvested at 24 hr and 72 hr post infection and washed three times with PBS. Approximately 5 x 10^6 cells were extracted with 200 µl of lysis buffer consisting of 0.05 M Tris hydrochloride, 0.15 M NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 0.1 mM phenylmethyl sulfonyl flouride for 30 min on ice. The cells were disrupted by 20 strokes in a 1 ml Dounce homogenizer (Wheaton, Mellville, NJ, USA) and then centrifuged at 10 000 g for 20 min. The supernatant was decanted and stored at -20°C.

The Bio-Rad protein assay was employed, which is based on the shift in the absorbance maximum of Coomassie Brilliant Blue (CBB) G-250 from 465 nm to 595 nm when binding to protein occurs (Bradford, 1976). The protein content of samples solubilised in lysis buffer could be accurately estimated by this method. In all cases, a standard curve of absorbance versus concentration of protein was drawn up for BSA in the concentration range of 0-50 mg/ml protein and the unknown sample protein concentrations were read off the curve.

2.11.2. Polyacrylamide Gel Electrophoresis

Proteins from ML-3 cells and monocytes were analysed by SDS-polyacrylamide gel elecrophoresis by the method of Laemmli (1970). The slab gels used in this work consisted of a running gel (appendix 3) of 12% acrylamide and a 4% stacking gel (appendix 3). A Bio-Rad vertical slab gel apparatus was used. Electrophoretic separation was carried out in a continuous Tris-glycine buffer system at a pH of 8.3 (appendix 3). Thirty µl of sample containing 20-25µg of protein was mixed with 30µl of 2X sample buffer (appendix 3) and heated to 95°C for 5 min. Two µl of bromophenol blue tracking dye solution (appendix 3) was added to each sample. The samples were loaded onto the gel and electrophoresis performed at room temperature for 5 - 6 hr at 100 V.

2.11.3. Western Blot Analysis

Proteins separated on SDS-polyacralamide gels were transferred electrophoretically onto nitrocellulose sheets by a modification of the original procedure described by Towbin et al. (1979). The electrode buffer used for transfer was composed of 25 mM tris base, 192 mM glycine and 20% methanol (v/v) at pH 8.3. A sheet of nitrocellulose (0.45µm pore size, Schleicher and Schuell Inc., Keene, NH, USA) and sheets of Whatman's number 1 filter paper were soaked with electrode

buffer. The gel was rinsed with electrode buffer and a sandwich prepared with the following successive layers

(i) two sheets of filter paper

(ii) the nitrocellulose sheet

(iii) the SDS-polyacrylamide slab gel with the stacking gel removed

(iv) two more sheets of filter paper

Care was taken to remove all air bubbles between the gel and the nitrocellulose. The assembly was placed in a Bio-Rad electrophoretic transfer apparatus with the gel facing the cathode. The buffer tank was filled with transfer buffer to cover the gel and electrophoretic transfer performed at 200 mA for 16 hr at 4°C.

The nitrocellulose sheet was washed in PBS at room temperature for 10 min with gentle agitation. To prevent non-specific background binding, the filters used in the transfer were incubated at 37°C for 1 hr in PBS containing 1% BSA. This blocking solution was aspirated and replaced with a 1:100 dilution of rabbit anti-human IL-1β polyclonal antibody (Cistron Biotechnology, Pine Brook, NJ, USA) in 10 ml of blocking solution. Incubation was continued for 1 hr at 37°C with gentle agitation. The immunoblotted filter was washed three times for 10 min each with PBS containing 0.05% Tween-20.

Goat antirabbit immunoglobulin alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AB, USA) at a 1:1000 dilution in blocking solution was used for detection of IL-1β antigen. The filter was incubated with the secondary antibody for 1 hr at 37°C with gentle agitation and then washed three times in PBS containing 0.05% Tween-20. The colour indicator substrate was prepared as follows: 3.3 mg 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) was dissolved in 1 ml N,N-dimethyl-formamide (DMF) and 6.6 mg nitro blue tetrazolium (NBT) dissolved in 50% DMF in water. One ml of each reagent was sequentially added to 200 ml of a 100mM Tris HCl (pH 9.5), 100mM NaCl, 5 mM $MgCl_2$ solution and immediately added to the filter. The filter was incubated at room temperature with gentle agitation until a purple colour developed (20-30 min). The filter was then transferred to another container containing water to halt colour development. Filters were air dried and stored between sheets of filter paper in the dark.

2.12.0. RNA PROCEDURES

2.12.1. Preparation of Total RNA from ML-3 Cells

PMA-stimulated or unstimulated ML-3 cells were infected or mock infected with HCMV and total RNA extracted at 1,2 and 4 days post infection. RNA was isolated by the

guanidine isothiocyanate method of Chirgwin et al. (1979). Cells were harvested by centrifugation and washed twice with PBS. The cell pellet was resuspended in 3.2 ml of 4 M guanidine isothiocyanate solution (appendix 4) and the suspension vortexed for 20 secs. The cells were homogenized at medium speed for 30 secs using a Polytron homogenizer (Brinkman Instruments Inc., Westbury, NY, USA). The lysate was carefully layered onto 1.2 ml of a 5.7 M CsCl solution in a 5 ml centrifuge tube (Beckman Instruments Inc., Palo Alto, CA, USA) and centrifuged for 18 hr at 36 000 rpm at 20° C in a SW 55 rotor (Beckman). The supernatant was carefully removed and the RNA pellet resuspended in a total of 400 µl of diethyl pyrocarbonate (DEPC) treated water (appendix 4). The RNA solution was transferred to a 1.5 ml microfuge tube and 40µl of 3 M sodium acetate, pH 6, added to the RNA preparation followed by 900 µl of 95% ethanol. The suspension was thoroughly mixed by vortexing. RNA was precipitated at -20° C overnight and the suspension spun in an Eppendorf microfuge for 30 min. The supernatant was discarded and the RNA pellet dried in a vacuum centrifuge (Savant Instruments, Hicksville, NY, USA). The pellet was resuspended in 50 µl of DEPC-treated water and the RNA concentration determined by O.D at 260 nm.

2.12.2. Electrophoretic Separation of RNA and Northern Blot

Total RNA extracted by the guanidine isothiocyanate method was resolved by agarose gel electrophoresis in the presence of formaldehyde according to the method of Lehrach et al. (1977). Agarose, 2g, (BRL, Gaithersburg,MD, USA) was dissolved by heating in 160 ml distilled water and cooled to 50°C before addition of 20 ml of 10X MOPS buffer (appendix 4) and 20 ml of 37% formaldehyde. A horizontal gel electrophoresis apparatus was used (Hoefer Scientific Instruments, San Francisco, CA, USA). Equal amounts of each RNA sample (20 µg) were applied to wells in 5 mm thick agarose formaldehyde gels. RNA was denatured in 25-30µl of loading buffer (appendix 4) by heating to 56° C for 15 min. Elecrophoretic resolution was carried out using 1 X MOPS as the running buffer at 4° C for 18-20 hr at 35-40 V per gel.

Total RNA resolved by agarose gel electrophoresis into 28 S rRNA and 18 S rRNA species was transferred onto nitrocellulose sheets using the procedure described by Maniatis et al. (1982). Nitrocellulose (0.45 µm pore size, Scheicher and Schull) and three pieces of Whatman 3MM paper cut to size were wetted in 2 X SSC buffer (appendix 3). Whatman 3MM paper was wrapped around a glass plate and the plate placed on a support in a large baking dish. The dish was filled with 20 X SSC to just below the top of the support and a sandwich

prepared on the glass plate with the following successive layers :

(i) two sheets of Whatman 3MM paper cut to the size of the gel and soaked in 2 X SSC

(ii) the agarose gel

(iii) a nitrocellulose sheet cut 1-2 mm smaller than the gel and soaked in 2 X SSC

(iv) one sheet of Whatman 3MM paper soaked in 2 X SSC

(v) paper towels cut to the size of the nitrocellulose to a height of 5-8 cm

This arrangement allowed for a flow of liquid from the reservoir through the gel so that the RNA was deposited on the nitrocellulose. Transfer was complete within 12 hr. The paper towel stacks and paper sheets were removed and the nitrocellulose sheet placed between two sheets of 3MM paper and baked for 2 hrs at 80°C under vacuum.

2.12.3. Dot Blot Analysis of Monocyte RNA

LPS-stimulated or unstimulated monocytes were infected (5 pfu/cell) or mock-infected with HCMV and total RNA extracted at 1,2 and 4 days post-infection using a modification of the method of White and Bancroft (1982).

Monocytes were cultured in 15 ml polypropylene tubes at a concentration of 1-2 x 10^6 cells/ml and transferred to Eppendorf microfuge tubes for RNA extraction. Cells

were harvested and washed twice with DEPC-treated PBS. Two hundred µl of lysis buffer (appendix 4B) was then added to the pellet. The cells were sheared using a 26G needle attached to a 1 ml syringe and the cell lysate incubated at 37° C for 15 min. Phenol:chloroform (1:1), 200 µl, was added to the lysate, the suspension vortexed and allowed to stand at room temperature for 3 min. The two phases were separated by spinning in an Eppendorf microfuge for 2 min. The aqueous phase was transferred to a new tube and extracted with 0.5 ml chloroform. The suspension was vortexed and spun for 2 min in an Eppendorf microfuge. The aqueous phase was collected and 0.5 ml cold ethanol added. The RNA was allowed to precipitate at -20°C overnight. The ethanol precipitates were spun in an Eppendorf microfuge for 10 min and the ethanol decanted. RNA pellets were dried in a vacuum centrifuge for 5 min. The pellet was dissolved in 100 µl of TE buffer (pH8) by occasional vortexing. One hundred µl of 4 M $LiCl_2$ was added and the tubes placed at 4° C overnight. Samples were centrifuged at 12 000 rpm for 20 min in an Eppendorf microfuge and the supernatants carefully removed. The RNA pellet was resuspended in 10 µl of DEPC treated water, 6µl of 20X SSC and 4µl of 37% formaldehyde. The samples were heated at 60 °C for 15 min and 80 µl of 15X SSC added to bring the volume to 100 µl. RNA was serially diluted two-fold in 15X SSC and applied to nitrocellulose. The dot blot manifold (Schleicher and Schuell Inc., Keene, NH, USA) was assembled as follows : Whatman 3MM paper

and 0.45µm nitrocellulose were cut to the size of the apparatus. The nitrocellulose was soaked first in DEPC-treated water followed by 15X SSC.This was placed on top of the Whatman 3MM paper also soaked with 15X SSC. The wells were rinsed twice with 15X SSC. The RNA samples were applied to the wells after which they were rinsed twice with 15X SSC. The nitrocellulose filter was baked in a vacuum oven at 80° C for 2 hrs.

2.12.4. Oligonucleotide and cDNA probes

2.12.4.1. Kinase Labelling of 5' ends of Oligonucleotide DNA Probes

Oligonucleotide probes for IL-1ß, CSF-1, TNFα, lysozyme and ß-actin were a gift from E. Kawasaki and were synthesized at Cetus Corporation (San Francisco, CA, USA) from published sequences: IL-1ß (March et al., 1985), CSF-1 (Kawasaki et al., 1985), TNF α (Wang et al., 1985), lysozyme (synthesized from a consensus sequence) and ß-actin (Ponte et al., 1984).

The 5' ends of the DNA probes were labelled with T4 polynucleotide kinase in a reaction mixture containing 1-50 pmoles of dephosphorylated DNA (5' ends), 10µl of 10X kinase buffer I (appendix 4), 150 uCi[^{32}P] ATP (specific activity=3000 Ci/mmole), 10-20 units T4 polynucleotide kinase and water to 50µl. The reaction

mix was incubated at 37 °C for 30 min. Following incubation, 2 µl of 0.5M EDTA was added, the DNA extracted once with phenol/chloroform (1:1) and precipitated with ethanol. The DNA was redissolved in 50 µl of TE buffer (pH 9) and labelled DNA separate from unincorporated ^{32}P-ATP by separation th ough small columns of Sephadex G-50.

