CELL MEDIATED IMMUNITY TO HUMAN CYTOMEGALOVIRUS INFECTION

CYTOTOXIC T CELL AND NATURAL KILLER CELL

MEDIATED LYSIS OF

HUMAN CYTOMEGALOVIRUS INFECTED CELLS

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ABSTRACT

Cell mediated immune responses are widely held to be important in limiting the dissemination of persistent virus infection, thereby helping to maintain the virus/host equilibrium. Hitherto, little was known of the nature of such responses against human cytomegalovirus (HCMV), which produces severe morbidity and mortality in immunosuppressed allograft recipients. The objective of these studies was to determine the nature and specificity of T and natural killer responses in peripheral blood lymphocytes of normal persistently infected individuals. These studies have shown:

1) lymphocytes from such individuals proliferated to both Early and Late HCMV antigens. When maintained in IL2, these cells grew into T4+ non-cytotoxic lines which even when cloned retained their specificity for and released IL2 in response to HCMV antigens.

2) MHC class I restricted, HCMV specific, T8+ cytotoxic T cell lines were established by co-culture of lymphocytes with HCMV infected autologous fibroblasts. At least a proportion of such cytotoxic T cells were specific for HCMV Early antigens.

3) natural killer cells mediating lysis of HCMV infected cells were IFN inducible large granular lymphocytes, expressing surface T11, T8, Leu 7 and Leu 11. Increased susceptibility of HCMV infected cells to NK lysis was evident when HCMV Early antigens were expressed, although the target structure(s) recognised were probably not virally encoded. One possible
host cell target structure - the transferrin receptor - was studied in detail. Although increased expression of this receptor in HCMV and other virus infected and uninfected fibroblasts was correlated with increased susceptibility to NK lysis, this was not inhibited by isolated receptor or excess transferrin.

Both T and natural killer cells from normal persistently infected individuals recognised HCMV infected cells, when non-structural viral antigens were expressed, prior to viral DNA replication, which may enable cytotoxic effector cells to directly restrict dissemination of HCMV infection in vivo.
To Gwenllian, Catherine and Rachel.
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ABBREVIATIONS

ACG  - 9-(2-hydroxyethoxymethyl)guanine
AD  -  actinomycin D
ADCC  - antibody dependent cell mediated cytotoxicity
AIDS  - acquired immune deficiency syndrome
APC  - antigen presenting cell
BME  - basal medium with Earle's salts
BSA  - bovine serum albumin
C  - complement
CFA  - complete Freund's adjuvant
cft  - complement fixation test
CHX  - cycloheximide
CMV  - cytomegalovirus
cpe  - cytopathic effect
DHPG  - 9-(1,3-dihydroxy-2-propoxymethyl)guanine
DMSO  - dimethylsulphoxide
DTH  - delayed type hypersensitivity
E  - sheep erythrocyte receptor
EA  - early antigen (CMV)
EBV  - Epstein-Barr virus
ELISA  - enzyme linked immuno-assay
FCS  - foetal calf serum
FITC  - fluorescein isothiocyanate
GPCMV  - guinea pig cytomegalovirus
HA  - haemagglutinin (influenza)
HCMV  - human cytomegalovirus
HCMV-NK  - NK cells lysing HCMV infected cells
HSV  - herpes simplex virus (type 1)
IE  - immediate early
IEA  - immediate early antigen (CMV)
IFN  - interferon
IL2  - interleukin 2 (T cell growth factor)
IS  - immunosuppressed
K562-NK  - NK cells lysing K562 cells
LA  - late antigen (CMV)
LCMV  - lymphocytic-choriomenigitis virus
LDL  - low density lipoprotein
LGL  - large granular lymphocyte
LYDMA  - lymphocyte determined membrane antigen (EBV)
M  - matrix protein (influenza)
MC MV  - murine cytomegalovirus
MEM  - Eagles' minimal essential medium
MLR  - mixed lymphocyte reaction
MOI  - multiplicity of infection
N  - neuraminidase (influenza)
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<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
<td>NP</td>
<td>nucleoprotein (influenza)</td>
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<td>NSE</td>
<td>non-specific esterase</td>
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<td>PAA</td>
<td>phosphonoacetate</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PBL</td>
<td>peripheral blood lymphocytes</td>
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<td>phytohaemagglutinin</td>
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<td>PPF</td>
<td>phosphonoformate</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SFV</td>
<td>Semliki Forest virus</td>
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<tr>
<td>SRBC</td>
<td>sheep red blood cells</td>
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<tr>
<td>Tc</td>
<td>T cytotoxic cell</td>
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<tr>
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<td>T cell mediating delayed type hypersensitivity</td>
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<tr>
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<tr>
<td>TfR</td>
<td>transferrin receptor</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<tr>
<td>Ts</td>
<td>T suppressor cell</td>
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<td>VZV</td>
<td>varicella zoster virus</td>
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SECTION 1

INTRODUCTION

The studies described have investigated the nature of human T and natural killer (NK) cell responses to human cytomegalovirus (HCMV) in normal persistently infected and uninfected subjects. Before proceeding to a description of the experiments performed, the first section reviews aspects of the biology of HCMV infection and the immune response against the virus, with particular reference to T and NK cells. This work commenced in 1980 and during the time it has been in progress, considerable advances have been made in our understanding of the molecular biology of HCMV and the immune response to virus infection. These studies, although published since 1980 are considered together with previously published reports to enable the work described to be placed in a current context.

1.1 HUMAN CYTOMEGALOVIRUS

Cytomegaloviruses are ubiquitous species specific herpesviruses that have been identified in a number of mammals including man. The characteristic cytopathological appearance of intranuclear inclusions associated with cytomegalovirus (CMV) were first identified at post mortem examination in the interstitium of the kidney, liver and lung of an eight month stillborn foetus with congenital syphilis (Jesionek and Kiolemenoglow, 1904). Ribbert (1904) described a similar case he observed.
in 1880, a neonate with congenital syphilis and similar inclusions in the kidney, but considered that these were not syphilitic, as similar changes were found in the parotid gland of another child (aged 1y). Subsequently further post mortem case reports appeared as reviewed by Vonglahn and Pappenheimer (1925). The intranuclear inclusions were initially attributed to a protozoan parasite and even though unidentified except in the 'intranuclear form' it was named 'Entameba mortinatalium' by Smith and Weidman (1914).

Goodpasture and Talbot (1921) first suggested a viral aetiology for these inclusions based on similarities between them and observations of similar inclusions in skin lesions from patients with varicella (Tyzzer, 1906). Vonglahn and Pappenheimer (1925) also drew attention to the similarity of these inclusions and those produced during experimental herpes encephalitis. Similar changes were observed in the parotid glands from 26 of 48 guinea pigs (Jackson, 1920). Cole and Kuttner (1926) showed that extracts of guinea pig submaxillary glands, containing such intranuclear inclusions, inoculated intracerebrally into 1 month old guinea pigs resulted in meningitis with intranuclear inclusions in 89% of animals. They also showed that the agent could be serially transferred, did not infect other species and was filtrable through a Berkefeld N filter that retained B. coli.

Farber and Wolbach (1932) found that intranuclear inclusions in salivary glands were widely distributed - 22 of 183 autopsies performed on infants under 17 months had submaxillary inclusions. They were unable to
identify any consistent clinical syndrome associated with these inclusions but, although they did not comment on it themselves, they described two neonates with disseminated inclusions, a haemorrhagic diathesis and 'erythroblastosis' consistent with cytomegalic inclusion disease.

Similar intranuclear inclusions were identified in a number of different species including monkeys and rodents but inter-species transfer in vivo and in vitro was unsuccessful (Andrewes, 1930). Following the observations of Goodpasture and Talbot (1921) and Cole and Kuttner (1926) the inclusions were presumed to be of viral origin in man even though the virus could not be isolated. This did not prevent the identification of 'cytomegalic inclusion disease' in infants with erythroblastosis and a haemorrhagic diathesis associated with disseminated intranuclear inclusions (Wyatt et al, 1950; Smith and Velios, 1950). In vitro isolation of CMV was achieved initially with murine cytomegalovirus (MCMV) (Smith, 1954), and then HCMV, independently by three groups. Firstly, from a 7 month infant with adrenal carcinoma and a 1 month child with cytomegalic inclusion disease (Smith, 1956), secondly from adenoid tissue of asymptomatic children aged 11, 7 (Ad 169 strain) and 6y (Rowe et al, 1956) and thirdly from 3 children with cytomegalic inclusion disease (Weller et al, 1956) (Davis, Kerr and Esp strains). (Weller, 1970). In each case the virus could only be isolated and propagated in human diploid fibroblasts. The ability to study the virus in vitro has led to the further characterization of its structure and function.
1.1.1 Virion structure

The similarities between the intranuclear inclusions suggested that HCMV was a herpesvirus. When HCMV virions were examined by electron microscopy they were morphologically indistinguishable from other herpesviruses. The virion consists of a 750Å diameter core containing viral DNA enclosed within a 960Å diameter icosahedral capsid made up of 162 capsomeres (Wright, Goodheart and Lielausis, 1964; Smith and De Harven, 1973), surrounded by a lipid envelope containing viral glycoproteins.

The exact number of HCMV structural proteins is unknown although about 30 different proteins can be identified. Most are found in low amounts and their function remains unknown (Sarov and Abady, 1975; Stinski, 1977; Stinski, 1978). The capsid itself has a major (150kDa) and minor (34kDa) protein. Between the capsid and envelope are at least 3 matrix proteins 200kDa, 72kDa and 68kDa that probably bind the capsid to the envelope. The single most abundant polypeptide in the virion is the 64–68kDa matrix protein which has been purified by reverse-phase high pressure liquid chromatography from HCMV infected cells (Clark et al, 1984), the gene encoding it identified and expressed in Cos cells (Davis and Huang, 1985). The envelope contains at least 6 viral glycoproteins (Stinski, 1976; 1977).

Isolated HCMV DNA from purified virions is a linear duplex and the molecular weight as computed by electron microscopic contour measurements...
and velocity sedimentation was approximately $155 \times 10^6$ Da (equivalent to 240 kilobase-pairs) with a coding capacity for up to 130 proteins (Stinski, 1983). This was about 50% larger than HSV DNA and 11% of the HCMV DNA is repeated, with 89% unique. The DNA is arranged in a manner similar to that of HSV DNA with a long and short segment (comprising 82% and 18% of the DNA respectively in Davis strain (Demarchi, 1981)). These segments are bounded by terminal and internal repeat units of DNA inverted with respect to each other (Fig. 1). This combination allows four isomeric forms of HCMV DNA to be present (Weststrate, Geelen and van der Noordaa, 1980; Demarchi, 1981; Stinski, 1983). In spite of its forbidding size work is in progress to obtain the complete DNA sequence of AD169 HCMV.

From the outset several strains of HCMV were isolated and a major question has been the degree of similarity between them. This has been studied by morphology of cytopathic effect (cpe), antigenically, by nucleic acid hybridisation and restriction enzyme mapping. Albrecht et al (1980) investigated the development of cpe following HCMV infection with standard laboratory 'strains’ (AD169, C-87, Davis, Esp and Kerr). They observed that there were no major differences in the cpe but the kinetics of development of cpe were different with different viruses, independent of multiplicity of infection (MOI) and source of fibroblasts. Antigenically, using virus neutralisation with polyclonal antisera against HCMV, Zablotney, Wentworth and Alexander (1978) and Waner and Weller (1978) observed both antigenic relatedness e.g. between AD169 and Kerr.
Fig 1. HCMV DNA indicating regions of IE transcription (AD169) and homologies with host cell DNA. Earlier studies, particularly with AD169 depicted the UL segment in the opposite orientation.
strain and heterogeneity particularly with Towne strain. However, these differences were slight and cross reactivity was observed between all isolates tested. Lymphocyte proliferative responses to AD169, Towne and Davis strains were comparable in normal seropositive individuals (Starr et al, 1980).

Using DNA reassociation, Huang and Pagano (1974) showed that there was no significant sequence homology between HCMV, HSV type I and II and Epstein-Barr virus (EBV), or between HCMV and simian or MCMV. Similarly, there was over 80%-90% homology between Towne and AD169 strain (Pritchett, 1980). When HCMV DNA from different isolates is compared by restriction endonuclease mapping, most restriction fragments are similar although slight differences may be detected and result in innumerable 'strains', similar to the observations made in HSV strains (Buchman et al, 1978). These differences may be of value in epidemiological studies (Huang et al, 1980; Spector, 1983), but their biological significance is unknown. Differences between recent clinical HCMV isolates and laboratory strains have been observed but their relationship to changes in the restriction maps is unknown (Rice, Schrier and Oldstone, 1984). However, it has also been observed that an individual patient (especially immunosuppressed patients) may shed more than one 'strain' as defined by restriction maps at different times (Huang et al, 1980) or simultaneously (Drew et al, 1985; Spector, Hirata and Neuman, 1985). The evidence to date would therefore suggest that studies using a standard laboratory strain, such as AD169, are relevant to the examination of
immune responses generated in vivo against 'wild type' virus.

1.1.2 Virus replication

Unlike HSV, HCMV is strongly species specific. Both in vivo and in vitro it has a restricted host range and in vitro only the diploid human fibroblast is fully permissive for virus replication. The progression of cpe was studied by McAllister et al (1963) and Albrecht et al (1980). Following infection of monolayers at high MOI cell rounding was observed within 5-24h post infection, but infected cells then regained their fibroblast shape. 8h following infection there was enlargement of the nuclei together with development of characteristic intracytoplasmic inclusions and intranuclear inclusions which increased in size up to 96h. Virus shedding was not detected until 72h following infection. This cycle of virus replication is much slower than that observed for HSV where shedding of virus occurs after 8h (Smith and De Harven, 1973; Stinski, 1983).

Smith and De Harven (1974) followed the early stages of virus entry into fibroblasts by electron microscopy. They observed that HCMV, like HSV, can enter the cell by direct fusion with the cell membrane or by endocytosis and this process occurs within 3 minutes of binding to the cell surface. Within 5 minutes the virus capsids migrate towards the nucleus, but coated HCMV capsids can remain within the cytoplasm for up to 36h following infection.

Like HSV, virus replication is preceded by regulated sequential
expression of nonstructural virus encoded proteins. These were identified serologically using human sera and termed 'immediate early antigens' (IEA) (Michelson-Fiske, Horodniceanu and Guillon, 1977) and 'early antigens' (EA) (The, Klein and Langenhuysen, 1974). IEA were expressed in the presence of idodeoxyuridine, cytosine arabinoside and phosphonacetate acid (PAA) (viral DNA synthesis inhibitors) and also in cycloheximide (CHX) pretreated cells following removal of the block i.e. they did not require de novo viral protein synthesis for IE mRNA transcription. EA were expressed when cells were infected in the presence of cytosine arabinoside but required de novo viral protein synthesis. Therefore in the permissively infected cell, HCMV IEA antigens are expressed first, without the requirement of de novo viral protein synthesis, followed by HCMV EA, which require synthesis of the IEA but are themselves a prerequisite for viral DNA replication. Virus structural or late antigens (LA) are expressed after virus DNA replication and their expression is inhibited by viral DNA polymerase inhibitors such as PAA and phosphonofornate (PPF) (Fig 2).

1.1.3 Immediate early antigens

HCMV IE proteins were studied in cells infected with HCMV (Towne), in the presence of CHX. By blocking translation of mRNA this allows accumulation of IE viral mRNA. This block is reversible but if medium containing actinomycin D (AD) to block further RNA transcription, is added only the accumulated mRNA is translated. $^{35}$S labelling revealed 3
Immediate Early antigens (α)
- first genes transcribed
- 4-5 proteins - major 72K
- nuclear distribution
- transactivation

Early (β)
- non-structural
- nuclear and membrane distribn.
- β genes
- DNA polymerase

Late (γ)
- structural
- 30+ proteins
- transcribed after DNA repln.

Fig 2. HCMV replication in vitro. Following HCMV infection of fibroblasts, virus encoded proteins are expressed in ordered sequence - termed immediate early, early and late antigens which are analogous to the and antigens of HSV as indicated. The function of the non-structural proteins is mostly unknown and is discussed (section 1.2). Viral DNA replication occurs 15h pi followed by expression of LA or structural proteins. Infectious HCMV is usually detectable in the medium after 48h. Restricted expression of HCMV antigens can be produced by use of CHX and AD or PPF as indicated and described in Section 2.4.3.
proteins at high concentrations (75, 72 and 68kDa) but lower amounts of other proteins were detected (Stinski, 1978). In vitro translation of IE mRNA revealed 9 proteins (75, 72, 59, 56, 42, 39, 27, 16.5, and 6.7kDa) (Wathen, Thomsen and Stinski, 1981). Many of these may correspond to the proteins detected by \(^{35}\)S labelling in infected cells when a CHX block was applied 2h post infection viz. 135, 59, 56, 53, 39, 27, and 19kDa (Stinski, 1978). Similar virus specific IE proteins were identified by immunoprecipitation with convalescent human serum (Michelson et al, 1979; Blanton and Tevethia, 1981). Monoclonal antibodies to the major 72kDa intranuclear, phosphorylated immediate early antigen have been produced, notably 6-E3 (Goldstein et al, 1982), which crossreacts between laboratory and clinical isolates and has been used to identify HCMV in biopsy specimens. Monoclonal antibodies to other IEA have been produced, and one was detected on the cell surface (Amadei et al, 1983).

The transcription of IE mRNA has been studied and its location on the restriction map for Towne (Wathen, Thomsen and Stinski, 1981; Wathen and Stinski, 1982), Davis (Demarchi, 1981) and AD169 (McDonough and Spector, 1983; Jahn et al, 1984) strains of HCMV, determined. There is a marked similarity in all three laboratory strains of the virus. Transcripts of different size (4.8-4.6, 2.2 and 1.9kb - Towne and 5.0, 2.3, 2.2 and 1.9kb - AD169) have been identified, the most abundant of which (1.95kbp up to 88% of total IE mRNA) maps to a restricted region - Xba-I-E fragment of DNA (0.739-0.755 map units - Towne strain) (Stenberg, Thomsen and Stinski, 1984) or the HindIII-E fragment of AD169 (Jahn et
al, 1984), in the U of the HCMV genome (Fig 1). This 1.95kbp HCMV IE mRNA (Towne strain), codes for the 72kDa major immediate early protein. It has been sequenced and a predicted primary amino acid sequence obtained. The predicted molecular weight is 64kDa and three features about its structure are interesting: 1) it contains a high number of charged amino acids and overall is slightly acidic, 2) it has a region with 36/38 hydrophobic or nonpolar amino acids and 3) there is a region rich in proline residues at both the -COOH and -NH₂ ends, similar to the major IE protein of HSV (Vmw 175) (Stenberg, Thomsen and Stinski, 1984).

The role of these HCMV IEA antigens is of particular interest for two main reasons. Firstly they are the first HCMV genes transcribed in the absence of de novo protein synthesis and therefore factors promoting their transcription are crucial to the establishment of productive infection. Secondly, IEA expression is a prerequisite for expression of EA and IEA may be responsible for some of the effects of the virus on host cell macromolecular synthesis.

Thomsen et al (1985) identified a promoter region upstream of the major IE gene that enhanced synthesis of 1.95kb mRNA, by RNA polymerase II in the HeLa lysate system. Stinski and Roehr (1985) used deleted promoter sequences upstream of indicator genes - thymidine kinase, chloramphenicol acetyltransferase and ovalbumin - and examined transcription of these by the HeLa lysate system or translation, following transfection of HeLa cells. The regulatory region was mapped to -465bp from the major IE gene and consisted of several 18 and 19 nucleotide
repeat units which acted in 'cis' on the adjacent genes. Stably transfected TK− cells were examined for the effect of infection with HCMV and HSV. HCMV specific 'trans'-activation occurred in the absence of HCMV specific protein synthesis; thus it was probably mediated by a HCMV structural protein. Similar results were obtained by Spaete and Mocarski (1985) except that using the E.coli B-galactosidase gene as an indicator, they found that HSV could also 'trans'-activate the IE promoter although less efficiently than HCMV. Batterson and Roizman (1983) have shown that transcription of the HSV-α genes is trans-activated by a structural protein, probably the tegument protein - V\textsubscript{MW} 65 (Campbell, Palfreyman and Preston, 1984). It appears likely that a HCMV structural protein similarly enhances transcription by host RNA polymerase II of the major IE gene and initiates the virus replicative cycle.

Progression of HCMV infection to EA and LA expression, is dependant on HCMV IEA expression. These IEA, especially the major 72kDa IE protein, may be chromatin associated (Stenberg, Thomsen and Stinski, 1984) and thus could affect not merely the rate of transcription of virus EA genes but also explain the increased host cell chromatin transcriptional activity (Kamata, Tanaka and Watanabe, 1978). These latter changes may affect host cell macromolecular synthesis (see below) and such alterations maybe important in allowing HCMV replication. The ability of IE gene products of HSV, VZV and HCMV to 'trans'-activate the rabbit β-globin gene was investigated by Everett (1984). The HindIIIIE region of AD169 HCMV (which codes for the major 1.9 mRNA and two less abundant species - 2.15 and
1.7kb (Fig 1)) was co-transfected into HeLa cells with plasmids containing the rabbit B-globin gene. HCMV IE gene products were able to enhance transcription of B-globin if either the HSV gD or B-globin promoter was used. However, if the Hind IIIE fragment was deleted so that only the major IEA could be expressed, the 'trans'-activating activity decreased, suggesting that, as is the case with HSV, the minor HCMV IEA mRNA transcripts are also important in enhancing transcriptional activity of a mammalian gene.

The 'trans'-activation of a HCMV IEA promoter by HCMV IEA was studied by Spaete and Mocarski (1985). They identified a HCMV early promoter region near the most abundant HCMV early transcripts in the internal repeat sequences (0.80 - 0.82 map units Towne strain (Fig 1)). This gene was placed upstream from the E.coli B-galactosidase indicator gene and transfected into cells. These cells were then infected with HCMV and if viral protein synthesis was allowed to continue, increased B-galactosidase activity was measurable 12h p.i. No increase in translational activity controlled by the HCMV early promoter, was observed following HSV infection. This contrasted with the increased transcriptional activity observed from the gD promoter following expression of HCMV IEA (Everett, 1984). Thus transcription and translation of HCMV IEA appears to be enhanced by a HCMV structural protein, and IEA themselves may 'trans'-activate the expression of early and host cell genes during permissive HCMV infection.
1.1.4 Early antigens

HCMV EA are expressed after de novo viral protein synthesis following infection, and require previous HCMV IEA expression, which may 'trans'-activate transcription of HCMV EA mRNA (Spaete and Mocarski, 1985). However, HCMV EA are themselves a prerequisite for viral DNA replication and, unlike the IA, are expressed if viral DNA replication is inhibited with PAA or PPF. The exact number of these proteins is unknown and many are expressed in relatively low amounts in the infected cell. Stinski (1978) identified a total of 10 immediate early and early proteins by $^{35}S$ labelling of HCMV infected cells. Three were identified as IEA and the remainder as EA - designated as 59, 56, 53, 39, 27, 21 and 19kDa. By immunoprecipitation, Blanton and Tevethia (1981) identified a possible 18 polypeptides that were produced prior to viral DNA replication but were not produced following CHX and AD treatment; thus the number of EA expressed in productively infected cells is probably close to 20.

In all the strains of HCMV tested, Towne, Davis and AD169 (Wathen and Stinski, 1982; DeMarchi, 1981; McDonough and Spector, 1983) EA mRNA mapped to the long repeat sequences and some lower abundance mRNA species to the long unique segment (Fig 1). HCMV IE mRNA does not persist for long in the permissively infected cell - 2h post-infection there was a marked reduction in the 4.8kbp IE mRNA (the 2.2 and 1.9kbp mRNA increased) but by 24h or in the presence of PAA, IE mRNA was no longer detected (Wathen,
Thomsen and Stinski, 1981). HCMV LA mRNA was not detected at 24h although viral DNA replication occurred 12-15h post infection.

mRNA from cells infected with HCMV was isolated from CMV DNA cellulose either after 6h infection followed by 8h CHX treatment, to permit accumulation of viral mRNA, or 24h post infection without use of inhibitors. The translation products of this mRNA were then studied in vitro using the rabbit reticulocyte lysate system. Although IEA antigens were translated from 6h infected cell mRNA, these had virtually disappeared in 24h infected cells corresponding to the observed decrease in mRNA. A series of EA were identified at 82, 44, 41, 20, 16, 11, 8, and 6.9kDa which were present in both the 6h and 24h samples. Thus in contrast to the transient expression of IEA mRNA, EA mRNA remains within the cell for prolonged periods. This is different to HSV and has been suggested as a possible explanation for the slower rate of replication of HCMV (Stinski, 1983). It is also interesting that in the same study Wathen, Thomsen and Stinski (1981) observed that 0.03% of total uninfected cell mRNA bound to their HCMV DNA cellulose. When this was translated only host specific proteins were detected. This may be related to the presence of regions of the HCMV genome that hybridise to human DNA although the precise significance of these observations is unknown (Ruger, Bornkamm and Fleckenstein, 1984; Shaw et al, 1985) (Fig 1).

The exact function of all but one HCMV EA is unknown although they are required for virus DNA replication and are probably involved together.
with IEA in inducing changes in host cell metabolism. One of the EA is the viral DNA polymerase (Huang, 1975). It differs from the cellular DNA polymerase-a by having a greater affinity for dGTP, dCTP and dTTP, as does HSV DNA polymerase (Mar et al, 1985). This enzyme is required for viral DNA replication and dissemination of infection, hence these differences are being exploited in an effort to produce specific anti-viral agents. HCMV and HSV DNA polymerase, in contrast to human DNA polymerase-a, is inhibited by the pyrophosphate analogues PAA and PPF. These agents inhibit viral DNA replication and expression of LA, although EA are expressed. PPF is being used clinically (Lancet, 1985) but the inhibition is reversible and infection may persist even in vitro - 5 weeks following in vitro HCMV infection in the continued presence of PPF, removal of the agent resulted in expression of LA and release of infectious virus (Wahren and Oberg, 1980). Purified herpesvirus DNA polymerases are also inhibited by the nucleoside derivatives 9-(2-Hydroxyethoxymethyl)guanine (ACG) and 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) (Mar et al, 1985). However, DHPG inhibits HCMV replication in vitro more efficiently than ACG. This is probably because cellular kinases produce the triphosphate derivative of DHPG more readily than with ACG. ACG is however more readily phosphorylated by the HSV induced virus thymidine kinase, which HCMV does not possess, explaining its particular effectiveness in vivo and in vitro against HSV (Balfour, 1984). The use of DHPG in HCMV infection is still being investigated.

In addition to the EA polypeptides, 2 HCMV EA are glycoproteins (MW
145 and 100kDa). These glycoproteins were identified 18h following HCMV infection (Stinski, 1977) and were found distributed on the plasma and microsomal membranes of HCMV infected cells by electron microscopy (Stinski et al, 1979). These membrane antigens may elicit an antibody response especially during primary HCMV infection (Middeldorp, Jongsma and The, 1985) although most of the EA identified by patients' sera are nuclear in distribution (The, Klein and Langenhuysen, 1974).

1.1.5 DNA replication

The precise details of HCMV DNA replication are unknown although most evidence would support a similar model for HCMV replication as proposed for HSV DNA replication (Stinski, 1983; Roizman and Batterson, 1985). HCMV DNA replication probably commences 12-15h after infection (Towne strain - MOI 100:1), but maximum viral DNA replication does not occur until 72-96h post-infection (Stinski, 1978). Several explanations have been proposed but to date none satisfactorily account for the difference between HCMV and HSV e.g. differences in the EA; HCMV DNA competes inefficiently with cellular DNA synthesis, thereby reducing the rate of HCMV DNA replication; the difference in the physical size of HCMV DNA as compared with HSV DNA.

In addition, the relationship between host cell and HCMV DNA synthesis in the infected cell is unresolved. This is exemplified by the conflicting observations of St. Joer and Hutt (1977) and Demarchi and Kaplan (1976). St. Joer and Hutt (1977) analysed the amount of viral and
cellular DNA synthesised at various times following infection and suggested that HCMV DNA replication is dependant on host cell DNA synthesis and therefore like replication of MCMV (Muller and Hudson, 1977) requires entry of the host cell into S-phase. In contrast, DeMarchi and Kaplan (1976) found that HCMV LA synthesis was reduced in S phase cells, thereby implying that viral DNA synthesis occurred independantly of the cell cycle, even though cellular DNA synthesis is activated after HCMV infection, which may be a property of defective HCMV virions (DeMarchi and Kaplan, 1977).

1.1.6 Late antigens

HCMV LA, the structural proteins of the virion, are expressed after viral DNA replication. However HCMV DNA replication (Towne strain) begins after 12-15h, but LA expression in the infected cell can only be detected at very low levels using a 2h $^{35}$S-methionine pulse, even 36h following infection. The rate of expression of these LA then increases simultaneously with the accumulation of HCMV DNA (Stinski, 1977). HCMV LA are immunoprecipitated by antisera raised against virions and have similar electrophoretic mobilities to proteins from virions and dense bodies. Although between 30-35 LA polypeptides have been identified in $^{35}$S labelled virions the majority of these are present in low amounts. The most abundant are the 68kDa (15.4% of total $^{35}$S labelled virus protein), 83kDa (8.4%), and 120kDa (7.1%) (Stinski, 1983).
HCMV LA mRNA in infected cells is likewise initially present in low amounts but accumulates with time after viral DNA replication. Some late mRNA's may be detected prior to viral DNA replication, although this observation has yet to be confirmed. The polysome associated mRNA consists of many different size classes and maps to most restriction fragments of the \( U_L \) segment (Wathen and Stinski, 1982; Demarchi, 1981; McDonough and Spector, 1983). The function of most LA is unknown, other than that they are present in the virion itself.

The most abundant transcripts in late infected cells map to the EcoRI G fragment of Towne strain HCMV at 0.37-0.39 map units (Fig 1) which encodes a 1.9kbp mRNA expressed after viral DNA synthesis. When this region was inserted into a plasmid and transfected into Cos cells a 67kDa protein was produced and identified by monoclonal antibodies recognising the major 67kDa LA present in virions (Davis and Huang, 1984). This protein is phosphorylated and has protein kinase activity similar to the kinase activity reported with the AD169 68kDa protein studied by Michelson et al (1984). No expression of the 67kDa protein was observed following transfection of WI38 cells. The nucleotide sequence suggested that the protein consists of 549 amino acids although this could be 19 amino acids longer as a second start and stop codon have been identified. It is a basic protein which the authors speculate may be consistent with DNA binding activity and by analogy with the major tegument protein of HSV, this major tegument protein of HCMV may mediate a similar trans-activating function on HCMV IE mRNA transcription although direct
experimental evidence for this is lacking (Davis and Huang, 1985).

8 to 9 HCMV glycoproteins have been identified in the late phase of virus infection and these glycoproteins are also present in the virion (Stinski, 1976; Stinski, 1977). Whereas HCMV glycoproteins were identified on the plasma membrane at early stages of infection, during late stages HCMV glycoproteins were also detected on the endoplasmic reticulum and nuclear membranes (Stinski et al, 1979). HCMV glycoproteins induced neutralising antibody responses in vivo (Rasmussen et al, 1985a) and monoclonal antibodies directed against such antigens were neutralising in the presence (Rasmussen et al, 1985b), or absence (Rasmussen et al, 1984) of complement, implying that such glycoproteins were important in binding and entry of the virion into the cell.

1.1.7 Encapsulation

In addition to examining the early events of HCMV infection by electron microscopy, Smith and De Harven (1973) also followed the course of HCMV and HSV infection in human fibroblasts. Within 12 to 24h following infection with Towne strain HCMV (MOI 10:1) they observed changes in the shape of the nucleus which became reniform and within the indentation a prominent golgi complex was observed. Input virus, initially observed in vacuoles, had disappeared by 48h. Capsid assembly was observed in the nucleus from 3d onwards, usually associated with the nuclear inclusions. Individual virus particles were surrounded by a filamentous coat and from 3 to 3.5d envelopment of the virus capsid was observed at the inner
nuclear membrane and the enveloped virus particles were then observed either in perinuclear cisternae or associated with sac-like protrusions contiguous with the cisternae. These enveloped virus particles were then observed in smooth vacuoles within the cytoplasm, and as no direct communication between the endoplasmic reticulum and the external surface of the cell was found, this was considered a discontinuous transfer of virions, via intermediate vacuoles.