Sephadex G-50 columns were set up as follows : the bottom of a 1 ml syringe was plugged with sterile glass wool. In the syringe a column (0.9 ml bed volume) was pre ared using Sephadex G-50 equilibrated in TE (pH 8) containing 0.1 M NaCl. The syrin s inserted plastic centrifuge tube and spu 000 rpm for 10 min. Sephadex was added entrif repeated untilthe column volum olumn was washed twice using 200 µl of T The DNA was applied to the column in a mal V 0.1 ml. The column was spun at 2 000 rpm for 5 min And the effluent from the syringe collected in d capped Eppendorf tube. The unincorporated ^{32}P dN remained in the syringe.

2.12.4.2. Nick Translation of cDNA probes

Three cDNA clones, MAD-2, MAD-6 and MAD-9 were isolated by Dr. Stephen Haskill, Chapel Hill, NC, USA, while some of the above studies were being carried out in his

laboratory. The clones were induced in monocytes in a rapid response to adherence. These cDNA clones were (i) MAD-9, which is identical to a proinflammatory protein that is chemotactic for human neutrophils. This protein is known as monocyte-derived neutrophil chemotactic factor (MDNCF) purified by Yoshimura et a. (1987) or as neutrophil-activating factor (NAF) (Peveri et al., 1988) or as neutrophil-activating protein (NAP-1) (Larsen et al., 1989), (ii) MAD-2 shows 88% nucleotide and amino acid homology with a melanoma stimulatory growth factor called h-gro or MGSA (Anisowicz et al., 1987; Richmond et al., 1988) and (iii) MAD-6 whose DNA sequence shows no homology to know gene..

The cDNA clones were labelled with [α-^{32}P] in a nick translation reaction containing 5 µl of 10X nick-translation buffer, 1 µg of DNA, 1 µl of a 1 mM solution of dNTPs, 100 pmoles of [α-^{32}P]dNTPs in a total reaction volume of 44 µl. The reaction mixture was chilled to 0 °C before addition of 0.5 µl of a DNase I solution (0.1 µg/ml). After vortexing, 5 units of E. coli DNA polymerase I (Promega Biotech., Madison, WI, USA) was added and the reaction mixture incubated at 16° C for 1 hr. The reaction was stopped by addition of 2 µl of 0.5M EDTA and the nick-translated DNA separated from the unincorporated dNTPs by chromatography on a Sephadex G-50 column.

A Sephadex G-50 column was prepared in a disposable 5 ml borosilicate glass pipette . The DNA sample in a volume of 200 µ l was applied to the column and a reservoir of TE (pH 8) connected to the column so thatthe flow rate was 0.5 ml/min. The leading peak of radioactivity consisted of nucleotides incorporated into the DNA. These fractions were pooled and stored at -20 ° C.

2.12.5. Hybridization

Baked nitrocellulose filters were prewashed for 1 hr at 42° C in a solution containing 0.1 M EDTA (pH 8), 5 M NaCl, 1 M Tris-HCl (pH 8) and 10% SDS. This was followed by prehybridization at 42 °C for 2 hr in hybridization fluid containing 50% formamide, 5X SSC, 5X Denhardts, 0.1% SDS, 10 mM NaH PO_4 and 100µg/ml salmon sperm DNA. [^{32}P]-labelled oligonucleotide probes or cDNA probes were then added to the hybridization fluid at 10^6 cpm/ml and filters hybridized for 18 to 24 hrs at 27 °C for oligonucleotide probes and 42° C for cDNA probes. Filters were washed to a final stringency of 0.2X SSC as follows :

(i) 6 X SSC, 20 mM NaH PO_4, 0.1% SDS for 10 min at 27° C.

(ii) 2 X SSC, 20 mM NaH PO_4 for 20 min at 42°C.

(iii) 1 X SSC, 20 mM NaH PO_4 for 20 min at 42 C.

(iv) 0.2 X SSC, 20 mM NaH PO_4 for 20 min at 42° C.

Filters were exposed to Kodak XAR X-ray film at -70° C.

2.13.0. In situ Hybridization

2.13.1. Cell Culture and Fixation

Monocytes were isolated by Ficoll-Hypaque separation and $2x10^5$ cells/well adhered to an 8 chamber Labtek culture slide for 30 min at 37° C. After removal of non-adherent lymphocytes, the remaining adherent monocytes were incubated overnight in RPMI 1640 supplemented with 10% AB serum. Mononcytes were then infected with HCMV at a MOI of 5 pfu/cell or mock infected with supernatant from uninfected fibroblast cultures as described previously.

At 1,2 and 4 days post-infecton, culture medium was removed and cells rinsed with RPMI 1640. Cells were fixed in 4% paraformaldehyde/PBS for 15 min at 4°C followed by a second incubation at room temperature for 15 min. Cells were then gently rinsed twice with PBS with 5 mM $MgCl_2$ added (PBSM). Slides were dehydrated at 22°C as follows: 70% ethanol for 3 minutes, 95% ethanol for 3 minutes, 100% ethanol for 3 minutes.

2.13.2. Hybridization

Slides were incubated sequentially in PBSM and 0.2M

Tris-HCl (pH 7.5), 0.1M glycine for 10 min at room temperature. Labelled cRNA probe was resuspended at $3x10^6$ cpm/ml in hybridization buffer containing 50% deionized formamide, 0,6 M NaCl, 10 mM Tris-HCl pH 7.5,

1 mM EDTA, 1% SDS, 10 mM dithiothreitol (DTT), 10% polyethylene glycol (PEG-6000), 1X Denhardt's solution and 0.25 mg/ml E.coli tRNA. A 15 µl volume of this solution was applied to each chamber of the slide and covered with a coverslip. Slides were incubated at 55° C for 20 hr in petri dishes containing moist Whatman filter paper circles (wetting solution contained 50% v/v deionized formamide, 50% v/v distilled water and 100 mg/ml PEG-8000). Petri dishes were sealed with plastic tape.

Coverslips were removed by gently dipping slides into PBSM. Slides were incubated for 30 min at 37°C in a 0.5 M NaCl, 10 m.. Tris-HCl (pH 8) solution containing 20 mg/ml RNAse A to facilitate digestion of non-hybridized ssRNA. Slides were then washed in 0.5 M NaCl, 10mM Tris-HCl pH 8 at 37°C for 30 min followed by four stringency washes as follows

(i) 2X SSC, 50% formamide, 10 mM DTT for 30 min at 55° C

(ii) 1X SSC, 50% formamide, 10mM DTT for 30 min at 55° C

(iii) 1X SSC, 50% formamide, 10mM DTT, 0.05% Triton X-100 for 30 min at 37° C.

(iv) 1X SSC, 50% formamide, 10mM DTT, 0.05% Triton X-100 for 30 min at 55° C.

Slides were then dehydrated with increasing concentrations of ethanol and air dried.

2.13.3. Autoradiography

NTB2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY, USA) was dissolved in 0.6 M ammonium acetate which had been prewarmed to 42° C. Slides were coated with emulsion solution which was kept at 42° C in a waterbath. After air drying for 1 hr, slides were exposed at 4°C in a light tight box containing dessicant chips for 5-10 days.

2.13.4. Development and Counterstain

Slides were warmed to room temperature and developed in Kodak D19 developer for 1 min and fixed in Kodafix (Eastman Kodak) for 5 min at room temperature. After two washes in distilled water for 5 min each, slides were counterstained as follows:

(i) Gil's haematoxylin, No.2 (Sigma Corp.,St.Louis, MO., USA) for 5 secs followed by three 10 second and one 5 minute wash in distilled water

(ii) water soluble eosin, 0.5% w/v, (Sigma) for 90 seconds, followed by three 10 second and one 5 minute wash in distilled water.

Slides were dehydrated in increasing concentrations of ethanol followed by three xylene dips of 3 minutes

each. For viewing, slides were mounted in Permount.

2.13.5. cRNA probe labelling

For the generation of sense and antisense RNA probes, the full length 1.8 kb IL-1ß cDNA was subcloned into the Bam HI site of the plasmid pGEM-blue (Promega Biotech, Madison, WI, USA) in two different orientations. Run-on transcripts of the template cDNA were synthesized using T7 RNA polymerase in a 20 µl reaction mix containing 0.2µg of linearized template DNA, 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine. 10 mmM NaCl, 10 mM DTT, 2.5 mM each of ATP, CTP and 0.04 mM ^{35}S-α-UTP (650 Ci/mmol; Amersham Corp., Arlington Heights, IL, USA). The labelled RNA was extracted with phenol:chloroform (1:1), ethanol precipitated and resuspended in hybridization buffer.

2.14.0. Transfection Studies

2.14.1. Plasmid DNA

Plasmids 3M-AT and 3ME-AT were obtained from Dr. P. Auron, MIT, Cambridge, MA, USA. These plasmids containing putative human proIL-1ß gene regulatory regions were derived by insertion of each of two different fragments derived from the BDC-454 clone described by Clark et al. (1986). The AvaI/ TaqI

fragment located between positions -512 and +12 (Fragment AT) was treated with the Klenow fragment of E. coli DNA polymerase and inserted by blunt-end ligation into the pSVCAT-3M plasmid described by La'mins et al. (1984). Therefore the resulting plasmid contained proIL-1β genomic DNA corresponding to positions -512 to +14 for AT (Figure 3). In addition, these fragments contain a TATA sequence at position -31 as well as other features which have been described by Auron et al. (1989).

The HCMV immediate-early gene region plasmids pHD101SV1, pHD101SV2 and 10142 (Davis et al., 1987) were constructed in the laboratory of Dr. Eng-Shang Huang. In pHD101SV1, the region 1 and region 2 of the HCMV IE region are under control of the HCMV IE promoter with the SV40 enhancer located further upstream. Plasmid pHD101SV2 is identical to pHD101SV1 except that the 5.5 kb Bam H1 fragment containing region 2 has been deleted. Plasmid 10142 has the exon sequence of region 1 deleted.

2.14.2. Plasmid Purification

An overnight culture of E.coli containing plasmids 3M-AT and 3ME-AT respectively, was grown at 37°C in 40 ml LB medium (appendix 5) supplemented with 50μg/ml ampicillin. The culture was added to 1 litre of

minimal medium (appendix 5) and grown with shaking in a Gallenkamp orbital shaker until the optical density (OD) at 600 nm was 0.6. When this OD was reached, 1 ml of a 100 µg/ml chloramphenicol solution was added to amplify copy number of the plasmid while stopping bacterial replication. The flask was shaken at 120 rpm overnight at 37 °C. Cells were spun down at 6000 g for 5 min at 4 °C. The pellet was resuspended in 10 ml of a solution containing 0.05 M Tris-HCl, 0.01 M EDTA and 20% sucrose together with 1 ml of 50 mg/ml freshly made lysozyme solution. Twenty ml of a solution containing 0.2 N NaOH and 1% SDS was then added with gentle mixing and the tube incubated on ice for 15-20 min. Fifteen ml of a 3 M NaAc solution was then added and the tube swirled very gently. The suspension was incubated on ice for a further 15 min. The white precipitate was spun down at 10 000 g for 20 min. The supernatant was extracted with an equal volume of phenol:chloroform (1:1) and the phases separated at 5 000 g for 10 min. The upper aqueous phase was transferred to a new tube, extracted with an equal volume of chloroform and the phases separated by centrifugation. The aqueous phase was removed and one tenth of this volume of 3M NaAc plus 2 volumes of 95% ethanol were added to the aqueous phase. The DNA was precipitated for 1 hr at -20° C and centrifuged at 10 000 g for 30 min at 4 °C. The pellet was dried at room temperature for 25 min. CsCl was made up at 1.58g/ml in TE buffer and the refractive index

adjusted to 1.395-1.400. The DNA pellet was resuspended in 36 ml of CsCl solution and the suspension transferred to Beckman quick-seal tubes (VTi 50; 25 x 89 mm tube). The remaining volume of the tube was completely filled with ethidium bromide in TE buffer (10 mg/ml), the tubes sealed and spun in a Beckman VTi 50 vertical rotor at 45 000 rpm for 18 hrs. After centrifugation, the lower band containing plasmid DNA was removed using a syringe needle attached to a syringe. The collected material was made up to 36 ml with CsCl and banded a second time as before. The plasmid band was removed in the same way and an equal volume of n-butanol added. This was mixed well and the upper butanol layer discarded. This extraction was repeated three more times until all traces of ethidium bromide were removed. The lower aqueous phase was transferred to a 30 ml glass tube and extracted once with phenol:chloroform (1:1) and once with chloroform. DNA was ethanol precipitated and centrifuged at 10 000 rpm for 30 min at 4 °C. The pellet was dried, resuspended in 1 ml sterile distilled water and the OD at 260 nm measured to determine the DNA concentration.

2.14.3. DNA Transfections

Plasmid DNA was reprecipitated with 3 M ammonium acetate, pH 6, and 95% ethanol prior to transfection and resuspended in sterile distilled water at a

adjusted to 1.395-1.400. The DNA pellet was resuspended in 36 ml of CsCl solution and the suspension transferred to Beckman quick-seal tubes (VTi 50; 25 x 89 mm tube). The remaining volume of the tube was completely filled with ethidium bromide in TE buffer (10 mg/ml), the tubes sealed and spun in a Beckman VTi 50 vertical rotor at 45 000 rpm for 18 hrs. After centrifugation, the lower band containing plasmid DNA was removed using a syringe needle attached to a syringe. The collected material was made up to 36 ml with CsCl and banded a second time as before. The plasmid band was removed in the same way and an equal volume of n-butanol added. This was mixed well and the upper butanol layer discarded. This extraction was repeated three more times until all traces of ethidium bromide were removed. The lower aqueous phase was transferred to a 30 ml glass tube and extracted once with phenol:chloroform (1:1) and once with chloroform. DNA was ethanol precipitated and centrifuged at 10 000 rpm for 30 min at 4 °C. The pellet was dried, resuspended in 1 ml sterile distilled water and the OD at 260 nm measured to determine the DNA concentration.

2.14.3. DNA Transfections

Plasmid DNA was reprecipitated with 3 M ammonium acetate, pH 6, and 95% ethanol prior to transfection and resuspended in sterile distilled water at a

concentration of 1 µg/ul. PMA-stimulated or unstimulated ML-3 cells were resuspended in RPMI 1640 and 10% AB serum at a concentration of 10^7 cells/0.5 ml for each transfection. ML-3 cells were transfected by electroporation with a Zapper Electroporation Unit (University of Wisconsin Medical Electronics Lab, Madison, WI, USA) at 1300 V. Cells and plasmid DNA were mixed in 0.5 ml RPMI 1640 and 10% AB serum in a 1 cm x 1 cm plastic cuvette to which aluminium electrode strips were glued. After voltage was applied to the samples, the cells were diluted into 10 ml RPMI medium containing 10% AB serum in 100 mm plastic petri dishes and incubated at 37°C in an atmosphere of 5% CO_2/95% air.

2.14.4. Chloramphenicol Acetyltransferase (CAT) Assay

Cell extracts were prepared and assayed for CAT activity by a modification of the method described by Gorman et al., (1982). Cells were pelleted at 1 000 rpm and washed three times with PBS. The cell pellet was resuspended in 200 µl of 0.25 M Tris-HCl, pH 7.8. ^{14}C chloramphenicol (0.2 µCi; specific activity 60 mCi/mmol; New England Nuclear, Boston, MA) was incubated with 0.7 mM acetyl coenzyme A (Sigma Chemical Co., St Louis, MO, USA) and 100 µl of cell extract in a final volume of 120 µl of Tris-HCl, pH 7.8 for 60 min at 37°C. Radioactive materials were extracted with ethyl acetate and the

non-acetylated chloramphenicol and acetylated reaction products separated by chromatography on thin layer chromatography (TLC) plates (Eastman Kodak Co., Rochester, NY, USA). Plates were exposed to Kodak XAR X-ray film overnight.

2.15.0. Staining of ML-3 Cells and Flow Cytometry Analysis

The method was adapted directly from the method of Mann et al. (1987). Cells were washed in PBS and the cell pellet resuspended in 1.5 ml of PBS containing 4% paraformaldehyde. Cells were incubated at 37°C for 15 min with constant agitation. The cell pellet was resuspended in PBS supplemented with 0.2% Triton X-100 and incubated on ice for 10 min. The cells were then pelleted by centrifugation, resuspended in PBS containing 0.1% Tween 20 and 1% BSA and incubated on ice for 10 min. Cells were then resuspended in 5 µl of anti-human IL-1β (IgG1) monoclonal antibody (1:20) (Cistron Biotechnology, Pine Brook, NJ, USA) or 5 µl of the T cell specific Leu 2a, an antibody non-reactive for macrophages of the IgG1 subtype (Becton Dickinson, Mountainview, CA, USA) and incubated for 30 min at 37 °C. The cells were then washed twice in PBS supplemented with 0.1% Tween 20 and 1% BSA, pelleted by centrifugation and resuspended in 10µl of a 1:20 dilution of phycoerythrin conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AB, USA) and

incubated at 37° C for 30 min. Cells were then washed twice and resuspended in PBS for flow cytometry analysis. Control cells were stained with secondary antibody only. Flow cytometric analysis was carried out on an ORTHO SYSTEM 50 operating with simultaneous 488 and 514 nm excitation.

2.16.0. Staining of Monocytes for Intracellular IL-1β

CMV infected and mock-infected monocytes were cultured in Labtek slides and stained for the presence of intracellular IL-1β at various times post infection. Cells were washed in PBS and fixed in PBS containing 4% paraformaldehyde at 37°C for 15 min. This solution was replaced with PBS containing 0.2% Triton X-100 and incubated on ice for 10 min. Slides were then incubated in 3% hydrogen peroxide on ice for 10 min to prevent endogenous monocyte peroxidase activity, followed by incubation in a 50% v/v normal goat serum (NGS)/PBS solution to prevent non-specific binding of monoclonal antibody to Fc receptors on monocytes.

Slides were then incubated with anti-human IL-1β (IgG 1) monoclonal antibody (Cistron Biotechnology, Pine Brook, NJ, USA) or Leu 2a (Becton Dickinson, Mountainview, CA, USA) for 30 min at 37°C and then washed in three changes of PBS, care being taken to prevent drying at any stage. Peroxidase-conjugated

incubated at 37° C for 30 min. Cells were then washed twice and resuspended in PBS for flow cytometry analysis. Control cells were stained with secondary antibody only. Flow cytometric analysis was carried out on an ORTHO SYSTEM 50 operating with simultaneous 488 and 514 nm excitation.

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CMV infected and mock-infected monocytes were cultured in Labtek slides and stained for the presence of intracellular IL-1β at various times post infection. Cells were washed in PBS and fixed in PBS containing 4% paraformaldehyde at 37°C for 15 min. This solution was replaced with PBS containing 0.2% Triton X-100 and incubated on ice for 10 min. Slides were then incubated in 3% hydrogen peroxide on ice for 10 min to prevent endogenous monocyte peroxidase activity, followed by incubation in a 50% v/v normal goat serum (NGS)/PBS solution to prevent non-specific binding of monoclonal antibody to Fc receptors on monocytes.

Slides were then incubated with anti-human IL-1β (IgG 1) monoclonal antibody (Cistron Biotechnology, Pine Brook, NJ, USA) or Leu 2a (Becton Dickinson, Mountainview, CA, USA) for 30 min at 37°C and then washed in three changes of PBS, care being taken to prevent drying at any stage. Peroxidase-conjugated

goat antimouse IgG1 (Cappel Labs., Cochranville, Pa, USA) (1:50 dilution) was then added for 30 min at 37° C. Slides were rinsed in three changes of PBS and incubated in PBS containing 0.02% diaminobenzidine (DAB) and 0.01% hydrogen peroxide in PBS at room temperature for 5-10 min . After a final rinse in PBS, stained cells were counterstained with a 0.1% solution of methyl green.

3.0.0. RESULTS

3.1.0. Comparison of the degree of infection of monocytes with a clinical isolate of HCMV and laboratory-adapted strain AD169

Unstimulated or LPS-stimulated monocytes challenged with HCMV strain AD169, which has been highly passaged in human fibroblasts, showed very low (0.01% - 0.1%) expression of HCMV immediate-early antigens, even when a multiplicity of infection of 5 pfu/cell was used. No differences in the number of IE-positive monocytes were observed for stimulated or unstimulated cultures. HCMV IE antigen expression in both unstimulated and LPS-stimulated monocytes was demonstrable in a higher percentage of monocytes after infection with the clinical isolate of CMV (Figure 4). The percentage of monocytes showing nuclear or perinuclear flourescence increased slightly with increasing MOI's (Table 2). For an MOI of 0.001 pfu/cell, as assessed by plaque forming assays on fibroblasts, 1-2% of infected monocytes showed detectable IE antigen expression, while for MOI's of 0.005 and 0.1 pfu/cell, the percentage of monocytes showing flourescence varied between 3-5% and 7-9% respectively (Table 2). Indeed,the results suggest that many more infectious virus particles were present in the clinical isolate, than could be detected by pfu assays on fibroblasts. When an MOI of 1 pfu to 1

Table 2

Percentage of monocytes expressing the major 72-kDa immediate-early CMV polypeptide after infection with CMV strain AD169 or a CMV isolate

CMV strain	Multiplicity of infection (PFU/cell)*	E-3 positive† (%)
AD169	0.5	0
	1.0	0-0.01
	5.0	0.01-0.1
V3474-84‡	0.001	1-2
	0.005	3-5
	0.01	4-6
	0.1	7-9

* Multiplicities of infection were estimated by plaque forming assays on fibroblasts.

† Monoclonal antibody E-3 detects the major 72-kDa immediate-early CMV polypeptide.

‡ CMV clinical isolate strain, V3474-84, was isolated from an adult with CMV mononucleosis.

monocyte, as assayed on fibroblasts, was used, cell lysis of monocytes was observed (unpublished data). No expression of IE antigen was observed in uninfected monocytes (Figure 4), or in infected monocytes where only secondary antibody was used for staining. Monocytes that were positive for IE antigen expression were observed after 3 days in culture, and thereafter for as long as cells remained viable (7-9 days).

3.2.0. Suppression of T Lymphocyte Proliferation by Autologous, LPS-stimulated or Unstimulated, HCMV-infected monocytes.

The number of unstimulated, uninfected monocytes needed to augment the proliferative response of purified autologous T cells was initially established (Table 3). For all donors tested the ratio of monocytes:T lymphocytes that gave a significant increase in lymphocyte stimulation was found to be 1 : 5 ($4x10^4$ monocytes : $2x10^5$ T lymphocytes). Therefore for all subsequent experiments, a 1:5 ratio was used, and the percentage stimulation of T lymphocytes in the presence of 4×10^4 unstimulated, uninfected monocytes taken as 100; all other increases or decreases detailed in Table 3 were calculated relative to this.

FIGURE 4

HCMV immediate-early antigen immunoflouresence staining of human peripheral blood monocytes infected with HCMV isolate V3474-84. Immediate-early antigen was probed with monoclonal antibody E3 and indirect immunoflouresence.

(a) HCMV IE antigen flouresence staini of uninfected monocytes. Cells were stained 3 days after mock-infection (500X)

(b) Peripheral blood monocytes infected with HCMV isolate V3474-84 stained positive for IE antigen expression 3 days after infection (500X)



a



b

Addition of LPS-stimulated monocytes to autologous T lymphocytes, in the same ratios as for unstimulated monocytes, significantly augmented the T cell response to PHA when compared to unstimulated monocytes plus T cells, to T cells alone (Table 3).

In the presence of both unstimulated and stimulated monocytes, the proliferative response of autologous T cells to PHA was significantly suppressed in the presence of monocytes infected with either the clinical isolate of HCMV or strain AD169 (Table 3). For both strains of virus, the higher the MOI used, the greater was the suppression of lymphocyte responses by infected monocytes. However, for all donors tested, monocytes infected with the clinical isolate were significantly more suppressive for T cell responses to PHA than were monocytes infected with strain AD169 ($P<.01$ by Student's t test).

LPS-stimulated monocytes infected with either strain of HCMV at the same MOI's used for unstimulated monocytes, were significantly more suppressive for autologous T lymphocyte proliferation than were infected, unstimulated monocytes. Infection of LPS-stimulated monocytes with the clinical isolate at MOI's of as low as 0.005 pfu/cell, completely abrogated the mitogenic response of T lymphocytes.

Table 3
Suppression of T lymphocyte proliferation by LPS-stimulated or unstimulated, CMV-infected monocytes.