After 4d, naked virus capsid were observed in the perinuclear cytoplasm, and these budded into vesicles particularly near the region of the golgi. These virions thus acquired a non-nuclear envelope. The vesicles into which such budding has been observed are rich in glycoprotein as shown by periodic acid-methenamine silver staining (Kanich and Craighead, 1974b) and HCMV glycoproteins were observed in such vacuoles (Stinski et al, 1979). In addition after 4d homogenous electron ‘dense bodies’ were present in the same region of the cell. These bodies also budded into vesicles and such bodies were present in virion preparations. Free ‘dense bodies’ have been separated from virions and consist of virus structural proteins without viral DNA (Sarov and Abady, 1975).

Vesicles containing both virions and ‘dense bodies’ were found under the plasma membrane, and occasionally ostia formed between these vesicles and the extracellular space with release of virions. The vesicles containing HCMV particles were probably lysosomal in origin, possibly from the GERL (golgi-endoplasmic reticulum-lysosome complex), as they stain
histochemically for arylsulfatase and acid phosphatase. Interestingly, the virions appear to be resistant to lysosomal enzymes, as very few damaged or degraded particles were observed in the sections (Smith and De Harven, 1978). Thus unlike many viruses HCMV and HSV appear to use lysosomes to transport virus particles to the surface of infected cells and the envelope surrounding the capsid is derived either from the nuclear membrane or from the lysosome itself, into which glycoproteins have been inserted, rather than the plasma membrane as observed with other enveloped viruses.

1.1.8 Effects of HCMV infection on the host cell

Prolonged maintenance of cellular integrity, cell death occurring 7-8d following high MOI infection in vitro, is required for the completion of the HCMV replicative cycle and unlike many viruses HCMV does not inhibit host cell macromolecular synthesis. The cell undergoes characteristic cytopathic changes following infection with HCMV, as described (McAllister et al, 1963; Kanich and Craighead, 1974a; Smith and De Harven, 1973; Albrecht et al, 1980), and these together with metabolic changes in the cell are induced predominantly by HCMV IEA and EA. The metabolic consequences of HCMV infection may be considered in terms of effects on cellular DNA, RNA and protein synthesis.

DNA synthesis

Host cell DNA synthesis is increased following HCMV infection. St.
Joer et al (1974) found that HCMV enhanced cellular DNA synthesis 5-fold, when arrested confluent monolayers of fibroblasts were infected with AD169 HCMV. This increased synthesis was not due to DNA repair and was blocked by UV or heat inactivation of the virus. In addition, Vero cells and guinea pig fibroblasts which were non-permissive for HCMV showed a similar increase (Furukawa, Tanaka and Plotkin, 1975). The increased DNA synthesis occurs in two phases reaching maxima at 16h and 96h respectively. The initial increase is predominantly due to mitochondrial DNA synthesis (Furukawa, Sakuma and Plotkin, 1976). A relationship between induction of host cell and viral DNA synthesis was proposed by St. Joer and Hutt (1977) but Demarchi and Kaplan (1976) suggested that viral DNA synthesis was independent of increased host cell DNA synthesis. Furthermore, they showed that preparations containing defective HCMV virions could stimulate host cell DNA synthesis more readily, and thought that increased host DNA synthesis may result from abortive infections either by defective virus or normal virus in non-permissive cells (Demarchi and Kaplan, 1977).

RNA synthesis

Increased host cell RNA synthesis occurred in HCMV infected cells, preceded viral DNA replication and was found in abortively infected guinea pig fibroblasts. Both the 28 and 18S ribosomal as well as 4S tRNA synthesis was increased in infected cells and this increase required IEA expression, as UV and heat inactivation of virus, and CHX treatment of cells 0-6h p.i. blocked increased RNA synthesis (Tanaka, Furukawa and
Plotkin, 1975).

**Protein synthesis**

Host cell protein synthesis is increased rather than suppressed following HCMV infection (Stinski, 1977). Immediately following infection host cell protein synthesis was suppressed for up to 6h but this was followed by a cyclical increase. Virus proteins were also synthesised in a cyclic manner. This enhanced protein synthesis required the presence of infectious virus but UV inactivated virus could mediate the initial suppression of synthesis. This suggests that an IEA or EA is required for the enhanced protein synthesis. The relative difference in the amount of virus as opposed to host cell specific protein synthesis following infection showed that at 12h only 10% of protein synthesis was virus specific and this gradually increased to a maximum of 60% at 48h. Thus even in 4d infected cells 40% of protein synthesis is host cell specific.

In addition to this general increase in protein synthesis certain host cell enzymes are expressed at higher levels in the HCMV infected cell, including: thymidine kinase (Estes and Huang, 1977), host DNA polymerase-α (Huang, 1975), host cell RNA polymerases I,III and particularly II (probably mediated by HCMV IEA) (Tanaka, Ihara and Watanabe, 1978) and ornithine decarboxylase associated with an increase in polyamine synthesis (Tyms and Williamson, 1982).

These changes in host cell macromolecular synthesis are probably
required for HCMV replication e.g. RNA polymerase II is required to transcribe IE viral genes, inhibition of polyamine synthesis blocks virus replication. Many of these effects on the host cell require the expression of HCMV IEA and/or EA but the mechanism by which these viral products cause such changes in host cell metabolism is unknown. Kamata, Tanaka and Watanabe (1978; 1979) showed that HCMV encoded functions expressed shortly after infection, were able to alter cellular DNA template activity and effect conformational change in host cell chromatin. These factors had properties of DNA binding proteins and were blocked by anti-IEA polyclonal antibody, suggesting that they may be a HCMV IEA. The ability of IE proteins to alter host cell DNA, coupled with the observation that IEA may trans-activate mammalian genes such as the β-globin gene (Everett, 1984), suggest a possible mechanism whereby HCMV IEA may mediate the host cell changes described.

1.1.9 Non-productive infection and virus persistence

Although HCMV will only productively infect human fibroblasts in vitro, a number of human and cell lines of other species may be non-productively infected. In most cases some expression of HCMV IEA occurs but virus replication is inhibited. Perhaps the best studied of these cells are guinea pig fibroblasts. Following infection HCMV IEA and some EA are expressed (Stinski, 1977) but virus DNA replication and expression of LA does not occur. In addition host cell DNA and RNA synthesis is enhanced in a manner analogous to the productively infected
human fibroblast, although the characteristic cell rounding associated with early infection in human fibroblasts does not occur (Furukawa, Tanaka and Plotkin, 1975).

HCMV persists following primary infection, but the anatomical site of persistence/latency and the nature of the persistent state is unknown. There are isolated reports that other human cells can be non-productively infected in vitro. Epitheloid cells (St. Joer and Rapp, 1973) and lymphoblastoid cells may be infected, in the latter case a slow persistent infection in the culture was reported with only 1% of the cells producing infectious virus at a given time (Tocci and St. Joer, 1979).

This limited cell tropism following in vitro infection contrasts with the widespread dissemination of HCMV in vivo during infection. Using in situ hybridisation with biotin-conjugated HCMV DNA probes which did not cross react with host cell sequences, Myerson et al (1983) showed HCMV DNA in obviously cytomegalic cells and in endothelial cells, cardiac myocytes, splenic and lymph node reticular cells, glomerular, interstitial and tubular cells of the kidney and in breast stroma in patients with active HCMV infection. No HCMV was identified in lymphocytes using this technique. Unfortunately this technique has not been applied to the examination of normal HCMV seropositive individuals with no clinical evidence of infection/reactivation.

The site of persistence of HCMV in the normal seropositive individual remains unknown. In the case of MCMV the virus persists in salivary gland and prostate (Cheung and Lang, 1977; Cheung et al, 1980),
spleen (Olding, Jensen and Oldstone, 1975; Wise et al, 1979) and kidney (Gould and Mims, 1980). The infection of spleen cells was particularly interesting as activation of MCMV was observed following antigenic challenge. The virus probably persists in B cells but when explants are placed into culture the infection is transferred to macrophages. In addition MCMV probably persists in at least a subpopulation of peritoneal macrophages (Jordan, 1983). These findings together with the observation of Mims (1982) that many viruses which establish persistent infection in vivo have the capacity to infect immunocompetent cells has focused attention on infection of such cells by HCMV.

HCMV infection can be transmitted by blood transfusion and this risk is reduced if blood from seronegative donors or leukocyte depleted blood is used (Adler, 1983). However, HCMV has not been isolated from blood of 1500 HCMV seropositive donors in the USA (Jordan, 1983), although in a smaller Roumanian study, HCMV was recovered from 2 of 21 donors (Diosi et al, 1969). In HCMV mononucleosis virus can be isolated both from peripheral blood mononuclear (PBM) (9/20) and granulocyte (19/20) fractions (Carney, Iacoviello and Hirsch, 1983). Attempts at productively infecting PBM in vitro have been mostly unsuccessful if expression of LA and release of virus was used as criteria of infection (Rinaldo et al, 1978; Wahren, Robert and Norlund, 1981). Recently Rice, Schrier and Oldstone (1984) showed that a small number (<10%) of monocytes in PBM infected with recent clinical HCMV isolates as opposed to
laboratory strains of HCMV expressed IEA although no productive infection occurs. Pagano (1975) showed HCMV DNA in blood cells from some normal bone marrow donors (0.5-6.5 genome equivalents/cell). In addition, using a sensitive in situ hybridisation technique, HCMV IE mRNA was found in <5% of T cells, from normal seropositive individuals, but no late mRNA could be detected (Schrier, Nelson and Oldstone, 1985). Thus although the evidence is incomplete, human PBM can probably be infected with HCMV, although this infection is non-productive with little or no secretion of infectious virus.

HCMV infection can also be transmitted in transplanted organs - kidney (Betts et al, 1975) and heart - and by HCMV DNA-DNA hybridisation has also been detected in colonic epithelium (Roche et al, 1981).

Although HCMV DNA has been detected at a number of different anatomical sites the question as to whether the virus is latent in a manner analogous to HSV or whether there is a continuous low level of virus shedding as has been shown in the case of EBV (Yao, Rickinson and Epstein, 1985) remains unanswered. The factors controlling HCMV shedding in the persistently infected host are unknown but allogeneic stimulation of MCMV infected lymphocytes caused shedding of MCMV. Replication of MCMV was blocked in a murine teratocarcinoma line but if cellular differentiation was induced with dimethylacetamide, infectious virus was released (Dutko and Oldstone, 1981). Gonczol, Andrews and Plotkin (1984; 1985) found that there was no expression of HCMV EA and IA or virus release following infection of human pleuripotent embryonal
carcinoma cells (2102Ep and TERA-2) However, differentiation of 2 clones (from the TERA-2 line) by retinoic acid, following HCMV infection, was associated with expression of HCMV EA, LA and virus release. Similarly, virus was released by these cells differentiating spontaneously in cultures of the parent line. It is possible that as with MCMV, cellular differentiation may provide a 'trigger' required for virus replication, although the precise block in the pleuripotent cell is unknown.

1.1.10 Transformation and malignancy

Herpesviruses have been associated with malignancy in animals and man: EBV with Burkitt's lymphoma and nasopharyngeal carcinoma. Similar associations have been proposed for HCMV e.g. Kaposi's sarcoma, prostatic carcinoma and colonic adenocarcinoma (Rapp, 1981). The evidence for HCMV being aetiologically implicated in these diseases is indirect and based predominantly on serological data and the presence of HCMV DNA by hybridisation in tumour cells. In the latter instance, particularly in the case of Kaposi's sarcoma conflicting results have been obtained.

Direct evidence for HCMV induced transformation of cells is more limited. HCMV transformed hamster embryo fibroblasts which expressed HCMV antigens, established tumours in hamsters (Albrecht and Rapp, 1973). Human fibroblasts have been transformed in vitro and these cells were able to establish tumours in athymic nude mice (Geder et al, 1977). Nelson et al (1982) identified a 2.9kb fragment in the HindIII E fragment of AD169 HCMV, which when transfected into NIH 3T3 cells caused loss of anchorage.
dependent growth and these cells established tumours in nude mice. Sequence analysis of this region identified a possible protein of 118 amino acids. In addition, this region hybridised to the 5.0kbp RNA transcribed during IE gene expression (Nelson et al, 1984; Jahn et al, 1984). Therefore although there is some evidence that HCMV may transform human and other cells in vitro, its association with any defined human malignancy is tenuous at present.

1.2 CYTOMEGALOVIRUS INFECTION IN MAN

Infection with HCMV, as with other human herpesviruses is widely prevalent. In most third world countries seropositivity rates approach 100% in the adult population, whereas in developed countries they are usually between 40-60% (Krech, 1973; Cabau et al, 1979; Gold and Nankervis, 1982). Isolated or remote communities such as the Tiriyo Amazonian Indians, who do not possess antibodies to measles and influenza are seropositive, suggesting that HCMV can maintain itself in small populations and has been a longstanding pathogen of man (Black et al, 1970). HCMV infection is more prevalent in lower socioeconomic groups in developed countries (Cabau et al, 1979; Gold and Nankervis, 1982), as has been observed with other human herpesviruses such as EBV (Evans and Niederman, 1982). HCMV infection is acquired earlier in developing countries, so that approximately 85-95% of children in
Melanesia and Tanzania are seropositive by 5-6y compared with 5-30% in the U.S.A. (Pass, 1985). The number of seropositive individuals gradually increases with age after the neonatal period without a convincing rapid increase during adolescence.

1.2.1 Transmission of HCMV

Identification of modes of transmission of HCMV is hampered by the lack of identified epidemics in open populations and the predominance of asymptomatic primary infection. Under conditions where primary infection is identified, virus is shed in saliva, urine, tears, semen, cervical secretions and breast milk. Reactivation during pregnancy with virus shedding in cervical secretions has been identified - 14% of HCMV seropositive pregnant women had cervical shedding of the virus and a particularly high number (35%) of those aged 21 or less shed the virus in the first trimester (Chandler, Alexander and Holmes, 1985). This together with virus shed in breast milk may account for the high number of infants acquiring infection in the first year of life (Pass, 1985).

HCMV may be transmitted horizontally as identified by restriction endonuclease analysis of virus isolated from children attending day care centres (Hutto and Pass, 1984). However, such transmission probably requires close contact as seroconversion in hospital workers caring for congenitally infected, HCMV shedding infants rarely occurred (Dworsky et al, 1983; Pass, 1985). The identification of virus in genital secretions and high seropositivity rates in promiscuous populations, suggest that
sexual transmission of the virus may also occur; as reviewed above, HCMV may also be transmitted in blood products.

1.2.2 Clinical syndromes of HCMV infection

The vast majority of HCMV infections are asymptomatic although certain well defined syndromes occur:

**Congenital and perinatal infection**

The frequency of intrauterine infection in the U.S.A. has been estimated at about 1% live births in the USA (Stagno et al, 1982). Symptomatic congenital infection is frequently associated with primary infection in pregnancy rather than reactivation (Stagno et al, 1982). In considering the pooled results of the 4 largest series, Ho (1985) found that 55% of children had congenital infection following primary maternal infection and 25% of these were symptomatic. Although symptomatic congenital infection as a result of recurrent infection is recognised Stagno et al (1982) found that all 20 such infections in their series were asymptomatic.

The full clinical picture of cytomegalic inclusion disease is characterized by jaundice, petichial rash and multi-system involvement particularly of the CNS. This syndrome was identified before the virus was isolated (Smith and Velios, 1950), although earlier descriptions of such infants appeared in the literature (Farber and Wolbach, 1932). In addition to these gross sequelae of congenital HCMV infection, subtle defects can
be recognised many years later - notably bilateral hearing loss (affecting up to 17% (10/59; 3 of 8 symptomatic and 7 of 51 subclinically infected), HCMV infected children (Stagno et al, 1977) and reduced IQ (Hanshaw et al, 1976). In the perinatal period, as in later life, HCMV infection may be asymptomatic but poorly developed infants are at particular risk and in these, infection may present as HCMV mononucleosis or with severe pulmonary involvement (Pass, 1985).