Cell type	CMV strain and MOI (PFU/cell)	^{3}H-THYMIDINE INCORPORATION* (ΔDPM X 10^{-3}) 4 x 10^4 monocytes	% stimulation	8 x 10^4 monocytes	% stimulation
T lymphocytes only (2 x 10^5)	-	109.82 ± 7.91	45.5	109.82 ± 7.91	45.5
T lymphocytes and unstimulated monocytes	-	241.12 ± 15.15	100†	275.17 ± 13.56	114
	AD 169 (0.5)	174.62 ± 12.46	72.4	223.82 ± 9.94	92.8
	AD 169 (1.0)	110.46 ± 10.01	45.8	133.43 ± 8.75	55.3
	AD 169 (5.0)	55.38 ± 8.25	22.9	60.10 ± 6.25	24.9
	V3474-84 (0.001)‡	150.41 ± 9.52	62.3	230.40 ± 9.46	95.5
	V3474-84 (0.005)	70.56 ± 6.48	29.3	120.55 ± 8.93	49.9
	V3474-84 (0.01)	28.81 ± 2.82	11.9	40.33 ± 5.21	16.7
T lymphocytes and LPS-stimulated monocytes	-	360.52 ± 24.9	150	380.27 ± 26.1	158
	AD169 (0.05)	210.21 ± 15.12	87.1	201.40 ± 14.32	83.4
	AD169 (1.0)	31.49 ± 4.97	13.0	21.20 ± 2.01	8.8
	AD 169 (5.0)	15.25 ± 2.44	6.3	10.01 ± 1.04	4.2
	V3474-84 (0.001)	300.61 ± 21.52	124.7	310.65 ± 21.08	128.8
	V3474-84 (0.005)	34.46 ± 4.09	14.3	45.63 ± 5.12	18.9
	V3474-84 (0.01)	22.39 ± 2.21	9.3	25.42 ± 2.71	10.5

* (^{3}H) thymidine incorporation is expressed as the mean dpm of triplicate samples ± 1 SD.

† % stimulation of T lymphocytes in the presence of 4 x 10^4 unstimulated, uninfected monocytes and PHA (2µg/ml) is represented as 100%. All other percentages are calculated relative to this value.

‡ CMV clinical isolate strain V3474-84 was isolated from an adult with CMV mononucleosis.

Differences in the degree of suppression that were observed for monocytes infected with either strain of HCMV, appear to be related to the extent of expression of the HCMV genome in infected cells, with monocytes infected with the clinical isolate showing detectable expression of immediate-early antigens and being significantly more suppressive for T lymphocyte responses than monocytes challenged with strain AD169.

3.3.0. Effect of HCMV Infection on Production of Functional IL-1 Activity by Unstimulated and LPS-stimulated Monocytes.

Infection of both unstimulated and stimulated monocytes with either the clinical isolate of HCMV or strain AD169 abrogated their production of functional IL-1 activity (Figure 5). Supernatants from unstimulated and stimulated HCMV-infected monocytes were significantly more suppressive for PHA-induced proliferation of mouse thymocytes than were uninfected monocytes. Uninfected, unstimulated monocytes produced low levels of IL-1 over the three days in culture, but IL-1 activity of monocytes infected with either strain of HCMV was 3-5 times lower than that of uninfected cells with the decrease in production of functional IL-1 activity being more marked in monocytes infected with the clinical isolate of HCMV (Figure 5). The net levels of IL-1 activity produced by monocytes stimulated with LPS

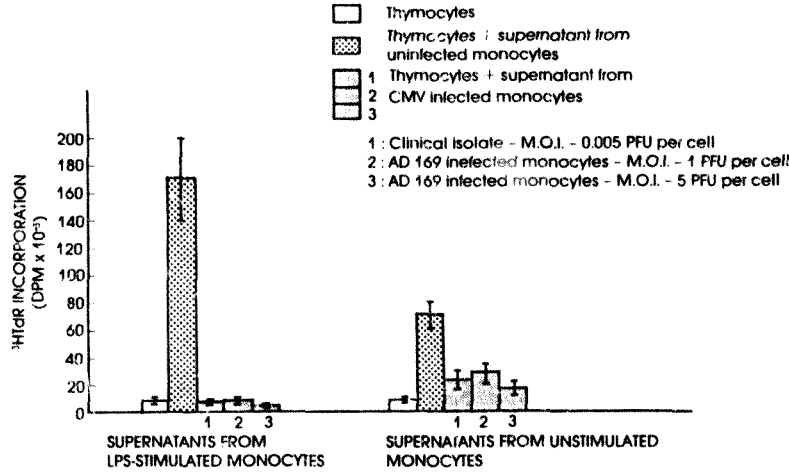


Figure 5
Effect of CMV infection on production of IL-1 activity by unstimulated and LPS-stimulated monocytes.
Supernatants from LPS-stimulated and unstimulated, uninfected or CMV-infected monocytes were assayed in the conventional bioassay which measures the ability of IL-1 to augment the PHA-induced proliferation of mouse thymocytes. IL-1 activity is expressed as the mean incorporation of (^{3}H) thymidine in dpm for triplicate samples ± 1 SD.

were considerably higher than those produced by unstimulated monocytes over the given culture period (Figure 5). The decreased production of IL-1 activity from stimulated monocytes as a result of infection by either strain of HCMV is more marked than for unstimulated cells, with levels produced by stimulated infected monocytes being 17-35 times lower than for stimulated, uninfected monocytes. Indeed, the supernatants of stimulated, infected monocytes did not augment [^{3}H]thymidine incorporation at all.

3.4.0. Effect of Addition of IL-1 Containing Supernatants or Pure IL-1 on T Lymphocyte Proliferation in the Presence of HCMV-infected Monocytes.

Having observed the suppression of proliferative responses of T lymphocytes to PHA in the presence of HCMV-infected monocytes, as well as a HCMV-induced abrogation of functional IL-1 activity, subsequent experiments in this study investigated whether this decrease was contributing to the hyporesponsiveness of lymphocytes, as IL-1 is an essential second signal for T cell activation (Oppenheim and Gery, 1982).

Addition of varying dilutions of pure IL-1 or IL-1-containing supernatant to stimulated, HCMV-infected monocytes, completely restored the proliferative response of autologous T lymphocytes to similar levels

measured in the presence of stimulated uninfected monocytes (Figure 6). This effect was observed for all concentrations of pure IL-1 used, ranging from 0.25 U/ml to 2 U/ml. Addition of IL-1-containing supernatant from LPS-stimulated monocytes undiluted and at a 1:2 and a 1:4 dilution, was able to restore the normal PHA response. At a 1:16 dilution, the response could not be fully restored. Proliferation at a 1:16 dilution was nevertheless significantly increased compared to levels in the presence of HCMV infected monocytes.

However, addition of varying dilutions of pure IL-1 or IL-1-containing supernatants to unstimulated, HCMV-infected monocytes was unable to restore the proliferative response of autologous T lymphocytes to levels found in the presence of unstimulated, uninfected monocytes (Figure 7). This was observed for the equivalent concentrations of IL-1 that were added to stimulated HCMV-infected monocytes.

3.5.0. Expression of HCMV IE Antigens in Differentiated and Undifferentiated ML-3 Cells

After treatment with PMA and infection with the Towne strain of HCMV, 10-12% of the cells were positive for IE proteins at 72h post infection (Figure 8). For undifferentiated cells infected with the Towne strain,

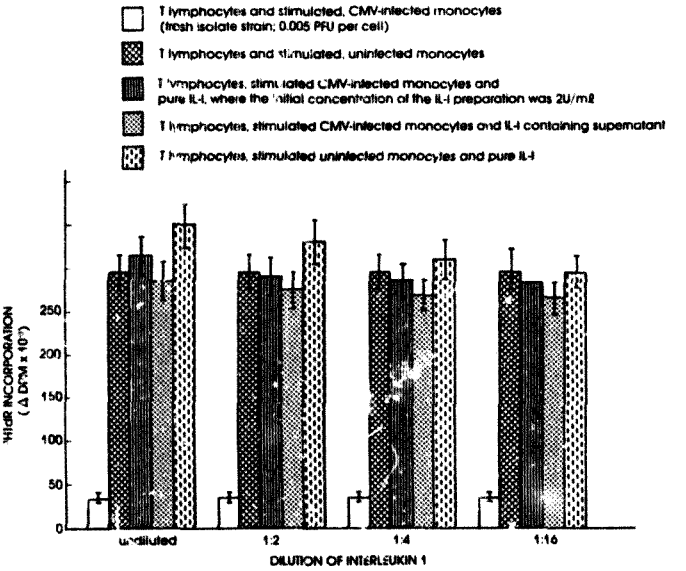


Figure 6
Effect of addition of IL-I containing supernatant or pure IL-I on the PHA-induced proliferation of T lymphocytes in the presence of PS-stimulated, CMV-infected monocytes. Culture supernatants from CMV-infected or uninfected monocytes were removed after 3-4 days and replaced with fresh autologous T lymphocytes in the presence of varying dilutions of IL-I containing supernatant or ultrapure IL-I. T lymphocyte proliferation was assessed 4 days later and (³H) thymidine incorporation expressed as the mean dpm of triplicate samples ± 1 SD.

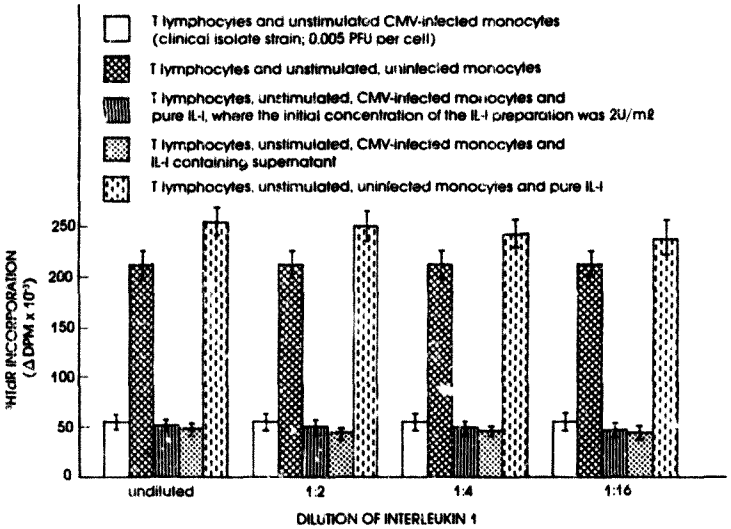


Figure 7
Effect of addition of IL-1 containing supernatant or pure IL-1 on the PHA-induced proliferation of T lymphocytes in the presence of unstimulated, CMV-infected monocytes. Culture supernatants from CMV-infected or uninfected monocytes were removed after 3-4 days and replaced with fresh autologous T lymphocytes in the presence of varying dilutions of IL-1 containing supernatant or ultrapure IL-1. T lymphocyte proliferation was assessed 4 days later and (^{3}H) thymidine incorporation expressed as the mean dpm of triplicate samples ± 1 SD.

the percentage of cells positive for IE proteins was 0-2% at 72h post infection. Pre-treatment of ML-3 cells with PMA for 24h prior to HCMV challenge, with subsequent washing to remove PMA, achieved the same effect as the continued presence of PMA in the medium throughout the 72h period after infection. This suggests that differentiation alone was responsible for increased expression of HCMV IE antigens. Treatment of ML-3 cells with PMA after infection also resulted in a greatly reduced frequency of cells expressing IE antigens (0 - 2%) at 72h, suggesting that pretreatment was essential for IE antigen expression.

3.6.0. HCMV Enhances Expression of Monocyte Inflammatory Mediator Genes in ML-3 Promyelocytes Following Induction of Differentiation by PMA

The results shown in Figure 9 indicate that exposure of the promyelocytic cell line, ML-3, to PMA prior to virus infection resulted in a high level of HCMV modulation of IL-1 ,TNF- and CSF-1. These three genes were all induced following PMA differentiation and their expression was further enhanced and sustained following HCMV infection. Within 24h of virus exposure, significantly higher levels of IL-1 , TNF and CSF-1 steady state mRNA were observed in HCMV-infected cells compared to uninfected controls. These levels were sustained for the next 3 days after infection. In ML-3

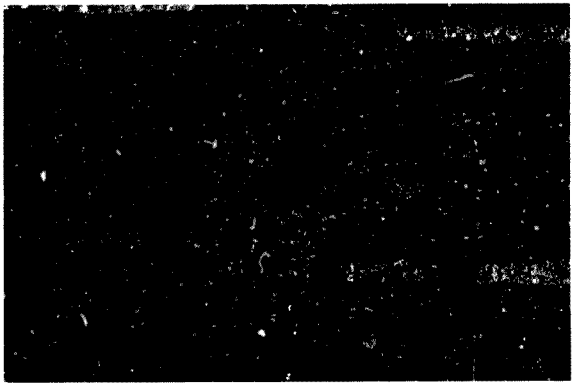
Figure 8

Indirect immunoflouresence staining of HCMV IE antigen in ML-3 cells following PMA-induced differentiation*

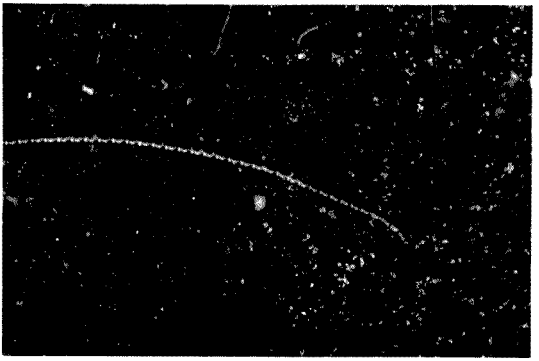
(a) HCMV IE antigen flouresent staining of uninfected ML-3 cells following PMA-induced differentiation. Cells were stained 3 days after mock-infection (500X)

(b) HCMV IE antigen expression in ML-3 cells following PMA-induced differentiation. Cells were stained 3 days after infection (500X)

* Cells were counterstained with methyl red to reduce non-specific flourescence



a



b

FIGURE 9

Northern transfer analysis demonstrating that HCMV infection modulates expression of IL-1β, TNFα and CSF-1 in ML-3 cells

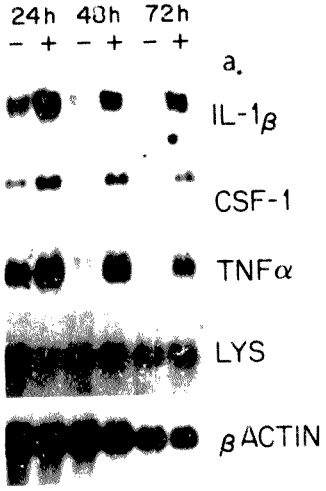
(a) induced to differentiate prior to HCMV challenge

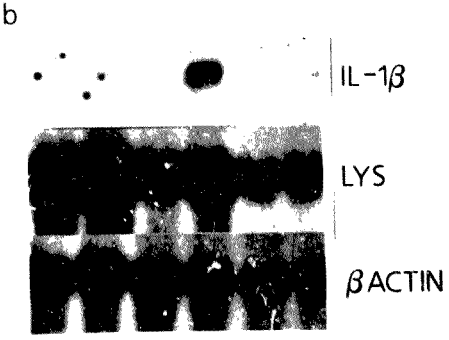
(b) undifferentiated prior to challenge with HCMV

Autorads were exposed overnight for IL-1β, TNFα CSF-1, lysozyme (LYS) and β-actin in (a). Exposure for LYS and β-actin was overnight for (b) and 2 weeks for IL-1β in (b).

(+) HCMV infected

(-) Uninfected controls


24h 40h 72h
− + − + − +
a.
IL-1β
CSF-1
TNFα
LYS
β ACTIN


b
IL-1β
LYS
β ACTIN

cells that were undifferentiated prior to HCMV challenge, only the IL-1β gene could be detected at very low levels in infected cells after a two week exposure of Northern blots (Figure 9B). CSF-1 and TNF-α were not detectable after this exposure period.

Results for Northern blot analysis obtained with the cDNA clones are shown in Figure 10. The only one of these inflammatory response genes to behave in the same way as IL-1β, TNFα and CSF-1 was MAD-9. Two days after infection enhanced levels of MAD-9 mRNA were still present in cells that were differentiated prior to infection by HCMV. Very low levels were detectable in uninduced ML-3 cells that were challenged with HCMV. PMA treatment of ML-3 cells did not result in induction of MAD-6 and MAD-2 mRNA and the expression of these genes was not enhanced and sustained after HCMV infection (Figure 10).

3.7.0. HCMV Dependent Enhancement Requires Induction of the Macrophage Phenotype

The results depicted in Figure 11 indicate that induction of the macrophage pathway of differentiation was required for enhancement of IL-1β steady-state mRNA levels. The low but significant level of IL-1β mRNA at 24h post infection, was markedly less than that observed in PMA-differentiated infected ML-3 cells at

FIGURE 10

Northern transfer analysis demonstrating that not all macrophage-associated inflammatory gene expression is up-regulated following HCMV challenge

Lanes (1) Human monocytes isolated by percoll gradient separation

(2) Monocytes adhered to plastic for 30 minutes

(3) ML-3 cells prior to PMA-induced differentiation

(4) ML-3 cells cultured with PMA for 8 hours

(5) Undifferentiated ML-3 cells cultured for 2 days after mock-infection

(6) Undifferentiated ML-3 cells cultured for 2 days after HCMV challenge

(7) PMA-differentiated ML-3 cells cultured for 2 days after mock-infection

(8) PMA-differentiated ML-3 cells cultured for 2 days after HCMV challenge

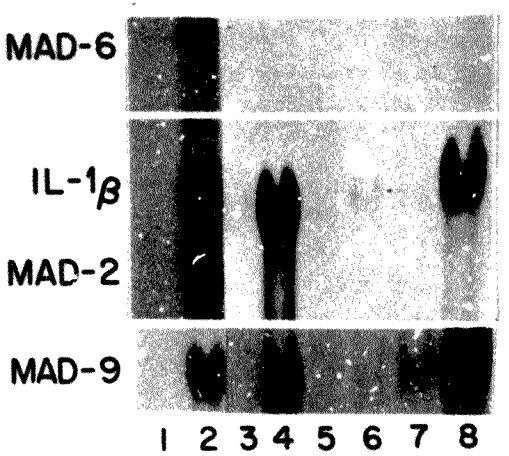


FIGURE 10

the same time point (Figure 11). Furthermore, this enhanced mRNA expression was not sustained in infected cells after 24h. By 72h post infection, the level of mRNA expression in infected cells had decreased and was comparable to that seen in uninfected cells. DMSO treatment resulted in depressed levels of lysozyme (LYS) mRNA at 72hr, but these were not further increased or decreased by virus exposure.

3.8.0. HCMV Infection of Monocytes Results in Enhanced and Longterm Expression of IL-1β mRNA

Cytoplasmic "dot blot" analysis of IL-1β mRNA levels in HCMV-infected and uninfected peripheral blood monocytes at 24h, 48h and 72h post infection is shown in Figure 12B and the densitometric analysis is shown in Figure 12A. Monocytes cultured in the absence of LPS and mock infected with culture supernatant from uninfected fibroblasts gave only a weak transient signal, whereas LPS-stimulated mock infected monocytes gave a strong transient response that was still detectable at 48h. In contrast, HCMV infection of LPS-stimulated monocytes resulted in a more sustained response that continued for 96 hr. The combination of HCMV and LPS gave a transient response not unlike LPS alone, except that the plateau phase was significantly elevated.

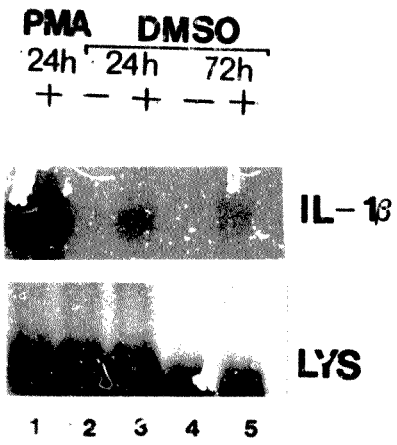


FIGURE 11

Northern transfer analysis showing that ML-3 cells require differentiation with PMA rather than DMSO in order to express competence for HCMV modulation of the IL-1β response

(+) HCMV infected

(-) Uninfected

PMA-differentiated HCMV infected ML-3 cells in lane 1 were used as a reference

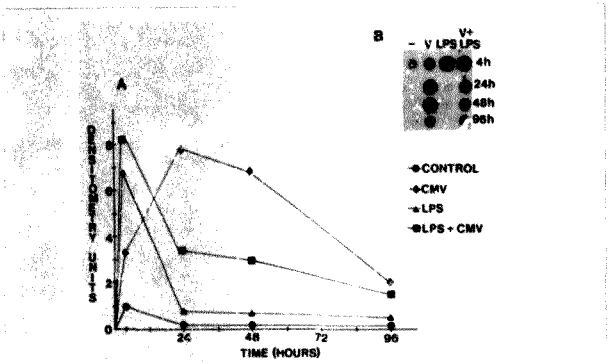


FIGURE 12

Total cytoplasmic dot-blot analysis of IL-1β mRNA expression in monocytes. Densitometric analysis (A) was derived from analysis of the dot blots in (B) and is intended as an approximation of the kinetics of the response rather than an exact quantitation.

In situ hybridization analysis to resolve IL-1 mRNA expression in HCMV-infected and uninfected monocytes at the single cell level is depicted in Figures 13 and 14. Data from 3 different donors is shown in Figure 13, as donor variations in levels of IL-1 mRNA expression were observed at the more sensitive single cell level. For all three donors however, it was observed that HCMV infection always had the same effect on IL-1 mRNA expression. The percentage of positive cells at 1, 2 and 4 days post infection was consistently higher in infected monocytes than in mock infected controls. For all donors, HCMV infection resulted in a small percentage of monocytes (1 - 8%) showing high levels of IL-1 mRNA expression (> 100 grains/cell for donor 2 and >200 grains/cell for donor 1 (Figure 14) and donor 3) which was sustained for up to 4 days post-infection (Figures 13 and 14). There was no difference in IL-1 mRNA expression levels in unstimulated versus LPS-stimulated monocytes (data shown for donor 2 only).

3.9.0. HCMV Dependent Enhancement of IL-1 mRNA Levels Leads to Transiently Enhanced Translation of IL-1

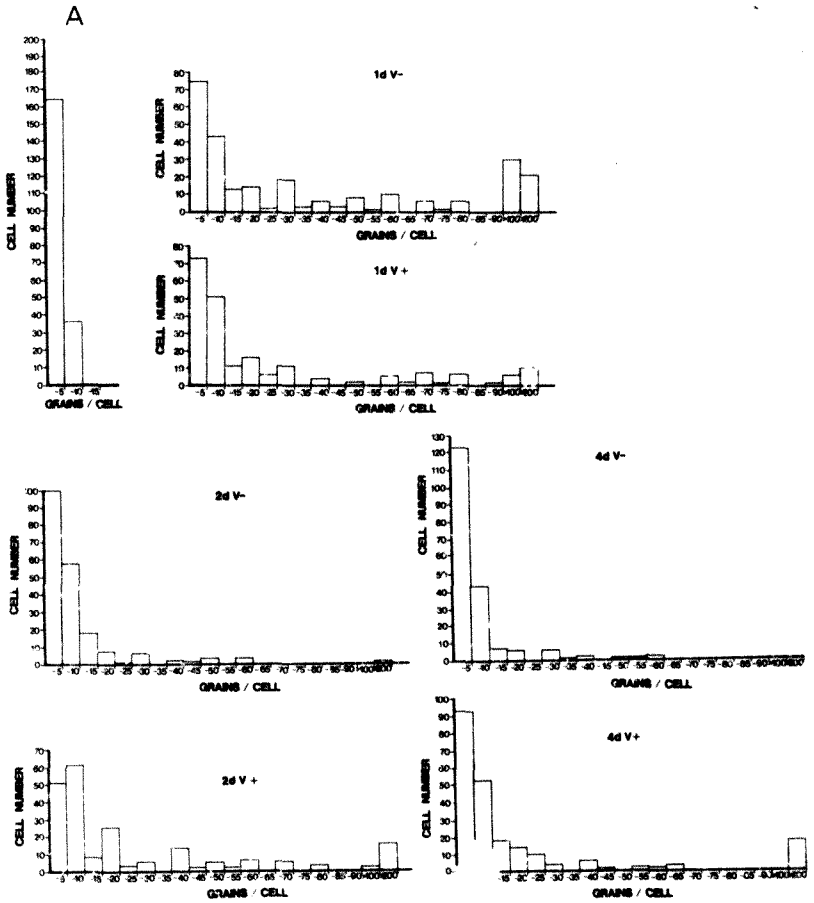
In the first study that was carried out to determine if the enhanced levels of steady state IL-1 mRNA were a reflection of enhanced translation of this important mediator gene, flow cytometric analysis demonstrated that although PMA treatment alone lead to expression of protein, exposure to HCMV following induction of