HCMV mononucleosis

Primary HCMV infection may present with the clinical features of infectious mononucleosis including biochemical evidence of hepatic involvement and atypical lymphocytosis, although heterophile antibodies are usually absent (Klemola, 1973; Jordan et al, 1973). A similar picture is seen in many cases of primary infection following open heart surgery with transfusions. Complications of HCMV mononucleosis are recognised although rare, and as the mononucleosis is often asymptomatic these may be presenting syndromes of primary HCMV infection. These include pneumonitis (although not as frequently as in the immunosuppressed (IS) patients), hepatitis, Guillain-Barre syndrome, meningoencephalitis, myocarditis, thrombocytopenia and haemolytic anaemia (Ho, 1985).

HCMV infection in immunosuppressed patients

HCMV infection is a major cause of morbidity and mortality in IS patients, both transplant recipients and those patients with an underlying
disease such as AIDS. Primary HCMV infection is often asymptomatic even in these patients, but symptomatic mononucleosis, accompanied by complications including choroidoretinitis occur more frequently. In addition symptomatic 'reactivation' of endogenous virus is more frequent.

In AIDS patients HCMV seropositivity rates are high (>95%) and the vast majority of these patients shed HCMV. Many of the clinical features of AIDS may be related to HCMV infection e.g. leucopaenia, reversed Th/Ts (Carney et al, 1981; Schooley et al, 1983; Lane and Fauci, 1985). The direct effects of HCMV such as pneumonitis often prove fatal in such patients (Macher et al, 1983).

HCMV infection is a major problem following renal and bone marrow transplantation. In renal transplant recipients primary infection is usually severe and is associated with loss of allograft function (Betts, 1984). Primary infection follows transplantation from a seropositive donor to a negative recipient in 65-75% of cases, suggesting that the virus is transmitted by the graft, however whether this is via the renal cells or passenger cells within the graft is unclear. The mechanism of graft failure is unknown but a specific HCMV induced glomerulopathy has been proposed (Richardson et al, 1981). HCMV pneumonitis is often a serious or fatal complication particularly if associated with hypoxia.

HCMV infection in bone marrow transplant recipients, is probably the commonest single infectious complication and is associated with a higher mortality than any other infection (Meyers, 1984). The major source
of HCMV in these patients appears to be the granulocyte or blood transfusions rather than the graft itself. The frequency of primary infection has been reduced by using HCMV seronegative blood products from seronegative donors. HCMV pneumonia is particularly important - in the Seattle study pneumonia occurred in 147 (15%) of 952 consecutive allogeneic transplants. This accounted for 44% of nonbacterial causes of pneumonia and the median survival of cases following onset was 3 weeks, with a case fatality rate of 88% (Meyers et al, 1983). In addition to pneumonia HCMV is also associated with a hepatitis and ulcerative lesions affecting all parts of the gastrointestinal tract (Meyers, 1984).

1.3 IMMUNE RESPONSE TO HUMAN CYTOMEGALOVIRUS INFECTION

The association of severe HCMV infection with immunosuppression focuses attention on the relationship between HCMV and the host immune response.

1.3.1 Antibody mediated immunity to HCMV

Following virus infection a large number of specific antibodies reacting with various viral determinants are generated. Although such antibodies can be directed against the virion and the virus infected cell, the former predominate. The major role of antibody appears to be the
elimination of virions thereby preventing virus reinfection of the host
and limiting dissemination of infection by virions released within the
host (Lachman, 1985).

Antibodies against HCMV, of different classes (Plotkin, 1982) and
subtypes (Linde et al, 1983) have been described, mediating a variety of
different responses. Complement fixing antibodies detected in the standard
complement fixation test (cft) test are the most frequently used marker of
HCMV seropositivity and a four-fold increase in cft titre is often used as
an indication of secondary infection. The cft antigen used in the assay
varies but the most sensitive is a glycine extract of solubilised HCMV
infected fibroblast monolayers containing predominantly LA between
66-85kDa (Waner, 1975), which cross react between strains.

Neutralizing antibodies can be detected in many seropositive
individuals, although the test is seldom used in view of the time
consuming plaque assay involved. The low titres observed may be partly
explained by the high particle/infectivity ratio of HCMV preparations. As
with other viruses the addition of 2% complement (C) enhanced neutralising
antibody activity (Andersen, 1972). Recently, monoclonal neutralising
antibodies against HCMV glycoproteins have been produced which have
a specificity for the virus envelope glycoproteins (Britt, 1984;

The antibodies against HCMV, present in normal seropositive sera
have specificity for many different viral antigens. Blanton and Tevethia
(1981) used human sera to immunoprecipitate not only the HCMV late
proteins but were also able to detect 4 immediate early and up to 18 early proteins in virus infected cells. These observations together with the detection of anti-HCMV EA antibodies both against nuclear (The, Klein and Langenhuiysen, 1974) and membrane (Middeldorp, Jongsma and The, 1985) antigens, suggest that virus infected cells may be susceptible to C and antibody dependant cell mediated cytotoxicity (ADCC) (Sissons and Oldstone, 1980). However, direct evidence for a protective role for ADCC in vivo is difficult to demonstrate even in experimental animal models let alone in HCMV infection in man.

The evidence for a protective role for HCMV specific antibodies is restricted to primary HCMV infection. The most compelling evidence is provided by the observation that passive immunisation of HCMV seronegative bone marrow transplant recipients with HCMV hyperimmune globulin prior to transplantation, reduced the frequency and severity of HCMV infection in such patients (Meyers et al, 1983). However, it is interesting that protection was only afforded to those patients who did not receive seropositive granulocyte transfusions. 7 of 8 hyperimmune globulin treated patients given such transfusions developed HCMV infection. This observation may also suggest that the transmission of virus by transfusion when it is probably intracellular (Adler, 1983), is not controlled by the presence of high titres of HCMV antibody alone or in association with C.

HCMV antibodies are also thought to be important in modifying HCMV infection of the foetus. Stagno et al (1982) found that 11 of 21 primary
HCMV infections in pregnant women resulted in congenital infection of the foetus, whereas 20 of 2330 seropositive women had congenitally infected infants. None of the latter group were symptomatic and this has been proposed as evidence that maternal IgG antibody protects the foetus during pregnancy. Symptomatic disease in children of seropositive mothers has however been documented (Ahlfors et al., 1981). This is not the only possible interpretation as a reduced dose of infectious virus during reactivation as opposed to primary infection, could also account for the observations. Such a reduced virus dose may be due not only to maternal antibody, but also to the presence of HCMV specific cell mediated immunity in seropositive individuals. In fact, Gehrz et al (1977) have shown that cell mediated immunity as measured by HCMV specific lymphoproliferation was reduced in those mothers who had congenitally infected infants.

1.3.2 Cell mediated immune responses to virus infection

Antibody responses are predominantly directed against the virion, whereas cell mediated responses, including virus specific MHC restricted T cells and non-specific natural killer (NK) cells, are directed against the virus infected cell. The importance of these mechanisms in protecting the host against virus infection is illustrated by patients with primary immunodeficiency (Rosen, Cooper and Wedgwood, 1984). Primary antibody deficiency is associated with increased pyogenic bacterial infection, although metastatic CNS infection with enteric viruses such as ECHO and poliovirus is recognised (Lachman, 1985). However, in primary T cell
deficiency there is a failure to clear established virus infection e.g. Hechts' giant cell pneumonia, progressive vaccinia. Many different types of effector cells, including T helper (Th), T suppressor (Ts), T cytotoxic (Tc), T cells mediating delayed type hypersensitivity (Td) and NK cells, are activated following virus infection. Their relative preponderance and importance in protecting the host, depends on the nature of the infecting virus, the route of infection and host factors such as the genetic background controlling T-T and antigen presenting cell (APC)-T cell interactions.

The importance of DTH in protecting the host against virus challenge has been shown both in murine influenza and HSV infection. In the latter, transfer of Td cells from draining lymph nodes following subcutaneous inoculation with HSV, was associated with protection of naive recipients against virus challenge (Nash, Field and Quartey-Papafio, 1981). Like other T cell responses Td were MHC restricted and it was probable that both MHC class I and class II restricted responses are required for maximum protection (Wildy and Gell, 1985). Challenge of mice with UV inactivated influenza generated class II restricted Td. In contrast to HSV, transfer of these cells 24h following live influenza infection in naive recipients not only failed to protect the animals but exacerbated the disease (Ada, Leung and Ertl, 1981). However, the role of virus specific Td cells in man, is difficult to determine particularly in the absence of an in vitro assay for DTH responses.

B cell and 'effector' T cell activity is downregulated by a number
of factors including anti-idiotypic antibodies, lymphokines such as IFN and Ts. The mechanism by which Ts mediate suppression is unknown, although it probably involves secretion of both non-specific and I-J containing antigen specific suppressor factors. Ts activity is itself regulated by T-T and APC-T interactions as suggested by Germain and Benacerref (1981). Ts have been identified in murine influenza and HSV infection. In the former MHC class II restricted Lyt1^+ ,23^- Ts were generated following intranasal challenge with live influenza, and they were able to inhibit the generation of the potentially detrimental class II restricted Td cells (Liew and Russell, 1983). Following HSV infection two populations of Ts cells were described by Nash (1985), which were virus specific and inhibited only the development of DTH responses but not Tc responses.

In persistent virus infection these cells may play an important role e.g. recrudescence of HSV II infection in the guinea pig is associated with reduced suppressor cell activity (Iwasaka, Sheridan and Aurelian, 1983). Herpesvirus infection, including HCMV, is frequently associated with increased T8^+ cell in PBM both during primary infection (EBV and HCMV mononucleosis (Reinherz et al, 1980)) and in transplant recipients (Schooley et al, 1983). Infectious mononucleosis is also associated with increased suppression of in vitro immunoglobulin synthesis (Tosato et al, 1979). However, as Ts particularly in the case of HSV, are virus specific and suppress particular 'effector' responses, the latter have to be first defined, before the activity of specific Ts can be studied.
1.3.3 Cytotoxic T cells

Tc have been described to different cell surface antigens including alloantigens, modified/haptenated cell surfaces and viral antigens. Zinkernagel and Doherty (reviewed 1979), identified a population of T cells, in the spleen of lymphocytic choriomeningitis virus (LCMV) infected mice, that killed LCMV infected cells, but only if the infected cells were identical to the Tc at the H-2K and H-2D regions. These observations led to the concept of MHC restriction i.e. Tc are restricted in their effector function by both virus and MHC class I determinants.

MHC restriction of virus specific Tc

Human virus specific Tc restricted by MHC class I determinants (HLA A and B) were identified by McMichael and Askonas (1978) against influenza virus, following secondary in vitro stimulation of PBL with UV irradiated, influenza infected autologous PBM. These studies were followed by identification of Tc specific for other human viruses including measles, mumps (Kreth et al, 1979; 1982) and herpesviruses such as EBV (Moss et al, 1981) and HSV (Sethi, Stroehman and Brandis, 1980; Yasukawa, Shiroguchi and Kobayashi, 1983). Virus specific Tc could be inhibited by blocking with anti-MHC class I antiserum and monoclonal antibodies (Wallace et al, 1981), suggesting that surface MHC determinants were recognised by Tc. This was supported by the observation that murine virus specific Tc and Tc clones killed virus infected L cells, in which H-2K,D
and/or L genes, matched with the Tc, were expressed following DNA-mediated gene transfer (Mellor et al, 1982). However, virus specific Tc may recognise different regions of the MHC compared with the epitopes identified by antisera, as shown by recognition of variant HLA-A2. Influenza infected target cells expressing serologically identified A2 antigens were not lysed by Tc from a number of HLA-A2 individuals. This discrepancy was resolved when HLA-A2 variants had different mobilities on SDS-PAGE with amino acid differences in the \( \alpha_2 \) domain were identified (Biddison et al, 1982; Krangel et al, 1982).

MHC class I determinants consist of a single protein, with an extracellular portion divided into three domains, a hydrophobic transmembrane portion and an intracellular tail. In the mouse this is encoded by 7 separate exons - the extracellular domains by 3 exons (N, C1 and C2), a transmembrane region (TM) and 3 exons corresponding to the intracellular domains (I1, I2 and I3). \( \beta_2 \) microglobulin is bound to the C2 domain (Hood, Steinmetz and Goodenow, 1982). The site(s) recognised by virus specific Tc on MHC class I determinants have been further investigated by two approaches. Firstly murine vaccinia specific Tc restricted by mutations in the \( K^b \) determinant (\( K^{bm} \)) did not kill vaccinia infected \( K^b \) cells when the mutations were mapped to the N and C1 domains, even when the differences involved single amino acid substitutions (Hurwitz et al, 1983).

The second approach involved exchanging the exons encoding the C1 and N domains between \( L^d \) and \( D^d \) genes. The ability of influenza
and vesicular stomatitis virus (VSV) specific, \(L^d\) and \(D^d\) restricted Tc to kill L cells transfected with these recombinants was studied. Tc recognised the appropriate Cl and N domains (Reiss et al, 1983). However not only are the extracellular domains involved in virus specific Tc lysis, the intracellular domains may also be of importance. The I1, I2 and I3 exons of the \(L^d\) gene were deleted, replaced by the the intracellular I-A\(_d\) genes and transfected into L cells. The truncated \(L^d\) was recognised as a restriction element by influenza specific Tc but there was a 5-fold reduction in susceptibility to lysis of VSV infected cells by VSV specific Tc (Murre et al, 1984). Thus at least in the case of VSV, interaction between the intracytoplasmic portion of \(L^d\) and a VSV product may be required for recognition by Tc or changes in the intracytoplasmic region might affect interaction between the surface domains and virus antigen.

Initial, Tc specific for different viruses were thought to be exclusively MHC class I restricted. However, class II restricted virus specific Tc clones have been described in man, including EBV (Meuer et al, 1983), HSV (Yasukawa and Zarling, 1984) and measles (Jacobson et al, 1984). This has raised the question of the relative precursor frequency of class II as opposed to class I restricted Tc, irrespective of antigen specificity. Human class II restricted Tc were \(T4^+\) whereas class I restricted cells were \(T8^+\) (Meuer et al, 1983). These surface phenotypes appear to define the nature of the MHC restricting element rather than function in terms of 'helper' and
'suppressor/cytotoxic' subsets as originally proposed (Reinherz et al, 1979; 1981). T4+ and T8+ lymphocytes were grown in limiting dilution culture and the precursor frequency of Tc determined irrespective of antigen and MHC specificity. Nearly 100% of T8+ cells were cytotoxic, compared with <10% of the T4+ cells (Moretta et al, 1983). Thus numerically most Tc are MHC class I restricted, at least as defined by the T8 phenotype.

**Generation of virus specific Tc**

It has proved difficult to generate a primary virus specific Tc in vitro, thus most studies refer to the requirements for secondary in vitro stimulation of Tc. Tc are generated in response to antigen in association with MHC determinants. Most attempts at secondary in vitro stimulation of Tc responses with isolated viral glycoproteins have been unsuccessful except for influenza haemagglutinin (HA) (Zweerink et al, 1977) and Sendai virus glycoproteins. These proteins can fuse with cell membranes and this may be related to their ability to generate virus specific Tc in vitro. However, Sendai virus glycoproteins (Hale, Lyles and Fan, 1980) and HSV glycoproteins (Lawman et al, 1981) can induce a secondary Tc response in vitro, when inserted into liposomes together with MHC determinants.

Th cells are probably required for the generation of virus specific Tc (Zinkernagel and Doherty, 1979). Th cells predominantly recognise antigen presented by APC in association with MHC class II. The augmentation of the Tc response by Th probably involves the release of
IL-2 by Th, and activated Tc expressing IL2 receptors can clonally proliferate (Smith, 1984). However, other studies have suggested that a Tc differentiation factor secreted by Th cells may also be required for the development of mature Tc (Raulet and Bevan, 1982).