FIGURE 13

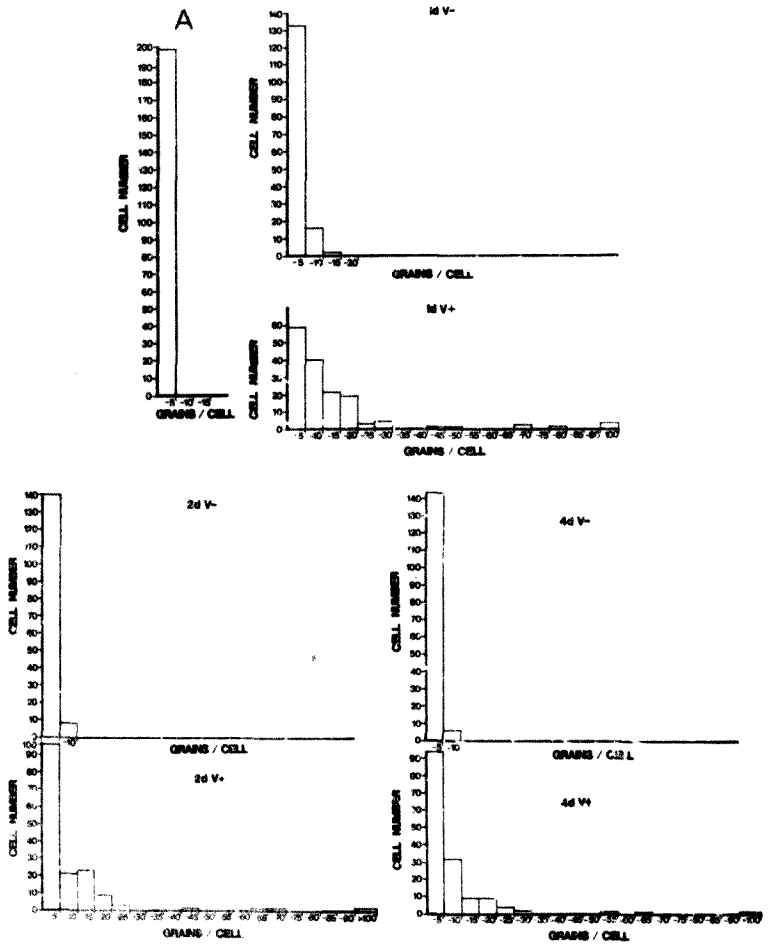
IL-1ß mRNA expression in HCMV infected and uninfected monocytes analysed by *in situ* hybridization. Monocytes from three different donors were analysed for IL-1ß mRNA expression 1,2 and 4 days after infection using a ^{35}S-labelled IL-1ß anti-sense RNA probe. Background staining was revealed by hybridizing cells to ^{35}S-labelled IL-1ß sense RNA sequences (A) for each donor. Numbers of positive cells and grain counts were determined by evaluating approximately 200 cells/slide.

(v-) uninfected

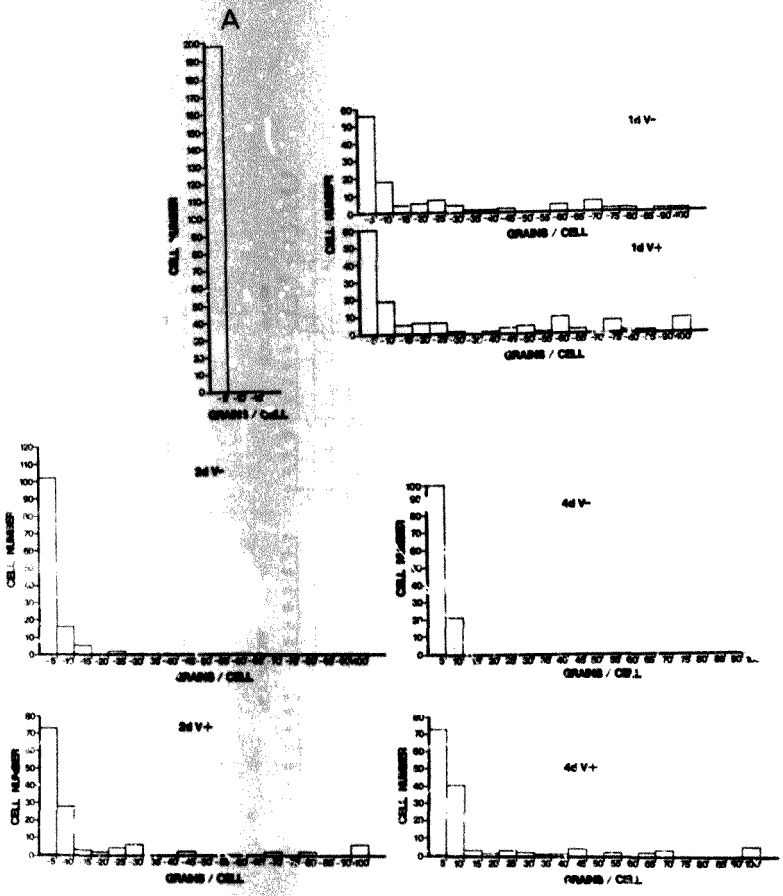
(v+) HCMV infected



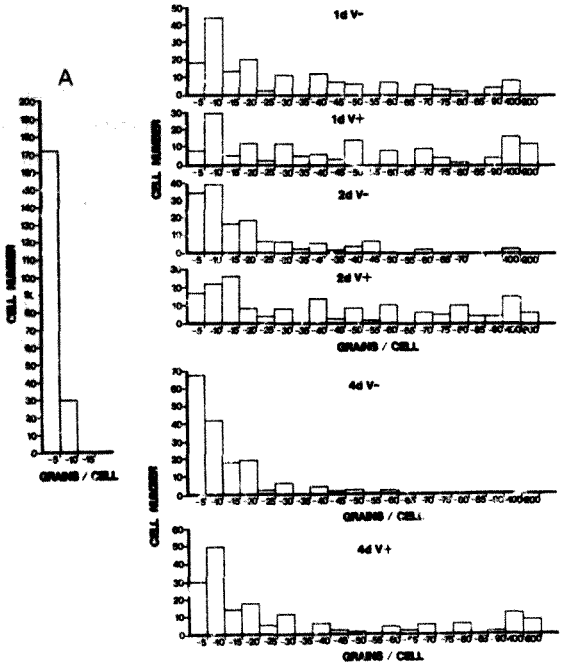
DONOR 1



DONOR 2



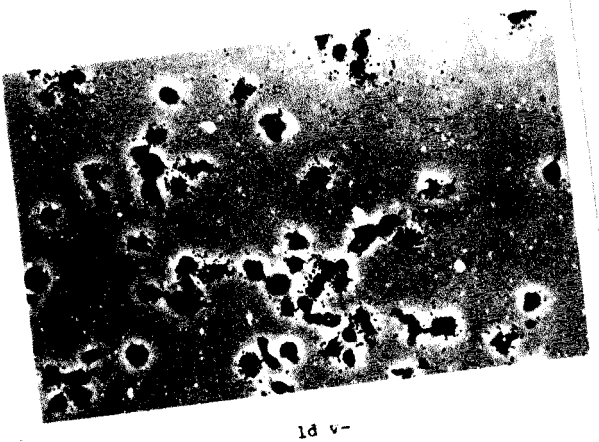
DONOR 2 + LPS



DONOR 3

FIGURE 14

In situ hybridization analysis of IL-1β mRNA expression in monocytes from Donor 1. HCMV infected (v+) or uninfected (v-) monocytes were cultured for up to 4 days post-infection. At 1,2 and 4 days, 14A,B and C respectively, cells were hybridized with a ^{35}S-labelled anti-sense RNA probe specific for IL-1β. Arrows indicate cells chronically expressing IL-1β mRNA. Control hybridizations were performed with a sense IL-1β probe (14D).

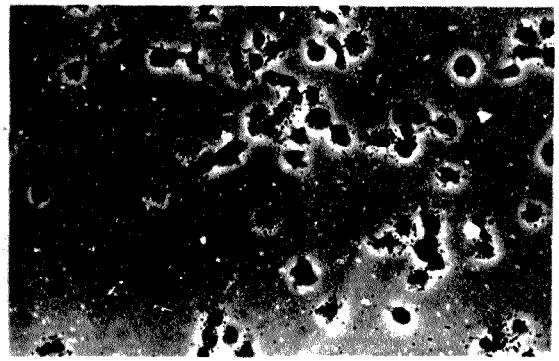


ld v-

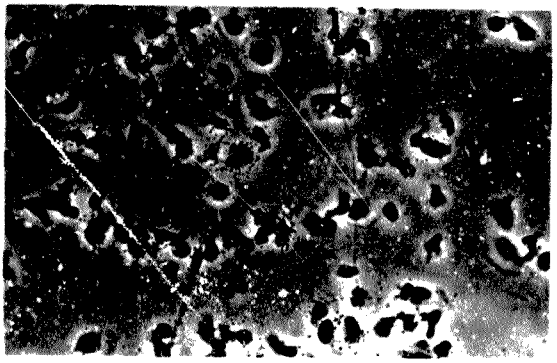


ld v+

FIGURE 14A

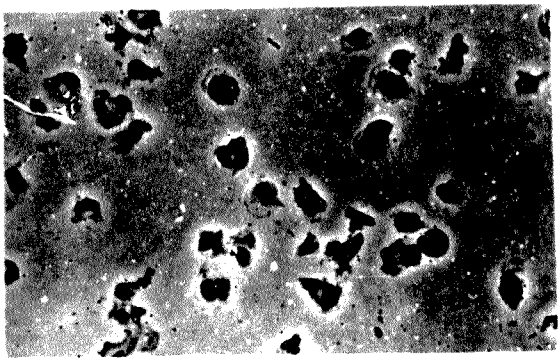


2d v-

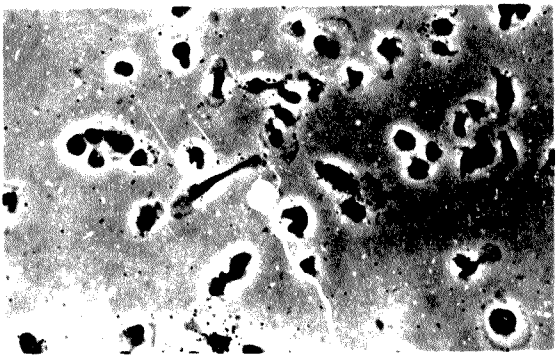


2d v+

FIGURE 14B



4d v-



4d v+

FIGURE 14C

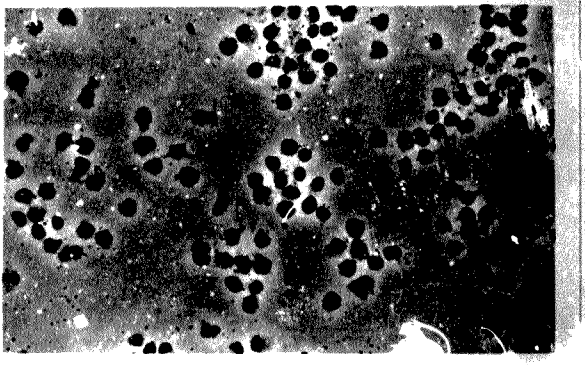


FIGURE 14D

differentiation (Figure 15) lead to a marked increase in the amount of cell associated intracellular IL-1β 24hr after infection. At later time points (72 hr after infection), no intracellular IL-1β was detectable. The isotype control monoclonal antibody that was used (Leu 2A) gave similar levels of flouresence on both cell preparations. HCMV- infected cells had the same levels of flouresence as controls (data not shown).

Staining for the presence of intracellular IL-1β in peripheral blood monocytes is shown in Figure 16. At 1d post infection, the majority of monocytes in both infected and uninfected cultures were positive for intracellular IL-1β. However, in the infected cultures there was a subpopulation of cells showing high IL-1β levels, as was observed for mRNA expression at the single cell level. At 2d post infection there was a decrease in the number of IL-1β positive cells, as well as a decrease in IL-1β levels. By 96h post infection intracellular IL-1β was no longer detectable.

To confirm the presence of intracellular IL-1β protein and to compare responses in ML-3 cells and monocytes, Western blotting analysis was performed, the results of which are shown in Figure 17 . In both cell types the intracellular form of IL-1β was only detected at 24 hr post-infection, indicating that sustained expression did not neccessarily lead to continued translation.

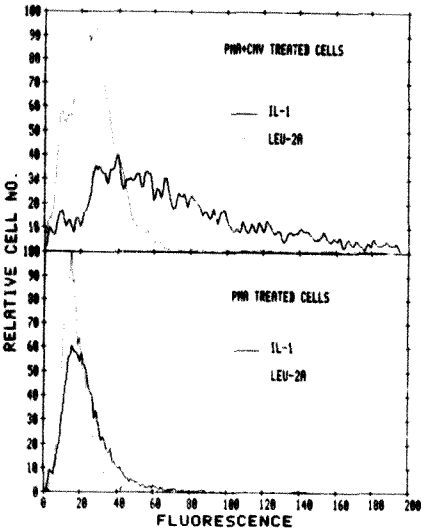


FIGURE 15

Flow cytometric analysis of intracellular IL-1β expression in PMA differentiated ML-3 cells with or without HCMV 24 hr after infection. Both the IL-1β monoclonal antibody and the Leu-2a control are IgG1 subclass.

FIGURE 16

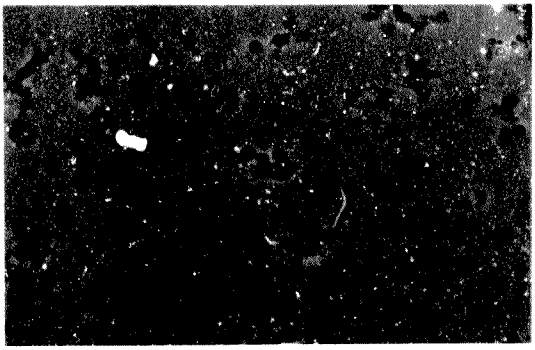
Indirect immunoperoxidase staining of cell associated IL-1 β in uninfected and HCMV-infected monocytes at 1 (16A) and 4 (16B) days after infection. Arrows indicate positive cells.

(v-) uninfected

(v+) HCMV infected

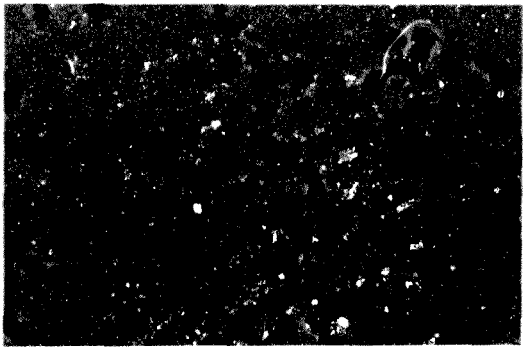


ld v-

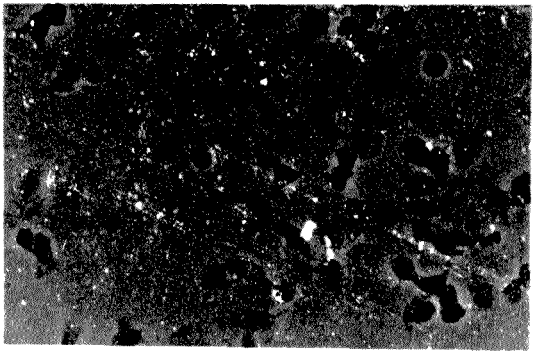


ld v+

FIGURE 16A



4d v-



4d v+

FIGURE 16B

FIGURE 17

Western blotting analysis of cell associated IL-1a translation products in monocytes and ML-3 cells. 1-5 (monocytes) and (6-11) ML-3 cells.

1 - Uninfected monocytes cultured for 24hr

2 - Monocytes infected with heat-inactivated virus 24hr after infection

3 - HCMV infected monocytes 24hr after infection

4 - Monocytes infected with heat-inactivated virus 72hr after infection

6 - HCMV infected monocytes 72hr after infection

7 - PMA treated ML-3 cells cultured for 24hr

8 - PMA treated ML-3 cells infected with heat-inactivated virus 24hr after infection

9 - HCMV infected PMA treated ML-3 cells 24hr after infection

10 -PMA treated ML-3 cells infected with heat-inactivated virus 72hr after infection

11 -HCMV infected PMA treated ML-3 cells 72hr after infection

* The blot was cut into individual strips to be developed, hence the overlapping of lanes in the photograph.

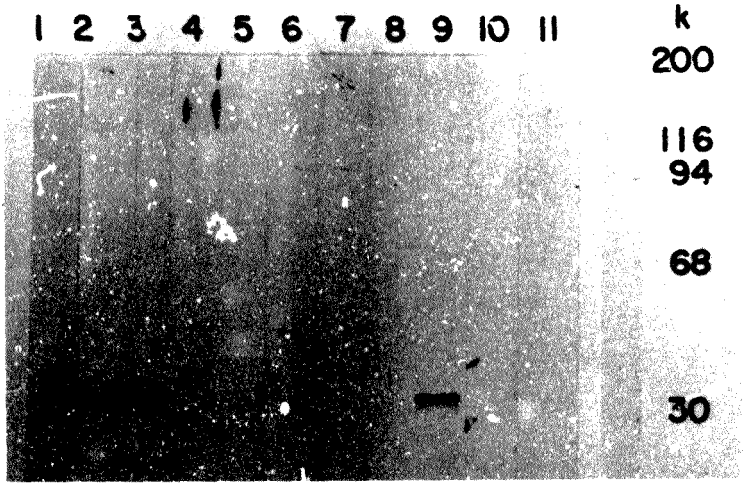


FIGURE 17

Unstimulated, mock infected monocytes cultured for 24h post infection showed, as expected, no pro-IL-1β protein production. HCMV infected monocytes, however, showed a significant level of the intracellular form of pro-IL-1β protein at 24h post infection. No activity was detected in uninfected or infected monocytes at 72h post infection. For ML-3 cells, only PMA-induced cells that had been infected with HCMV gave a detectable signal at 24h post infection.

Results in Figure 18 show that supernatants from HCMV infected unstimulated monocytes, at 24h post infection, contained significantly higher levels of secreted IL-1β than supernatants from uninfected unstimulated monocytes. The marked increase in intracellular IL-1β that was observed by Western blot analysis and monoclonal antibody staining was reflected in increased secretion of IL-1β up to 48 hr post infection. However, for LPS-stimulated monocytes, levels of secreted IL-1β were significantly lower in supernatants of infected monocytes compared to uninfected monocytes.

3.10.0. The Immediate-Early Gene of HCMV Transactivates the IL-1β Promoter in PMA Treated Cells

To examine the possibility that enhanced expression of IL-1β seen in ML-3 cells could result from transactivation of the IL-1β gene by HCMV IE gene

FIGURE 18

Quantitative measurement of IL-1 levels in supernatants from LPS-stimulated and unstimulated CMV-infected or uninfected monocytes. Supernatants from uninfected (-) and CMV-infected (+) monocytes were removed 1d after infection (1- and 1+) and replaced with fresh medium. Supernatants were removed after a further 24h (2- and 2+) and replaced with fresh medium. After a further 48hr supernatants were removed again (4- and 4+).

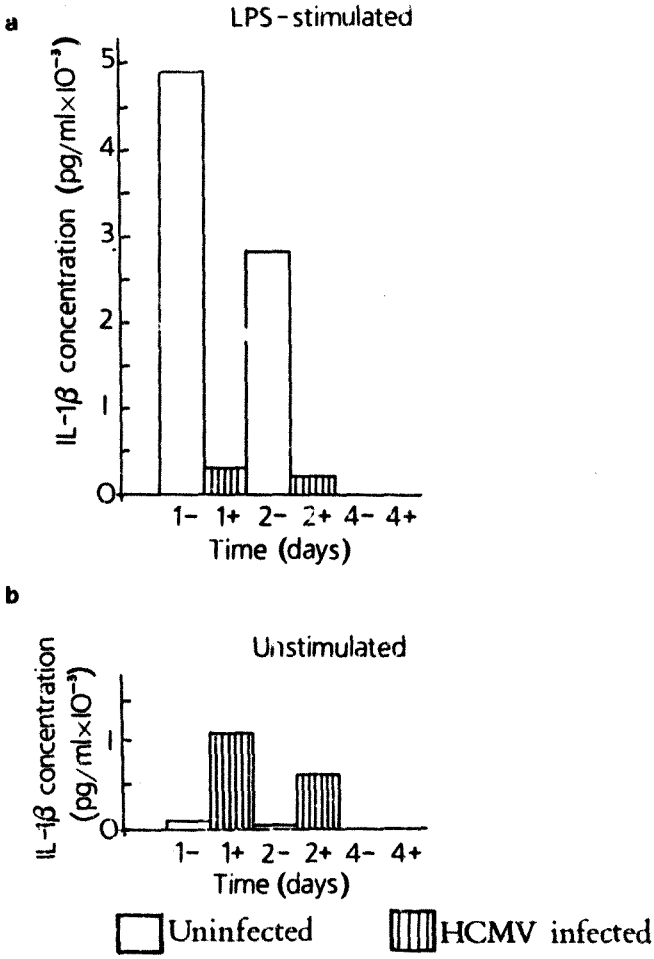

a
LPS-stimulated
IL-1β concentration (pg/ml×10⁻³)
5
4
3
2
1
0
1- 1+ 2- 2+ 4- 4+
Time (days)
b
Unstimulated
IL-1β concentration (pg/ml×10⁻³)
1
0
1- 1+ 2- 2+ 4- 4+
Time (days)
Uninfected
HCMV infected

FIGURE 18

products, chimeric plasmids containing proIL-1 sequences fused to the CAT gene were co-transfected into undifferentiated and PMA-differentiated ML-3 cells with various HCMV IE gene region plasmid constructs. The data in Figure 19 shows the effect of the HCMV IE gene region when tested in cotransfection experiments with the positive control plasmid 3ME-AT. The HCMV IE gene region plasmid contained both regions 1 and 2 of the IE coding sequences. In PMA-differentiated cells, increasing additions of the IE gene region DNA led to a marked expression of CAT activity. Co-transfection with 10ug each of plasmid pHD101SV1 and 3ME-AT DNA gave maximum activity in the dose response as shown in Figure 19. In undifferentiated ML-3 cells there was no trans-activation of the IL-1β gene by the HCMV IE gene region. The apparent requirement for differentiation suggests the need for a host derived competence factor that appears to be derived from PMA stimulation.

To confirm that the trans-activation of the IL-1β gene by HCMV IE gene products was not due to an enhancing effect of the SV40 enhancer located adjacent to the SV40 promoter in plasmid 3ME-AT, HCMV IE region gene constructs were co-transfected into ML-3 cells with plasmid 3M-AT. Results in Figure 20 show the effect of HCMV IE region expression plasmids on IL-1β promoter function in ML-3 cells that were induced to differentiate with PMA prior to transfection. When

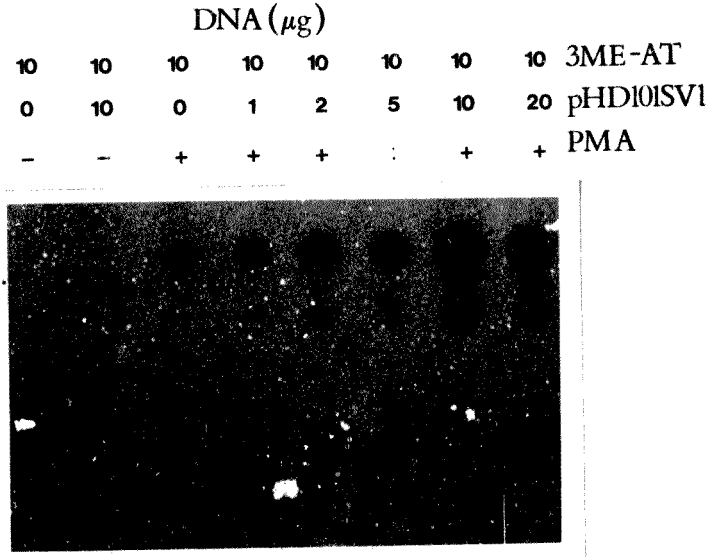


FIGURE 19

Effect of HCMV IE region gene products on the IL-1β gene in undifferentiated and PMA-differentiated ML-3 cells. Cells were cotransfected with the positive control IL-1β-CAT plasmid DNA (3ME-AT) and pHD101SV1 coding for regions 1 and 2 of the HCMV IE region. CAT activity was assayed for 48hr after transfection.