**Virus antigens recognised by virus specific Tc**

Virus specific Tc, will only kill cells infected with one virus, but the nature of the virus component recognised is mostly unknown. It was found that whereas Tc could be inhibited by antibodies against MHC determinants, anti-viral polyclonal or monoclonal antibodies were rarely able to inhibit Tc lysis. Inhibition of murine reovirus specific Tc (Finberg, Spriggs and Fields, 1982) and SV40 T antigen specific Tc (Pan and Knowles, 1983) by monoclonal antibodies has been described. Following reovirus infection in vivo, serotype specific Tc are detected after 3-5 days. Serotype specific antisera and neutralising monoclonal antibodies against the sigma-1 protein inhibited Tc activity. Anti-idiotypic antibodies, prepared against these blocking monoclonal antibodies, when incubated with Tc also inhibited Tc activity (Ertl et al, 1982). Furthermore these anti-idiotypic antibodies were able to generate reovirus specific Tc in the absence of virus (Sharpe et al, 1984). Thus in reovirus infection the Tc response is directed against a virus structural protein, which is involved in virus/receptor binding and shares the same epitope recognised by neutralising antibodies.

However, other virus specific Tc are not serotype specific
e.g. human and murine influenza specific Tc. 4 major antigenic proteins are produced during influenza infection – HA, neuraminidase (N), nucleoprotein (NP) and matrix protein (M). Influenza serotype specific Tc which recognise HA have been described, but these are less frequent following murine infection than cross-reactive Tc (Kees and Krammer, 1984). Murine and human influenza A specific Tc lysed different influenza A infected but not influenza B or C infected cells. This suggested that they recognised determinants other than HA and N, and such murine Tc clones recognised NP (Townsend and Skehel, 1984) and killed fibroblasts transfected with the NP gene (Townsend et al, 1984). NP is not normally found on the surface of influenza infected cells, thus it raises the possibility that either MHC altered by interacting with an intracellular viral protein may be the antigen recognised, or that normally intracellular proteins such as NP could undergo some form of processing by cells other than classical APC and be presented on the surface as a peptide fragment.

In both influenza and reovirus infection, the virus antigens recognised by Tc are constituents of the virion, in addition to being expressed in virus infected cells. In the case of EBV specific Tc the nature of the virus antigen recognised is unknown. These Tc are generated by coculture of PBM with autologous EBV transformed lymphoblastoid B cells. They can be assayed either by the $^{51}$Cr release assay or by their ability to induce 'regression' of such EBV transformed cells in vitro (Moss, Rickinson and Pope, 1977; 1978; Moss et al, 1981). The
majority of such Tc and Tc clones are MHC class I restricted (Wallace et al., 1981) although class II restricted Tc are recognised (Meuer et al., 1983). The virus antigen is unidentified but has been termed LYDMA - lymphocyte determined membrane antigen. It is expressed in non-permissive EBV transformed B cells and its presence can only be determined by the susceptibility of such target cells to EBV Tc, as no serological identification of this determinant has been achieved. Mann, Staunton and Thorley-Lawson (1985) have produced monoclonal antibodies to a 63kDa membrane protein expressed in EBV transformed cells although whether this will prove to be the LYDMA determinant is unknown.

Virus specific Tc in vivo

Tc have been found in spleen and draining lymph node after virus infection, and their ability to protect the host against virus challenge can be passively transferred (Zinkernagel and Doherty, 1979). A murine influenza A specific Tc clone transferred to naive recipients protected against virus challenge as shown by a reduction in the amount of virus isolated from the lung (Lin and Askonas, 1981). The mechanism of protection may not necessarily involve direct cytotoxicity as these cells also secreted IFN (Morris, Lin and Askonas, 1982) and mediated delayed type hypersensitivity (DTH) reactions (Lin and Askonas, 1981).

The role of Tc in persistent virus infection may be somewhat different. Unlike influenza specific Tc activity in man, which decreases
in the absence of a fresh antigenic challenge (McMichael et al, 1983), EBV specific Tc remain at a high precursor frequency in PBL in all seropositive individuals (Rickinson et al, 1981; Yao et al, 1985b). If the EBV specific Tc precursor frequency is reduced as following transplantation (Yao et al, 1985b) or in the X-linked lymphoproliferative syndrome (Harada et al, 1982) there is increased virus shedding and an appreciable increase of EBV positive B cell lymphomas. It has therefore been proposed that such Tc may be important in limiting episodes of reactivation or dissemination of the virus thereby maintaining a virus/host balance in the seropositive individual (Rickinson, 1985).

1.3.4 Helper T cells

Th cells are required not only for antibody synthesis in response to T dependant antigens such as viruses, but also for the generation of other T cell responses. Th may also be directly protective e.g. by secreting lymphokines such as IFN which may mediate DTH responses and enhance NK activity. In vitro these cells are identified by their ability to proliferate in response to virus antigen although their ability to provide help to virus specific antibody production in vitro is documented in some systems. Virus specific Th are MHC class II restricted by the DR, DC(DQ) and SB(DP) loci (Eckels et al, 1983). APC and/or B cells are required to present antigen to Th in association with appropriate class II antigens, although there is continuing controversy as to the degree of 'antigen processing' required for stimulation of Th (Unanue, 1981) - in
the case of alloantigens insertion into a membrane in association with class II antigens may suffice (Burakoff et al, 1984).

The specificity human of Th cells to influenza virus has been investigated in detail (Lamb et al, 1982c; 1982d). Like Tc to influenza both subtype specific and cross reactive Th cells have been described. Human Th clones have specificity for the different influenza antigens M, NP, HA and N. However, Th are markedly cross reactive – M specific Th clones augmented the production of HA specific antibodies in vitro (Lamb et al, 1982a). The specificity of HA specific Th clones was analysed using synthetic peptides of the HA molecule. There were two broad types of response: firstly Th clones proliferated in response to determinants remote from the antibody binding sites and also responded to HA from different influenza serotypes and secondly a Th clone that was specific for the immunising HA alone. When whole PBL were assayed against the same peptides, there was a proliferative response to peptides from all regions of the HA molecule, implying that many different clones were present each reacting with a different specificity to the HA molecule (Lamb et al, 1982b). Regions within these peptides have been identified which were particularly important in inducing Th clones to proliferate (Lamb and Green, 1983).

Thus the Th response to an individual viral protein such as HA recognised different sites on the molecule and these differed from antibody binding sites. However such differences did not prevent Th cells augmenting HA specific antibody production when different peptides of the
same molecule or even different proteins were used to stimulate Th cells. The cross reactivity of Th activity suggests an important antigen non-specific component, probably mediated by release of lymphokines such as B cell growth factor, IL2 and B cell differentiation factor (Moller, 1984). Antigen specific helper factors have also been described although their role is at present poorly defined.

1.3.5 HCMV specific T cell mediated immune responses

Investigations of the role of cell mediated immune responses to HCMV have predominantly studied lymphocyte proliferation to HCMV antigens. Ten Napel et al (1977) and Moller-Larsen et al (1976) showed that PBM from normal seropositive adults proliferated to HCMV antigens in vitro. The antigens used in these assays were late viral antigens or gradient purified virions and dense bodies. Ten Napel et al (1977) suggested that live virus as opposed to heat or UV inactivated virus was not effective at inducing lymphocyte proliferation. Maximum proliferative response was observed as with other viral antigens, after 6d culture but the nature of the responding cells was not defined. These techniques have been applied to a number of clinical situations including congenitally infected children (Gehrz et al, 1977; Reynolds et al, 1979) who had reduced responses compared with age matched controls, acquired infection in infancy (Pass et al, 1981), who similarly had a marked delay in the development of such responses. During HCMV mononucleosis (Levin et al, 1979) HCMV specific antibody responses developed soon after the onset...
of symptoms but, HCMV specific lymphocyte proliferation was delayed for several months. Following bone marrow transplantation, Meyers, Flournoy and Thomas (1980) found that HCMV proliferative responses were suppressed and recovery of the response was related to the occurrence of active HCMV infection.

The nature of the antigen used by different authors has varied. Starr et al (1980) investigated both HCMV infected WI38 cell sonicates and gradient purified HCMV virions of three different HCMV strains. Lymphocytes from seropositive individuals proliferated in response to both the purified and crude antigen and no difference between the HCMV strains was observed. In addition they noted that crude antigen sonicates were able to induce pH2 stable IFN from both seropositive and seronegative PBMC but the purified inactivated antigen could only induce pH2 unstable IFN from PBMC of seropositive subjects.

The response to 'free' HCMV antigens has also been compared with the proliferative response to HCMV infected fibroblasts (Schrim, Roenhorst and The, 1980). In these studies the fibroblasts were infected with HCMV at an MOI 1:1, for 4d prior to mitomycin C treatment, therefore, predominantly HCMV LA were probably expressed. A stronger proliferative response with HCMV infected fibroblasts than with cell free virus was observed (this was in contrast to Moller-Larsen et al (1977)) but there was no correlation between cft antibody level and proliferative response. The proliferative response was dependent on fibroblast/stimulator ratio, dose of infecting virus, duration of culture (optimum was 6d) and was
reduced by heat treating the stimulator cells. Although the authors suggested that different populations of lymphocytes were responding against the antigen preparations no analysis of the nature or function of responding lymphocytes was performed.

Cell mediated cytotoxicity of HCMV infected cells was first studied by Thong et al (1976) using a $^{51}$Cr release assay with cryopreserved HCMV infected target cells (expressing predominantly IA). These studies together with the observations of Rola-Pleszczynski et al (1977) were probably observing non-specific or NK lysis as no other virus infected cells were used as controls, and the HCMV infected fibroblasts and lymphocytes were not HLA matched.

Sethi, Stroehman and Brandis (1980) studied HCMV and HSV specific, HLA class I restricted Tc from patients with recent episodes of virus reactivation. PBM from 3 patients with recent HCMV infection were cultured with UV inactivated HCMV (AD169) (MOI equivalent to 0.1) for 5d and T cells seperated. These were then cultured in IL2 at $10^5$ cells/ml and after 15 to 45d in vitro used in in 4h $^{51}$Cr release assays with 8h HCMV infected HLA matched fibroblasts. High levels of MHC-restricted lysis were observed in the cultured T cells in addition to non-specific lysis.

Quinnan et al (1981;1982;1984) used HLA typed cryopreserved HCMV infected fibroblasts as target cells matched at one or more loci to PBM, obtained from bone marrow transplant recipients. By directly measuring HLA-restricted cytotoxicity of HCMV infected cells this group has suggested that the development of such cytotoxic cells in PBM, correlated
with favourable outcome following HCMV infection in bone marrow transplant recipients (Quinnan et al, 1981; 1982; 1984) and renal transplant recipients (Rook et al, 1984). Furthermore, reduced HLA-restricted cytotoxicity was also found in AIDS patients who were shedding HCMV (Rook et al, 1983; 1985).

Using this technique however, Tc killing HCMV infected cells have not been found in PBM of normal seropositive individuals. In addition the nature of the assay - using high effector:target ratios and direct lysis by PBM has prevented detailed characterization of the effector cells. HLA restricted cytotoxicity of HCMV infected cells was mediated by $E^+$ cells although exceptions were observed (Quinnan et al, 1982). The virus specificity of the responses described has not been established, as no other virus infected target cells have been used. Furthermore, the interpretation of the results must be cautious as all the patient groups studied were immunosuppressed, therefore the failure to develop Tc as measured by this assay could reflect a general lack of immune responsiveness rather than implying a specific role for such Tc in recovery from infection. Tc activity against virus or class I MHC antigens by MLC was not measured, and Con A proliferative responses were lower in the patients who died following HCMV infection as compared with the survivor group - stimulation index 2.0 (+/-1.3) vs 5.1 (+/-1.4) respectively (Quinnan et al, 1982).
1.3.6 Natural killer cells

In addition to virus specific T cell responses described above a number of non-virus specific responses are activated following virus infection, including NK cells, which have the capacity to kill HCMV (Starr and Garrabrant, 1981; Kirmani et al, 1981) and other virus infected cells in vitro.

These lymphocytes are functionally defined by their ability to kill virus infected and tumour cells in vitro without requirement for MHC restriction or previous exposure to the antigen. The cells mediating this activity have proved difficult to characterize but Timonen, Ortaldo and Herberman (1981) identified them as large granular lymphocytes (LGL) - slightly larger than resting PBL with a reniform nucleus and characteristic azurophilic granules on 10% Giemsa staining (lymphocytes with this morphology were first described by Pappenheim and Ferrata (1911)). They were present in the 'null' fraction of PBM (lacking characteristic T and B cell markers) (Ferranni et al, 1980; Roder, Karre and Kiessling, 1981), but could be separated by density gradient centrifugation (Timonen and Saksela, 1980; Timonen, Ortaldo and Herberman, 1981) and by adherence to target cells (Timonen and Saksela, 1978; Timonen et al, 1979a; 1979b; Saksela et al, 1979). These lymphocytes express surface Fc receptors for IgG, and low levels of E receptor. Studies with T cell and monocyte monoclonal antibodies have shown variable expression of a number of other markers e.g. T11 (E receptor), T3, T8 and OKM1 making their assignment to a particular lineage difficult.
In addition a number of monoclonal antibodies have been developed against surface determinants present on these cells - HNK-1 (Leu 7) originally produced against HSB-1 cells, identifying a structure on the surface of LGL (Abo and Balch, 1981). This antigen is not restricted to LGL but is also present as myelin-associated glycoprotein, a component of peripheral and central myelin sheaths thought to be important in cell-cell interactions within the nervous system (McGarry et al, 1983; Kruse et al, 1985). In addition anti-Leu 11 antibodies which recognise the Fc receptor for IgG also define this subset of lymphocytes (Lanier et al, 1983); considerable heterogeneity of function has been observed when using these antibodies to select cell populations (Lanier et al, 1983; Abo and Balch, 1982; Abo, Cooper and Balch, 1982). The ontogeny and lineage of human NK cells is also unknown, although they are bone marrow in origin but they do not appear to require thymic processing (Roder, Karre and Kiessling, 1981).

NK cytotoxic activity is enhanced by a number of factors notably interferon (IFN) (Trinchieri and Perrusia, 1985) and interleukin 2 (IL2) (Trinchieri et al, 1984). As NK cells themselves are capable of producing large amounts of α-IFN or β-IFN in immune individuals, this may enable LGL to self regulate NK cytotoxicity following virus challenge (Djeu et al, 1982). IL2 enhanced NK cytotoxicity directly, but NK cells can be grown in vitro in IL2 culture. When NK cells, from the null fraction of peripheral blood were cultured, they retained their non-MHC restricted
cytotoxicity but acquire T3 and other T cell markers (Timonen et al, 1982). Such human \textquoteleft NK lines\textquoteright have been cloned and the functional activity of these clones studied. Although some of these clones killed a number of different tumour cell targets there was a degree of target cell selectivity; some \textquoteleft NK clones\textquoteright also mediated ADCC (Hercend et al, 1983; Allavena and Ortaldo, 1984).

Interpretation of these results is not straightforward, as Brooks (1983) reported that if in vitro conditions under which murine MHC restricted Tc clones were maintained were altered, e.g. by adding IFN to the conditioned medium or increasing the concentration of IL2, Tc clones lysed other target cells in a non-MHC restricted manner. Moreover, Tc clones maintained in long term culture developed into non-specific cytotoxic cells indistinguishable from \textquoteleft NK\textquoteright cells (Hengartner et al, 1982; Binz et al, 1983) or changed their specificity (Simon et al, 1984). Thus the exact relationship between Tc, \textquoteleft NK clones\textquoteright, \textquoteleft activated NK cells\textquoteright and NK cells directly from PBM has yet to be established. In this context it is interesting that whereas in NK clones the \( \beta \) gene of the T cell receptor is rearranged, no \( \alpha \)-chain rearrangement is observed thus surface expression of the T3-Ti complex does not occur (Ritz et al, 1985).