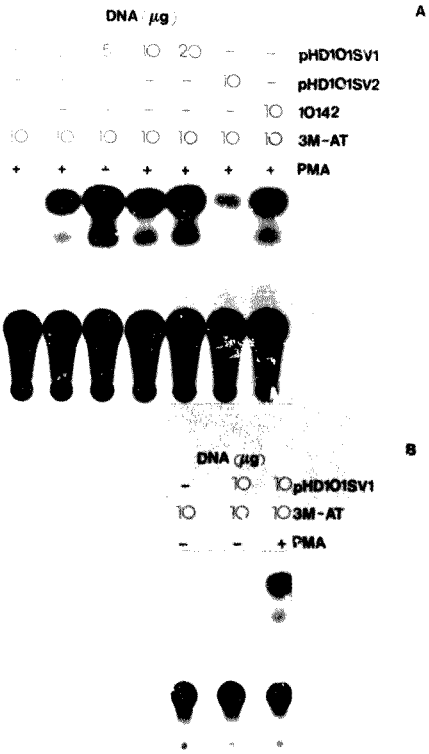


FIGURE 20

Effect of HCMV IE gene products on the IL-1β gene in undifferentiated (B) and PMA-differentiated (A) ML-3 cells. Cells were cotransfected with IL-1β-CAT plasmid DNA (3M-AT) and HCMV IE gene region plasmids, pHD101SV1 containing regions 1 and 2, pHD101SV2 containing region 1 only or 10142 containing region 2 only. CAT activity was assayed for 48hr after transfection.

3M-AT DNA was transfected singly into PMA-differentiated ML-3 cells there was no CAT activity. When plasmid 3M-AT containing 524 bp of pro-IL-1β genomic DNA was cotransfected with the HCMV IE region 1 and 2 expression plasmid, pHD101SV1, there was stimulation of activity of the IL-1β promoter as judged by CAT activity (Figure 20). To ascertain whether regions 1 and 2 of the HCMV IE region were both required for transactivation of 3M-AT, HCMV plasmids encoding either region 1 or region 2 were cotransfected with 3M-AT. The region 1 products alone (pHD101SV2) do not support IL-1β promoter activity (Figure 20A). In contrast, region 2 products resulted in activity equivalent to that obtained with regions 1 and 2 together. Figure 20B shows that cotransfection experiments in ML-3 cells that were not induced to differentiate prior to transfecttion, resulted in no stimulation of IL-1β promoter activity.

4.0.0. DISCUSSION

In this study the functional effect of HCMV infection on production of IL-1 activity was investigated in both unstimulated and LPS-stimulated monocytes in an attempt to examine more closely the mechanisms of immunosuppression that may accompany HCMV infection *in vivo*. Previous reports have described effects of HCMV infection on unstimulated monocytes only.

At a functional level, infection of monocytes with laboratory-adapted strains and clinical isolates of HCMV were also compared, as previous reports had described biological differences between such strains with regard to degree of infection of peripheral blood mononuclear cells (Rice *et al.*, 1984; Einhorn and Ost, 1984). Results obtained in this study confirmed that strains AD169 and Towne failed to initiate expression of detectable immediate early antigen in the majority of infected monocytes even when these cells were stimulated with LPS (Table 2). However, a small fraction of monocytes infected with the clinical isolate of HCMV expressed IE antigen (Figure 4) with the percentage of apparently infected cells rising with increasing MOI (Table 2).

A possible explanation for the detection of viral antigens or genes in such a limited proportion of cells is that immunological and molecular biological probes studied and used to date might not be able to detect latent HCMV infection at a sensitive enough level. Monoclonal antibodies for detection of HCMV IE antigens enable detection of region 1 and 2 gene products collectively, but not independent of one another. Recent studies by several different investigators have proposed that the HCMV IE region 2 gene products are involved in the regulation of viral or host cell promoters, either independently or in combination with HCMV IE region 1 gene products (Hermiston et al., 1987; Pizzorno et al., 1988). Gene amplification experiments using probes for specific IE region gene products will allow for an investigation of this phenomenon.

In addition to their effector role, monocytes play an essential role in the initiation of immune responses through antigen presentation and production of IL-1. Impairment of these accessory functions could be a major factor in the immunosuppression that is associated with HCMV infection. Changes in IL-1 regulation could contribute to the pathology of the infection. The investigation in this study into the role of monocyte infection in T lymphocyte responses to mitogens showed that virus-challenged monocytes were suppressive for T cell blastogenesis. LPS-stimulated

monocytes challenged with either strain of HCMV were more suppressive for autologous T lymphocyte responses than were virus-challenged unstimulated monocytes when tested under identical assay conditions (Table 3). Stimulated monocytes produce significantly higher levels of functional IL-1 activity than do unstimulated monocytes, but virus infection abrogated production of functional IL-1 bioactivity, as assessed by the mouse thymocyte bioassay with monocyte-conditioned medium (Figure 5). Monocytes infected with the clinical isolate were more suppressive than AD169 infected monocytes, with a MOI of 0.005 and 5 PFU/cell, respectively, resulting in a similar abrogation of released IL-1 activity (Figure 5).

The next stage in this study sought to determine whether the decrease in production of IL-1 bioactivity by HCMV-infected monocytes was contributing to the suppression of T cell proliferative responses. Exogenous IL-1 was therefore added to HCMV infected monocytes to determine whether this impaired accessory cell function could be reconstituted. Addition of supernatant containing IL-1 from LPS-stimulated monocytes or of ultrapure IL-1, fully restored the proliferative response of T cells cultured in the presence of stimulated HCMV-infected monocytes to that observed in the presence of control monocytes (Figure 6). In contrast, however, addition of IL-1 to T cells

cultured with unstimulated HCMV-infected monocytes did not restore proliferation (Figure 7), a result suggesting that unstimulated, HCMV-infected monocytes release functional IL-1 inhibitory activity. To assess whether unstimulated, infected monocytes were in fact releasing IL-1 activity that was being masked by the concomitant production of functional IL-1 inhibitory activity in the mouse thymocyte bioassay, quantitative measurements of IL-1 levels were made using ELISA assays.

Supernatants from infected stimulated monocytes contained significantly less secreted IL-1 activity than uninfected stimulated monocytes (Figure 18), which suggested that the decreased proliferation of mouse thymocytes was not due to inhibitory activity masking release of functional IL-1 . Also, the observation that normal T cell proliferative responses could be reconstituted by addition of exogenous IL-1 to stimulated, infected monocytes further substantiates the observation that suppression of lymphocyte responses by stimulated infected monocytes was not due to release of IL-1 inhibitory activity. However, in the unstimulated system, supernatants from infected monocytes contained increased amounts of IL-1 compared with those of controls (Figure 18) at 24h and 48h post-infection. The functional activity of this IL-1 could not be detected in the mouse thymocyte

proliferation assays, suggesting that IL-1 inhibitory activity was being concomitantly released. The release of IL-1 inhibitory factors by unstimulated, infected monocytes also explains the inability to reconstitute the PHA-induced proliferative response of T lymphocytes by addition of exogenous IL-1.

In an earlier study of HCMV-mediated immunosuppression, Carney and Hirsch (1981) showed that monocytes from HCMV-infected patients as well as monocytes infected *in vitro* inhibited the con A-induced proliferation of lymphocytes in subsequent culture. Rodgers *et al.* (1985) proposed that HCMV-infected unstimulated monocytes were defective in IL-1 production. They associated this with the release of a monocyte-derived inhibitor of IL-1. However, a subsequent report by Scott *et al.* (1989) has suggested that this inhibitory activity was due to mycoplasma contamination of the cell lines used.

Although the possibility exists that the stocks of strain AD169 used in the functional studies reported in Dudding and Garnett (1987), were contaminated with mycoplasma, the clinical isolate was passaged directly from a patient into HELs that were rigorously checked for mycoplasma. Furthermore, at a functional level, the clinical isolate had a greater immunosuppressive effect than strain AD169. The above observations (Dudding and Garnett, 1987) have since been corroborated by others.

Kapasi and Rice (1988), using stocks of Towne strain that were passaged in mycoplasma-free cell lines found that prior infection of either monocytes or lymphocytes with HCMV in vitro followed by reconstitution with uninfected lymphocytes or monocytes respectively, abrogated the proliferative response of lymphocytes to PHA in subsequent culture. Infection of either cell type reduced IL-1 and IL-2 production. Normal proliferative responses of lymphocytes cultured in the presence of unstimulated infected monocytes could similarly not be reconstituted by exogenously supplied IL-1 or IL-2. Their data suggested that decreased proliferative responses of PBMC were not solely due to decreased IL-1 production or production of IL-1 inhibitors, as PBMC should have responded to IL-2, which they did not. Furthermore, no evidence of an IL-2 inhibitor was found. A generalised metabolic depression of PBMC activity was proposed.

Further support for results obtained in this study is found in other virus systems. Roberts et al. (1986) showed that IL-1 inhibitors were produced concomitantly with substantial IL-1 activity by influenza and respiratory syncitial virus (RSV) infected monocytes. Much attention has recently been focused on IL-1 production by HIV-infected monocytes, in view of the fact that monocytes can be infected by HIV and that impairment of monocyte accessory cell functions could

be a major factor in the immune deficiency and pathogenesis of AIDS. Findings for IL-1 production by HIV-infected monocytes have been similar to those reported in this study. Berman et al. (1987) examined monocyte production of IL-1 activity from patients with AIDS and quantitated production of endogenous macrophage-derived IL-1 inhibitors. Culture supernatants from unstimulated peripheral blood monocytes were fractionated to assess spontaneous IL-1 release. PBMCs from patients with AIDS produced increased amounts of IL-1 activity compared with those of controls, together with marked increases in factors which inhibited this functional IL-1 activity, as assessed in the mouse thymocyte proliferation assay. Enk et al. (1986) found depression of T lymphocyte responsiveness to IL-1, but enhanced production of IL-1. Roux-Lombard et al. (1989) measured production of IL-1 and TNF in supernatants of monocytes obtained from HIV 1-infected patients that had been cultured in vitro for 18h. IL-1 levels were elevated in symptomatic patients compared with controls and intermediate in asymptomatic HIV 1-infected individuals.

The inhibitory factors produced by unstimulated HCMV-infected monocytes in this study and by HIV-infected monocytes are perhaps common endogenous mediators of virus-induced immunosuppression which are present in healthy individuals but increased in

infection. Larrick (1989) proposes that these macrophage-derived inhibitors associated with viral infections are likely to contribute to the pathogenesis of the disease either by direct inhibition of IL-1 secretion or by binding to receptor sites on IL-1 responsive cells, thereby interfering with accessory functions of IL-1.

At the molecular level, experiments done to assess the effect of HCMV infection on steady state IL-1β mRNA expression showed that HCMV infection of monocytes resulted in enhanced and long-term expression of steady state IL-1β mRNA. Dot blot analysis of total cytoplasmic RNA (Figure 12) showed that monocytes cultured in the absence of LPS gave only a weak transient signal, whereras LPS addition stimulated a strong transient response that was weakly detectable at 24 to 48hr. Similar kinetics have been described by Fenton et al. (1987). In contrast, HCMV exposure resulted in a more sustained response that continued for 96hr. The combination of HCMV and LPS gave a transient response, not unlike LPS alone, except that the plateau phase was significantly elevated.

In order to assess if these enhanced levels of steady-state IL-1β mRNA were due to increased expression by the entire population of monocytes or a certain fraction only, in situ hybridization was used

Author Dudding Lindsay Rose
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