Both Tc and NK cells kill target cells by degranulation and insertion into the target cell membrane of protein channels (Podack, 1985). Therefore one major difference between Tc and NK lysis of virus infected cells is the nature of the target structure(s) recognised.
on the infected cell. The nature of the NK target structure(s) is unknown although several candidates have been proposed. Initially the ability of NK cells to kill tumour and virus infected cells suggested that a retroviral glycoprotein may be the NK target structure. However, non-retroviral tumour cell lines were readily lysed (Roder, Karre and Kiessling, 1981) and viral glycoproteins, with the possible exception of HSV (Bishop, Glorioso and Schwartz, 1983), are unlikely to be direct NK target structures. Subsequent observations have focused on differences between NK susceptible and insusceptible tumour cells, and numerous target structures e.g. foetal antigens, surface sugars (Stutman et al, 1980), glycolipids (Young et al, 1981) and the transferrin receptor (Vondinelich et al, 1983) have been proposed.

Previous studies of NK mediated lysis of HCMV infected cells had shown that this lysis was mediated by non-B, predominantly non-T, Fc receptor bearing cells, was not HLA restricted and could be enhanced by pretreating the effector cells with IFN (Starr and Garrabrant, 1981; Kirmani et al, 1981). In addition NK activity as measured by lysis of the standard tumour cell target K562, was shown to be depressed in bone marrow transplant recipients who had fatal HCMV infection (Quinnan et al, 1982). Again whether this observation represents a measure of generalised immunosuppression in those who developed widely disseminated HCMV or a defect which allowed dissemination of the infection is impossible to determine.

The importance of the role of NK as opposed to specific T cell
immunity with respect to virus infection remains unknown. NK activity, measured in vitro, is rapidly increased following infection, often associated with increased IFN release which in turn augments NK activity. It is possible that these cells may serve to protect the host against acute virus challenge before specific Tc, Td and antibody responses develop, as might be suggested by their protective role in MCMV infection (see below). However this protection alone is imperfect, as virus infection occurs in the face of NK activity. In addition to their cytotoxic activity a number of different immunoregulatory roles have also been suggested for these lymphocytes including IFN release (Trinchieri and Perussia, 1985) and suppression of immunoglobulin synthesis (Tilden, Abo and Balch, 1983). Thus like Tc, their protective role in vivo may not necessarily depend on direct cytotoxicity.

1.3.7 Immune response to murine cytomegalovirus infection

The failure of antibody to protect the host from CMV infection was first suggested by Andrewes (1931) who found that injection of immune serum in the contralateral flank of guinea pigs, following subcutaneous inoculation of CMV, did not inhibit the development of salivary gland lesions. Although GCMV has certain homologies with HCMV infection in man (Bia et al, 1983), the immune responses to CMV in vivo have been investigated in more detail in the mouse.

The development of cell mediated and antibody responses following intranasal inoculation with MCMV have been described by Quinnan,
Manischewitz and Ennis (1980). IFN was increased within 24h following intra-peritoneal infection and increased levels of splenic NK activity were detected from 3-6d post-infection (Quinnan and Manischwitz, 1979; Quinnan, Manischewitz and Kirmani, 1982). Tc were present in draining cervical lymph nodes 5d after infection and preceded the development of significant levels of neutralising MCMV antibodies (Quinnan and Manischewitz, 1979; Quinnan, Manischewitz and Ennis, 1980). The generation of MCMV specific Tc following primary infection with MCMV was described by Quinnan, Manischewitz and Ennis (1978) and partly confirmed by Ho (1980). Starr and Allison (1977) showed that adoptive transfer of T cells from the spleen of immunised mice protected naive recipients as judged by a reduction of virus titre in the spleen of recipients. Ho (1980) generated MCMV specific Tc, by secondary in vitro stimulation using UV irradiated MCMV infected autologous fibroblasts as stimulator cells, and these cells could protect against virus challenge on adoptive transfer. Interestingly, in this study he was also able to show that MCMV suppressed the development of ectromelia specific Tc in vivo and secondary Tc responses in vitro.

MCMV Tc have also been shown to be virus stage specific. Reddehase and Koszinowski (1984), Reddehase, Kiel and Koszinowski (1984a; 1984b), detected a high precursor frequency of MCMV-IEA specific Tc in the popliteal lymph nodes of mice following footpad inoculation with virus. In addition to these Tc which did not require secondary in vitro stimulation, memory Tc which recognised LA expressing target cells were
also present. Not only may such cells have a protective role against MCMV infection as determined by reduction of virus titres, they may be important in limiting spread of MCMV infection in vivo.

Following MCMV infection by footpad inoculation, mice were irradiated and interstitial pneumonia was evident both histologically by in situ hybridisation to MCMV DNA, and by the isolation of high virus titres from the lung. Transfer of $10^4$ cells from lymph nodes of immunised recipients reduced virus titres in the lung even 24h following infection, provided that Lyt 2+ were not depleted (Reddehase et al, 1985).

Tc are not the only cell mediated response generated following MCMV infection, although they remain the best characterized. Chong and Mims (1982) described DTH in response to MCMV. The response was greater to attenuated than 'wild' virus challenge and was reduced in pregnancy. Secondary challenge with live virus was associated with greater swelling and no virus was recovered from the inoculation site. Furthermore, they have subsequently shown that adoptive transfer of Thy-1+ spleen cells from mice previously infected with live MCMV to mice infected sub-cutaneously, resulted in an antigen specific suppression of the DTH response (Chong and Mims, 1983). However, direct transfer of protection has not been demonstrated and the effector cells have not been fully characterized. It thus remains possible that such DTH may be mediated by Tc, as cloned influenza specific Tc were able to mediate DTH to influenza in vivo (Lin and Askonas, 1980).
In addition NK cells also have a protective role in MCMV infection. Natural resistance of different strains of mice to MCMV directly correlated with their ability to secrete IFN and NK activity (Shellam et al, 1983). When mice were treated in vivo with anti-asialoGM1 antibody, NK activity was reduced and these mice were more susceptible to MCMV (Bukowski, Woda and Welsh, 1984). In addition transfer of NK depleted spleen cells failed to protect these mice but a cloned NK line transferred into syngeneic mice protected against MCMV challenge (Bukowski et al, 1985). These cells may provide protection during acute virus challenge and it should be noted that large doses of MCMV injected intraperitoneally were used in these studies in contrast to the studies of MCMV Tc where subcutaneous or intranasal challenge was often used.

1.4 AIMS AND SCOPE OF THE STUDY

The studies described were performed with the ultimate aim of determining the nature and role of cell mediated immune responses to HCMV, to identify the immune defects associated with increased susceptibility to endogenous HCMV infection in IS patients. However, to approach this problem a prerequisite was an understanding of the mechanisms involved in maintaining the virus/host relationship in normal asymptomatic persistently infected individuals and in particular the role of the immune response in maintaining this equilibrium.
This study investigated several questions:

1) MCMV specific Tc were present in vivo following primary infection (Quinnan, Manischewitz and Ennis, 1977) and secondary MCMV specific Tc could be generated in vitro. These Tc were protective against virus challenge (Ho, 1980) and similar mechanisms may be important following HCMV infection in man. Other human virus specific Tc, notably influenza, were identified following acute virus infection but declined in the absence of re-challenge (McMichael et al, 1983). Tc specific for another human herpesvirus - EBV - remained at a high precursor frequency in seropositive individuals (Rickinson et al, 1981; Moss et al, 1981; Yao et al, 1985). In addition Quinnan et al, (1981; 1982; 1984) directly and Sethi, Stroehman and Brandis (1980) by secondary in vitro stimulation, identified HCMV and HSV specific Tc activity following clinical infection. If HCMV specific Tc were important in limiting endogenous HCMV infection, then as for EBV, precursor Tc may be present in PBM of seropositive individuals. Alternatively, it was possible that the persistence of EBV specific Tc could be unique, either because of the particular tropism of EBV for B cells or because EBV transformed B cells and the Tc may be related to a transformation-linked event, therefore not necessarily applying to a lytic human herpesvirus such as HCMV. These investigations were performed to investigate:

a) whether precursor HCMV specific Tc were present in clinically asymptomatic seropositive individuals.
b) the conditions required for secondary in vitro stimulation of these cells.

c) the surface phenotype, MHC restriction, virus specificity and in particular the nature of the HCMV antigens recognised by Tc.

2) HCMV infected cells were lysed by NK cells and this interaction was investigated to determine the nature of the susceptibility of HCMV infected cells to NK lysis. This included:

a) identification of effector lymphocytes mediating NK lysis of HCMV infected cells.

b) the relationship between NK cells killing HCMV infected and tumour cells.

c) the role of IFN in the interaction of NK cells with HCMV infected cells.

d) the nature of HCMV induced susceptibility to NK lysis.

e) identification of the target structure(s) recognised on HCMV infected cells.
2.1 SUBJECTS

96 different subjects participated in various aspects of these studies and relevant details of HCMV status, HLA type etc. are referred to in Results. A number assigned to a subject has been retained in the T cell studies described to allow direct comparison with different investigations. HCMV serological status was determined using the cft (Krech, 1973; Booth et al, 1979) by the Dept. of Virology, Hammersmith Hospital and seronegative status confirmed by a more sensitive enzyme linked immunoassay (ELISA) (Burroughs, 1984). HLA typing of PBM was performed by Prof. R. Batchelor, Dept. of Immunology, RPMS.

2.2 MEDIA

2.2.1 Tissue culture media

In the experiments described three different culture media were used:

i) RPMI 1640 (Flow) supplemented with 2mM L-glutamine, penicillin 100,000 IU/l, streptomycin 100 mg/l and either 10% foetal calf serum (FCS - Flow Laboratories or Seward Laboratories) (RPMI) or 10% HCMV
seronegative human AB serum, prepared as described below, for the establishment and maintenance of T cell lines and clones, K562, Molt 4, CEM-6 and CTL-D lines. The FCS concentration was occasionally modified to support maximum growth of the established lymphoid lines.

ii) Eagle's minimal essential medium (MEM) (Gibco) supplemented as for RPMI but with 1% nonessential amino acids (Gibco), for the establishment and maintenance of primary explant human fibroblast lines.

iii) Basal medium with Earle's salts (BME) (Flow Laboratories), supplemented as MEM, for culture of MRC-5, Flow 5000, Flow 2000, Hep-2, Vero, Vero-6 and with 15% FCS for the establishment and maintenance of guinea pig fibroblast cultures.

In addition serum free medium, phosphate buffered saline (PBS) (Difco PBS-A) or PBS supplemented with 2%FCS was used to wash cells etc. as detailed in individual experiments.

2.2.2 Foetal calf and human HCMV seronegative AB serum

Mycoplasma free, heat inactivated (56°C for 30 min) FCS aliquots were tested for their ability to maintain growth of established and primary human fibroblast and lymphoid lines, phytohaemagglutinin (PHA) (Sigma - purified) induced lymphocyte proliferation and support HCMV infection in fibroblasts in the plaque assay, prior to use.

Heat inactivated serum from 5 normal AB+ HCMV seronegative volunteers was tested for its ability to support maximum PHA (2μg/ml final concentration) induced proliferation at 48h. Serum from subject 9 was used
in subsequent experiments. 250ml aliquots were prepared, heat inactivated (56°C for 30min) and stored at -20°C until use. Batches were comparable in their ability to support maximum PHA induced proliferation, and mycoplasma free by direct culture.

2.2.3 Preparation of rabbit complement

Young rabbits (<6 months) were bled and the blood allowed to clot on ice. The serum was separated by centrifugation at 4°C and incubated for 45min with 10^7 Molt 4 cells/ml and 10mg ml^-1 agarose (Miles) (Mishell and Shiigi, 1980). The agarose and cells were removed by centrifugation at 4°C, the serum aliquoted into 1ml volumes and stored in liquid nitrogen until use. Each batch of complement was checked for non-specific lysis and generally used at a dilution of 1/8.

2.3 ESTABLISHMENT AND MAINTENANCE OF FIBROBLAST AND OTHER CELL LINES

2.3.1 Establishment of primary HLA defined fibroblast lines

HLA defined human fibroblast lines, for use as stimulator and target cells in MHC restricted cytotoxicity assays, were established by primary explant culture from subjects of known HLA type. Having obtained informed consent, a 3-5mm^2 skin biopsy was taken from the upper arm under 1% lignocaine anaesthesia. The biopsy was subdivided into 4-5.
smaller fragments, placed in 35mm petri dishes (Falcon) and allowed to adhere. 2ml MEM was added to the dish which was then incubated at 37°C in 5% CO₂ in air. Outgrowth of epithelial cells was observed after 5-6d expanding around the biopsy, followed by outgrowth of fibroblasts after about 10-14d. The cultures were refed at weekly intervals and confluent layer of fibroblasts was obtained after 8-10 weeks culture.

Difficulties were encountered using this system particularly desiccation of the biopsy during the adherence step and detachment of the explant. Because of these problems, only 6 lines were established from 28 donors.

The technique was modified and a sterile square 22mm glass coverslip (Chance) was placed over the biopsy, medium introduced under the coverslip by capillary action and then 2ml medium added to the petri dish. This overcame the previous problems but in addition outgrowth of cells was faster and petri dishes were often confluent after 6 weeks. Interestingly the fibroblasts often grew attached to the undersurface of the coverslip rather than on the petri dish, which aided passage of the cells. Following these modifications 29 fibroblast lines were established from 32 biopsies; 3 biopsies were lost because of fungal infection. A total of 56 such primary fibroblast lines have now been established.

Confluent petri dishes were passaged into 25cm² and 75cm² tissue culture flasks (Nunc; Falcon). Aliquots of cells were screened for mycoplasma contamination by DNA staining (Chen, 1977) and direct culture,
before the cells were stored in liquid nitrogen between passage 4 to 12. All experiments were performed with cells of passage 16 or less, although cell lines could be propagated beyond passage 25.

2.3.2 Passage of adherent cells

Confluent monolayers of fibroblasts or other adherent cells were passaged by removing the medium, washing the cell layer with PBS and then incubating in trypsin/EDTA in PBS (Gibco) for 5-15 minutes at 37°C. A single cell suspension was divided 1:2 for primary fibroblasts or 1:4+ for established cell lines.

2.3.3 Cryopreservation of cells

Adherent cells were trypsinised as described, washed in PBS and resuspended in cold (4°C) 'freezing' solution at 10^6 cells/ml (>95% viable). Cells grown in suspension culture were washed in PBS and suspended in 'freezing' solution at 10^7/ml. 1ml aliquots of the cells were dispensed into freezing vials (Nunc), placed in polystyrene containers at -70°C for 18-24h and then transferred to the liquid or vapour phase of liquid nitrogen. To reestablish cultures cells were rapidly thawed to 37°C, excess dimethylsulphoxide (DMSO) (BDH) removed by washing in medium and cultured as required.

The optimum concentration of serum and DMSO in the freezing solution on different cells was studied by freezing cells as described, thawing 5 days later and examining cell yield, viability and proliferation.
(Table 1). A 'freezing' solution containing 10% DMSO, 50% FCS and 40% medium was subsequently used.

2.3.4 Screening for mycoplasma contamination

All cell lines and virus stocks were screened for mycoplasma contamination by DNA staining (Chen, 1977). Adherent cells to be stained were grown on coverslips in 35mm petri dishes, and when confluent were fixed without removing medium in Carnoy's fixative (3:1 v/v methanol:glacial acetic acid). After 2 min the medium was decanted and 5ml fresh fixative added for 5 min at room temperature. The cover slips were air dried and stained with Hoechst 33258 (0.05ug ml⁻¹ in balanced salt solution without phenol red pH7.0 (Gibco)), rinsed in distilled water and mounted in 10% PBS-glycerol prior to examination for mycoplasma by UV microscopy. Suspected mycoplasma contaminated fibroblast lines and suspension cell cultures were examined by direct mycoplasma culture (Flow).

2.3.5 Establishment of guinea pig fibroblast lines.

Guinea pig embryo fibroblasts were prepared from asceptically removed from the uterus and disrupted, 30day embryos. The fragments were incubated in 0.25% trypsin/EDTA solution for 30min at 37°C on a magnetic stirrer, allowed to stand for 10min and the supernatent collected. This was washed, cells resuspended in BME and allowed to adhere. Adult guinea pig fibroblasts were grown by primary explant culture.
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\( ^a \) - % by volume of freezing solution; M = RPMI (serum free).

\( ^b \) - \( 10^6 \) PBM and \( 10^5 \) MRC-5 cells were frozen, thawed after 48h and cell number/viability (expressed as \( x10^6 \) cells or % of input cell number) determined.

\( ^c \) - proliferative response to \( 2\mu g \text{ ml}^{-1} \) PHA determined for \( 2x10^5 \) viable cells and % of cpm compared with proliferation of PBM from the same individual in parenthesis (cpm \( x10^4 \)). SD <5% of cpm in all cases.

\( ^d \) - NK activity determined at E:T = 100:1 (viable cells) against K562 and compared with NK activity of PBM from the same subject (% of control).
from heart tissue. These cells were used in experiments at passage 3-5.

2.3.6 Maintenance of established cell lines

MRC-5 and Flow-5000 (F5000) (Flow Laboratories), human embryonic lung fibroblasts were maintained in BME either in 175 cm$^2$ flasks or in 500 ml roller bottle cultures. These cell lines were used to propagate virus stocks, plaquing etc and were used between passage 22-30 and 14-28 respectively. K562 cells - a human erythroleukaemia line (from Dr P. Ewan, St. Mary’s Hospital); Molt 4 - a T cell lymphoma line (from Prof. R. Batchelor), and CEM-6 (from Dr. D. Scott) were grown in suspension at 0.5-2x10$^6$/ml in RPMI 1640. CTL-D cells (from Dr. B. Roser), an IL2 dependent murine cytotoxic T cell clone, was maintained at 2.5-10x10$^4$/ml in RPMI 1640 further supplemented with 2mM 2-mercaptoethanol in the presence of exogenous IL2.

2.4 VIRUSES

2.4.1 Propagation and plaque purification of HCMV

HCMV, strain AD169, seed stock was purchased from the American Typed Tissue Culture Collection (Cat No. VR-538), grown in MRC-5 cells and harvested when 80-90% cpe was present in the monolayer. The cells were sonicated and aliquots of virus stored in liquid nitrogen. The infectious virus titre was determined (see below). Monolayers of MRC-5 cells were
infected at $\log_{10}$ dilutions and grown under 0.5% agar/medium. After 10-14 days culture when distinct plaques were present, individual plaques were harvested by aspiration through the agar layer. This was inoculated on to a further monolayer of cells and the procedure repeated.

The twice plaque purified HCMV AD169 stock had CPE typical of HCMV and infected cells stained with monoclonal and polyclonal anti-HCMV antibodies (Fig 3). The virus was then propagated by inoculation at low multiplicities of infection (MOI) - 0.01 pfu/cell and harvesting the monolayer of cells as described above. Titres of stocks prepared in this way varied from $5 \times 10^5$-10$^7$ pfu/ml.

In order to improve virus yield and to examine whether supernatents from infected monolayers contained significant quantities of infectious HCMV, a number of fibroblast lines were tested for their ability to release infectious virus (Fig 4). There were marked differences between fibroblast lines; because of their ready availability and rapid growth, F5000 fibroblasts were routinely used, infected in roller bottle cultures (MOI 0.01:1) and supernatent collected from 7 to 11d post-infection, aliquoted and stored at -70°C or liquid nitrogen until use. As shown in Fig 4 adequate levels of HCMV were obtained from the supernatent and this rather than sonicated cell suspensions was used particularly in later experiments. No sorbitol or other agent was added to the virus, as a stock was used usually within 3 months; when tested virus titres fell by approximately one log unit in 9-12 months storage at -70°C but there was no reduction in liquid nitrogen over 1y.
Fig. 3

a)

b)

c)
Fig 3. Expression of IEA, EA and LA in HCMV infected cells. Fibroblasts were infected with HCMV as described (Section 2.4.3) and then examined by indirect/direct immunofluorescence using monoclonal antibodies E3 (IEA), H11 (EA), H9/2 (EA), H7/12 (LA) and polyclonal human FITC-F(ab)_2 (LA).

a) uninfected fibroblasts did not stain with any of the antibodies - cells stained with E3 and peroxidase conjugated goat anti-mouse Ig (x400).

b) fibroblasts infected with HCMV and treated with CHX and AD as described only showed HCMV IEA - shown stained with E3 and peroxidase conjugated goat anti-mouse-Ig (x200).

c) fibroblasts infected with HCMV in the presence of PPF showed similar distribution of E3 as in b) but there was no staining with the LA antibodies. Cells are shown stained with H11 and FITC-goat anti-mouse Ig (x400).

d) fibroblasts infected with HCMV for 4 days showed similar staining with E3 (as in b)) and H11 (as in c)). In addition (i) they expressed EA as shown with H9/2 and peroxidase goat anti-mouse Ig (x400); (ii) LA as shown with H7/12 and peroxidase goat anti-mouse Ig (x400) and (iii) polyclonal FITC-F(ab)_2 (x400).
Fig 4. Production of HCMV by different fibroblast lines.

a) Primary fibroblasts F43 (●), F3 (▲) and MRC-5 (■) were infected with HCMV (MOI 5:1). Samples of supernatent medium (—) and cell layers, disrupted by freeze-thawing (x3) (---) were sequentially harvested and plaqued on F5000 fibroblasts. HCMV was released into the medium after 48-72h culture but there was a marked difference in release of virus between different fibroblasts. In addition F43 cells were infected and maintained in presence of PPF at 200µg ml⁻¹, the PPF washed off, cells harvested, disrupted and then plaqued on F5000 cells. At this concentration there was inhibition of HCMV replication as measured by infectivity (.....).

b) F5000 cells infected with HCMV (MOI 0.1:1) were used to produce virus stocks (batch 1 (●); batch 2 (◆)) by harvesting supernatent from cultures from 6-10d post infection. The peak release of infectious virus is delayed at MOI=0.1:1 compared with MOI=5:1.
2.4.2 HCMV plaque assay

HCMV infectivity was determined by a modification of the plaque assay described by Wentworth and French (1971). Confluent monolayers initially of MRC-5 cells in 35mm petri dishes but later modified to F5000 cells in 24 well plates (Falcon), were inoculated, in triplicate, with 200ul (35mm PD) or 100ul (24 well plate) of sequential log_{10} dilutions of virus stock. The virus was allowed to adsorb for 1h at room temperature and then overlaid with equal volumes of 2xBME, prewarmed to 37°C and 1.0% aqueous agarose (95200-4 Miles) dissolved in a boiling water bath and cooled to 45°C. The cells were incubated at 37°C in 5% CO₂ in air for 6 days, then a second layer of medium and agar was applied followed by a further 6 days incubation. When easily distinguishable plaques were present (usually 12-16d), the cells were fixed in 10% formalin in PBS for 1h, overlays removed and the cell layer stained with 0.03% aqueous methylene blue for 1h. Excess stain was removed and the plates allowed to dry before counting the plaques with the aid of an inverted microscope (x40 magnification).

2.4.3 Preparation of cells expressing HCMV IEA

Fibroblast monolayers were infected with HCMV at MOI 20:1. Cells expressing HCMV LA were prepared by allowing the fibroblasts to grow for 4 days. Formalin and Bouin´s solution fixed cells showed typical cytomegally with characteristic nuclear and cytoplasmic inclusions (Albrecht et
Infectious virus was released from these monolayers, >90% of the cells stained with polyclonal F(ab)_2 and monoclonal antibodies detecting the 67K major structural (late) protein (Fig 3), in addition to antibodies against early and immediate early antigens. These cells were also lysed by polyclonal rabbit anti-HCMV serum and complement (Fig 5) and they expressed Fc receptors for IgG. These cells, as did uninfected and HCMV EA and IEA expressing fibroblasts, expressed class I MHC antigens but no class II antigens, as detected by W6/32 (class I) and IR2, I2 and anti-DR (class II).

Cells expressing HCMV EA and IEA only, were prepared by infecting fibroblasts as above but in the presence of 200μg ml⁻¹ PPF (Sigma) (Stinski, 1983; Wahren and Oberg, 1980). Release of infectious virus was inhibited at 200μg/ml (Fig 4) and in these cells HCMV IEA and EA antigens but not LA were detected (Fig 3). Morphologically these cells were rounded in culture but the characteristic nuclear and cytoplasmic inclusions were absent.

Enhanced expression of HCMV IEA alone was achieved by infecting cells as described above but in the presence of 400μg ml⁻¹ CHX (Sigma) to block translation and after a further 3-6h incubation at 37°C, the cells were washed free of CHX and incubated for a further 12h in medium containing 2μg ml⁻¹ AD (Sigma) to block further transcription but allow translation of accumulated message (Stinski, 1977; Jeang and Gibson, 1980). These cells did not release infectious virus and no late or early antigens were detected, but the 72K
Fig 5. Complement and antibody mediated lysis of HCMV infected fibroblasts. 4d HCMV infected and uninfected ⁵¹Cr labelled F29 fibroblasts were incubated with antibody (45min at 4°C) and C (20min at 37°C) as indicated. Only HCMV infected cells were lysed by adsorbed polyclonal rabbit anti-HCMV and complement.
immediate early protein was expressed in the nuclei of these cells (Fig 3). Morphologically no difference was discernable between these and similarly treated uninfected cells.

In addition, time course experiments were performed. Following infection at MOI 10:1, further transcription was blocked by treating the cell layer with AD after the required incubation period. This technique was also employed to prevent cross infection in cold target competition studies using HCMV infected and uninfected cells.

2.4.4 Preparation of isolated HCMV virions

Virus particles were purified from supernatant of infected cells as described by Stinski (1977). Supernatant from F5000 cells, infected with HCMV at MOI 0.1:1 and allowed to grow until 80-90% cpe was observed, was harvested and clarified at 10,000g for 30 min on a Sorvall GSA rotor. The clarified supernatant was underlayed with a 5ml cushion of 20% D-sorbitol in 0.05M Tris HCl, pH 7.2, and 0.001M MgCl₂ and then centrifuged at 70,000g for 1h. The pellet was resuspended in 0.05M Tris HCl, pH 7.4, 0.15M NaCl and 100ug/ml bacitracin. Urea was added to a final concentration of 0.5M and the mixture sonicated for 10sec. This preparation was then layered on to a 6 stage discontinuous D-sorbitol gradient in 0.05M Tris HCl, pH 7.2, 0.001M MgCl₂, 100ug/ml bacitracin from 20-70%, allowed to equilibrate overnight. The gradient was spun at 61,400g for 1h in a MSE 65, 6 x 5.5ml swing out rotor and 400ul fractions collected from the bottom of the tubes. Intact virus particles as observed
by electron microscopy and $^{35}$S labelled virus was obtained in fractions 3, 4 and 5 - equivalent to 55-60% D-sorbitol.

2.4.5 HSV

Herpes Simplex type I (HSV) (from Dr C. Lancashire, RPMS), Semliki Forest Virus (SFV) (from Dr. K. Apostolov, RPMS), measles virus - Edmonston strain (from Dr P. Casalli, Scripps Inst., La Jolla) and tissue culture adapted vaccinia (from Dept. of Virology, RPMS) were grown in Vero cells and supernatents of infected monolayers collected. 1ml aliquots were stored at -70°C. The viruses were plaqued on Vero cells using techniques (Lennette and Schmidt, 1979) with titres (pfu ml$^{-1}$) of $10^9$-HSV, $10^{10}$-SFV, $10^7$-measles and $10^9$-vaccinia. Live poliovirus vaccine (strains A,B and C - Wellcome) was used directly - pfu equivalent to $10^6$ ml$^{-1}$.

2.5 ISOLATION AND PURIFICATION OF PBM AND PBM SUBSETS

2.5.1 Preparation of PBM

Blood samples were anticoagulated with 50IU/10ml blood, preservative free heparin (Evans). Blood was diluted 1:2 with sterile PBS and PBM were isolated by density gradient centrifugation on Ficoll/Hypaque in conical 30ml universal containers (Sterilin) (Hudson and Hay, 1980). Interface cells were harvested and washed in PBS or PBS supplemented with 2%FCS, three times prior to use. Polymorph contamination was <5% when
May-Grunwald Giemsa stained cytospin preparations were examined.

2.5.2 Removal of adherent cells

PBL were prepared from PBM by removal of adherent cells using plastic adherence, nylon wool columns and G10 columns. PBM were suspended in serum free RPMI at $2 \times 10^6$/ml and 15-20ml pipetted into sterile non-tissue culture grade, 100mm petri dishes (Sterilin). The plates were incubated at $37^\circ C$ for 1hr and non-adherent cells removed by vigorous pipetting. Additional removal of adherent cells was achieved by incubating $2 \times 10^7$ adhered PBM/ml in RPMI at $37^\circ C$, on 10ml nylon wool (Travenol) columns (maximum of $10^8$ PBM/column). The nylon wool was previously washed with PBS, sterilised, washed and preincubated with RPMI and 10%PCS for 1h at $37^\circ C$ prior to use. The non-adherent PBL were eluted with RPMI, washed, resuspended at the same concentration and the procedure repeated (Julius, Simpson and Herzenberg, 1973). Following these preparative procedures T cells were relatively increased ($T_{ll}^+ = 72\%$ to $88\%$) but relative numbers of $T_4^+$ and $T_8^+$ cells were unchanged. Cells of $Mo_2^+$ phenotype were absent ($8\%$ to $<1\%$) and $Bl^+$ cells were also depleted. Monocytes detected by NSE staining were also markedly depleted ($10\%$ to $<1\%$).

Adherent cells were also depleted by adherence to Sephadex G-10 (Pharmacia) (Mishell and Shiigi, 1980). G10 washed in PBS to remove finings, was autoclaved and packed in 10ml columns. PBM at $10^7$/ml were incubated on the columns for 1h at $37^\circ C$ and then eluted with
RPMI. Using this procedure B cell numbers were reduced in addition to monocytes (<1% NSE⁺), but T cell numbers were again increased with no relative change in T4⁺ and T8⁺ numbers.

2.5.3 E-rosette separation of T cells

Sterile SRBC in Alsever's solution (MCS) were washed three times in PBS and a 1% suspension of SRBC in RPMI with 20% SRBC absorbed FCS was prepared. This was added to pelleted PBM and resuspended to a final concentration of 10⁷ PBM/ml. The mixture was incubated at 37°C for 5 minutes, and centrifuged at 200g for 10 minutes. The pellet was incubated at 4°C for 1h, and resuspended in 5ml RPMI. This was layered on Ficoll/Hypaque and centrifuged at 250g for 40min. Interface cells were collected and the red cell pellet incubated on ice 5min, in fresh SRBC lysis fluid (8.29g/l ammonium chloride, 1.0g/l KHCO₃ and 0.2g/l EDTA to pH 7.4 with acetic acid). The E⁺ cells were pelleted and washed with PBS. The E⁺ cells were >95% T11⁺ but the E⁻ fraction was not entirely depleted of T11⁺ cells. Better depletion of E⁺ cells was obtained by repeating the procedure, overnight incubation of the SRBC/PBM mixture or use of AET treated SRBC (3ml of packed SRBC incubated with 0.5g AET in 12.5ml water at 4°C for 5 minutes) (Falkoff, Peters and Fauci, 1982).
2.5.4 Percoll gradient separation of lymphocytes

PBM depleted of adherent cells were separated on percoll gradients as described by Timonen, Ortaldo and Herbermann (1981). 2x10^8, plastic nonadherent, nylon wool passed PBL were placed on a 10ml discontinuous Percoll-PBS (Pharmacia) gradient of 9 fractions from 42.5% to 62.5% and centrifuged at 550g for 30min. 1ml fractions were harvested from the top of the gradient and washed in RPMI prior to use.

2.5.5 K562 rosetting and adherence depletion of PBL

PBL were separated into K562 adherent and non-adherent cells by culture with K562 cells (5:1), at room temperature for 2h, as described by Timonen et al (1979b). The cells were resuspended and layered on 17% Percoll-PBS and centrifuged (40g for 8min). Pelleted cells - K562 adherent PBL - were collected and the interface cells again cultured with fresh K562 cells. The pooled pelleted cells were vigorously pipetted to dissociate the conjugates, centrifuged (40g for 8min) on 10% Percoll-PBS. Both K562 adherent and non-adherent PBL were incubated overnight at 10^6/ml and then used in cytotoxicity studies.

Adherence of PBL to fibroblast monolayers was studied by incubating nylon-wool passed IFN pretreated PBL (10^7 PBL/confluent 35mm petri dish. Non-adherent PBL were removed by pipetting the surface of the fibroblast layer after 1h incubation at room temperature. The procedure was sequentially repeated as indicated in the text.
2.5.6 Depletion of lymphocyte subsets by antibody and complement lysis

1-2x10^7 adherent cell depleted PBM were incubated with 50ul of monoclonal anti-Leu 7a or Leu 11a (both murine IgM antibodies), for 45 min at 4°C. Numbers of fluorescent cells were checked by indirect immunofluorescence or flow cytometry and the remainder of the cells washed in PBS. Both populations of cells were incubated with the appropriate concentration of rabbit complement (see Section 2.2.3) for 30min at 37°C. The cells were washed free of complement and % viable cells determined. Non viable cells were removed by ficoll/hypaque centrifugation and % viability again determined. A further sample was examined by indirect immunofluorescence for the presence of positive cells.

2.5.7 Analysis and sorting using the fluorescence activated cell sorter

PBM were prepared and stained with monoclonal antibodies as described. The FITC-stained cells were then resuspended at 10^7/ml in RPMI and sorted on the EPICS C (Coulter Electronics) according to the manufacturer's protocols. The positive and negative populations to be sorted were identified by 'forward angle light scatter' (cell size) and 'green fluorescence' (GFL) as parameters when excited by 488nm light from a 5W argon laser. Positive and negative cells were simultaneously sorted at 2000 cells/sec using PBS as sheath fluid. The purity of sort was determined by reanalysis and UV microscopy of the collected populations.
2.6. INTERLEUKIN 2

2.6.1 Production of IL2 containing conditioned medium from tonsils

Tonsils were obtained following routine tonsillectomy, divided into 8-10 segments and incubated overnight in RPMI containing 200,000 IU penicillin and 200mg/l streptomycin. The tissue was disrupted by a coarse sieve and a 21G sterile syringe needle. Medium containing released lymphocytes was allowed to stand for 10min in a conical tube to remove larger debris and the supernatent cells were separated by ficoll hypaque. The interface cells were washed in PBS, counted and suspended at 2x10^6/ml in RPMI with 2% FCS, containing 2-4ug/ml PHA.

Interleukin 2 (IL2) activity was released into the medium after 12h incubation and maximum levels of IL2 were present after 36-48h. In order to improve IL2 yields various modifications of this protocol were investigated. Inoue et al (1980) suggested that partial depletion of adherent cells, low dose irradiation and addition of indomethicin to the culture enhanced IL2 release. The effect of these procedures was studied (Fig 6) and significant improvement noted when cells were irradiated (1000rad). Addition of phorbol myristate acetate enhanced PHA induced IL2 release at a final concentration between 10-15ng ml^{-1}. Irradiation was routinely employed but although phorbol ester enhanced IL2 release, this was not used routinely because of possible effects on other lymphokines released into the medium and later difficulties in removing the additive during purification.
Fig 6. The effect of adherent cell depletion, irradiation and indomethicin on IL2 production by tonsillar lymphocytes. Tonsillar lymphocytes were separated (□) or separated and depleted of adherent cells on plastic petri dishes (□). Aliquots of the cells were then irradiated (1kRad) (□) and incubated at 2x10^6 ml^-1 and 2ug ml^-1 PHA with or without 20ug ml^-1 indomethicin (indometh.) (MSD Pharmaceuticals). The supernatent was harvested and assayed for IL2 activity on TCL1 cells (+/- 1SD). Low dose irradiation of tonsillar lymphocytes enhanced IL2 production but not removal of adherent cells and addition of indomethicin alone.
2.6.2 Purification and delectination of conditioned medium

After 48h incubation with PHA, tonsillar lymphocytes were removed by centrifugation (1000g for 10min) and the supernatant collected. It was stored at -20°C and pooled into batches of 5l. The conditioned medium was precipitated sequentially in 50% and 80% saturated (NH₄)₂SO₄ (Welte et al, 1982). The precipitate from the 80% solution was resuspended in PBS, dialysed against three changes of PBS over 48h at 4°C, concentrated to 50ml using Aquacite (Calbiochem) and then recycled on an anti-PHA affinity column.

This was prepared by immunising rabbits with PHA in CFA (x4) restimulating the animals with PHA alone 5d prior to sampling. IgG was separated by sodium sulphate precipitation and DEAE cellulose (DE52 Whatman) ion exchange chromatography (Hudson and Hay, 1980). The IgG was coupled to Sepharose 4B (Pharmacia) using cyanogen bromide and its binding activity tested using ¹²⁵I labelled PHA. A 20 ml column with a binding capacity of 4-5mg PHA was prepared and the dialysed IL2 preparation recycled slowly for 24h at 4°C.

The PHA depleted IL2 was sterilised through a 0.22μ filter and 10ml aliquots stored at -70°C until use. PHA attatched to the column was removed with 0.2M glycine HCl (pH 2.8). The removal of PHA, as studied with trace-¹²⁵I-PHA, from IL2 preparations showed that the bulk of PHA was removed by (NH₄)₂SO₄ precipitation but following aquacite the concentration of PHA was again 2ug ml⁻¹. This
was efficiently removed (final concentration <0.1µg ml⁻¹) on the column. At the concentrations used in lymphocyte cultures, no PHA proliferative effect on unstimulated PBMC was observed.

For further purification the 80% ammonium sulphate precipitate was resuspended in 0.05M Tris HCl pH 8.0 and dialysed against 6 changes of buffer at 4°C. 70ml was applied to a 35ml DEAE cellulose (DE52-Whatman) column equilibrated with 0.05M Tris-HCl (pH7.8) at a flow rate of 15ml/h. The sample was eluted with a 0 - 0.3M gradient of NaCl in Tris buffer. IL2 activity was measured and the 7ml fractions with peak activity pooled and concentrated down to a final volume of 5ml using PEG 6000/PBS (50% w/v) (Welte et al, 1982)

A 4ml aliquot was then applied to a 2.5x100cm AcA54 (Pharmacia) column equilibrated with PEG 6000/PBS (0.1%). 5ml fractions were collected at a flow rate of 15ml/h and were assayed for IL2 activity. The column was calibrated using BSA (68kDa), chymotrypsinogen (25kDa) and ribonuclease A (14kDa) (Sigma). Peak activity was eluted in fractions between a molecular weight of 14-25kDa (Frank et al, 1981).

This material was further studied (S.Morris personal communication) by iso-electric focusing, and IL2 activity was present with an isoelectric point 6.7 and 7.2. Iodinated material from the AcA54 peak had 15K and 20-22K bands by SDS-PAGE.
2.6.3 Assays for IL2 activity

IL2 activity was measured by proliferation of activated T cells in vitro in the presence of varying concentrations of IL2 containing material. Three different cells were used in the assays:

a) a one way mixed lymphocyte culture was established by incubating responder cells with irradiated HLA mismatched stimulator cells (4krad) at 1:1 ratio. After 6d the cells were expanded by culture in the presence of 20% conditioned medium supplemented RPMI and an irradiated layer of stimulator cells. Medium was replaced at 3day intervals and fresh feeder cells at weekly intervals for 4 weeks. At this stage the T cells were unresponsive to PHA but responded to exogenous IL2 by proliferation. However, the magnitude of the proliferative response was variable even to the same batch of IL2, thus making comparison between assays difficult.

ii) to overcome this variability in response, 5x10^8 PBM were cultured in the presence of 2ug/ml PHA at 2x10^6 cells/ml. Exogenous IL2 was added every 3 days and after 10 days the viable cells were harvested and stored in 10^7 aliquots in liquid nitrogen. Although these cells were not entirely lectin unresponsive, reproducibility of assays was much improved (Gramatzki et al, 1982).

iii) murine CTL-D, IL2 dependant T cells

10^4-10^5 cells were aliquoted into flat bottomed 96 well plates and medium alone or doubling dilutions of supernatent containing IL2 added to the culture. The plates were incubated for 24h or 3days and a
6h pulse of $^3$H Thymidine, luCi/well applied. The cells were harvested on to filter discs using a mesh harvester (Titrtek, Flow), and incorporated radioactivity measured in a Beckman B counter (Fig 7).

2.6.4 Commercial IL2 preparations

Later experiments described were performed using commercial IL2 (serum and lectin free IL2, Cell Products, NY) and the supernatent from the constitutive IL2 producing gibbon cell line - MLA 144 (from Dr J.Lamb). The commercial IL2 was prepared by phorbol ester stimulation of pooled human PBM and then delectinated. The relative potency of tonsillar, commercial and unconcentrated MLA IL2 is shown in Fig 7. Higher levels of IL2 activity were present in the commercial and MLA supernatents than in the best batch of tonsillar supernatent and thus these sources are routinely used.

2.7 ESTABLISHMENT AND MAINTENANCE OF LYMPHOCYTE CULTURES

2.7.1 Proliferation of PBM to HCMV and other antigens

PBM were prepared and suspended at $2 \times 10^6 \text{ml}^{-1}$ in RPMI with 10% HCMV seronegative AB serum. 100ul aliquots were cultured, in triplicate, in 96 well flat bottomed plates (Costar) with varying concentrations of HCMV and other antigens prepared as described. After 6 days culture, luCi $^3$H Thymidine was added to each well, for 18h and the wells harvested on to filter discs.
Fig 7. IL2 content of commercial (batch 1 ●; batch 2 ○), tonsillar (▲) and MLA 144 cell supernatent (■) preparations. IL2 content was estimated by incubating doubling dilutions of the preparations for 24h with $10^5$ 10d PHA-blasts/well, pulsing with $^3$H Thy (1uCi/well) and determining the difference between uptake in test wells those grown in medium alone. There was consistently greater IL2 activity in commercial as compared with tonsillar supernatents. However the amount of IL2 in the MLA supernatent (unconcentrated) was also much greater than in the tonsillar preparations which had been x50 concentrated.
Antigens used were:

i) PPD (Evans) at a final concentration of 2.5 - 20ug ml\(^{-1}\). Optimum responses were observed at 10ug ml\(^{-1}\).

ii) Free HCMV antigen - virus released from sonicated infected fibroblasts was plaqued and the stock diluted to contain the equivalent of 10\(^5\) pfu ml\(^{-1}\). This material was used either untreated, UV inactivated or heat (56°C for 30min) inactivated as described.

iii) HCMV LA - F5000 monolayers were infected with HCMV at MOI of 20:1 and incubated for 4 days. The cell number was determined in a comparable culture; cells were physically detached, sonicated for 1min, filtered through a 0.22u nitrocellulose filter and aliquoted into vials containing the equivalent of 10\(^5\) infected cells ml\(^{-1}\).

iv) HCMV IFA and EA - antigen was prepared as above except cells were cultured in the presence of 200ug ml\(^{-1}\) PPF.

v) HCMV IFA - antigen was prepared by infecting monolayers at 20:1 in the presence of 400ug ml\(^{-1}\) CHX for 6h, thoroughly washed free of the inhibitor and then incubated in medium containing 2ug ml\(^{-1}\) AD for a further 12h. The cell layer was washed free of excess AD and antigen prepared as for HCMV LA.

vi) purified virus particles - see Section 2.4.4.

vii) Proliferative responses with purified PHA (optimum dose 2ug ml\(^{-1}\)) were also studied but maximum proliferation was measured with a 6h pulse after 48h culture.
2.7.2 Establishment and maintenance of T cell lines to HCMV antigens

IL2 dependant T cell lines were established by culturing $2 \times 10^6$ PBM ml$^{-1}$ with free HCMV (MOI equivalent 1:1) or by coculture with 2 h HCMV infected (MOI 5:1) fibroblast monolayers (PBM:fibroblast approx= 50:1) in RPMI with 10% HCMV seronegative AB serum. The cells were incubated for 4-6 days, fresh medium being added to prevent cultures becoming acidic. The medium was then replaced with medium and IL2 together with $2 \times 10^6$ irradiated autologous PBM as feeder cells (3500 rads). Cells were refed every 3 days with fresh medium and IL2, and subcultured to keep the cells at approximately $10^5$-$10^6$/ml. Fresh HCMV antigen or HCMV infected cells were added at day 7 of culture. Prior to use in cytotoxicity assays viable cells were separated by Ficoll/Hypaque centrifugation (Davidson and Parish, 1975).

2.8 CYTOTOXICITY ASSAYS

2.8.1 $^{51}$Cr release assay

Throughout the study $^{51}$Cr release assays were used to measure cytotoxicity. Target cells were prepared by overnight incubation with $^{51}$Cr (CJS-1, Amersham) 100uCi/10$^6$ cells, when using adherent cells, or by incubation for 2h in 200ul medium for cells maintained in suspension culture. The adherent cells were then washed free of excess $^{51}$Cr, detached with trypsin/EDTA again washed 3x to remove
residual free $^{51}$Cr. Following removal of free $^{51}$Cr (if viability was >90%), target cells were resuspended in supplemented RPMI at $5 \times 10^4$ cells ml$^{-1}$. 100ul of target cells were aliquoted into round-bottomed 96 well plates (Corning) together with 100ul of effector cells. Each test was performed at least in triplicate, and 6 wells were incubated with 100ul medium without effector cells to determine spontaneous release. Maximum release was determined by addition of 100ul 1% aqueous Triton X 100 (BDH). Specific $^{51}$Cr release was calculated by the standard equation:

$$\text{% specific } {^{51}}\text{Cr release} = \frac{(\text{test cpm} - \text{spont cpm}) \times 100}{(\text{max cpm} - \text{spont cpm})}$$

This assay has been predominantly used in experiments measuring lysis of non-adherent cells (Perlman and Cerottini, 1979; Bonavida and Bradley, 1980), therefore studies were performed to examine this assay with HCMV infected and uninfected fibroblasts as target cells. They presented 3 major difficulties as target cells in $^{51}$Cr release assays:

1) previous studies with fibroblast target cells used a prolonged assay time - either overnight or 24h - as fibroblasts were thought to be less susceptible to lysis (Quinnan et al, 1981; 1982; Trinchieri and Santoli, 1978 Timonen et al, 1979; Saksela et al, 1979). This presented a potential problem as prolonged assays could permit activation of
non-specific NK cells by intra-assay IFN release.

2) spontaneous release was much higher than with tumour lines which may cause difficulties in interpretation of results. Several factors could contribute to this higher spontaneous release and their relative importance was examined. Different methods of performing the release assay were investigated to determine the optimum method of labelling target cells to produce lowest spontaneous release (Table 2).

   a) as expected the maximum release increased with duration of incubation with $^{51}$Cr.

   b) trypsin may have been important in increasing spontaneous release and destroying cell surface determinants required for Tc and NK cell recognition. To avoid trypsinisation, $10^4$cells/well were placed in a 96-well flat bottomed plates. 24h later half were infected with HCMV at MOI 5:1. 24h and 4h prior to assay cells were incubated with $^{51}$Cr. The wells were washed 3x with PBS and then incubated with medium (200ul) or medium (100ul) and 1% aqueous Triton X 100 (100ul). After 6h 100ul were harvested and to the wells incubated with medium alone, 100ul 1% aqueous Triton X added for 2h before a further 100ul was harvested from these wells. The maximum release was thus calculated both in 5 similar wells and by the method of Trichieri and Santoli (1978) from the same wells in which spontaneous release was measured (Table 2).

   c) fibroblasts were grown to confluency in a 25cm² culture flask, infected with HCMV (MOI=5:1), and incubated at similar times with $^{51}$Cr (50uCi/flask). The cells were then detached with trypsin/EDTA.
TABLE 2 Comparison of the effects of trypsin, methods of $^{51}$Cr labelling and methods of estimation on spontaneous and maximum release by HCMV infected and uninfected primary fibroblast lines.

<table>
<thead>
<tr>
<th>HCMV inf</th>
<th>$^{51}$Cr</th>
<th>Spont Rel cpm (SD)</th>
<th>Max Rel cpm (SD)</th>
<th>%spon rel</th>
<th>%spon rel-T</th>
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<tr>
<td>-</td>
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<td>434 (49)</td>
<td>892 (230)</td>
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<tr>
<td>-</td>
<td>4</td>
<td>1078 (45)</td>
<td>5044 (406)</td>
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<td>18.8</td>
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<tr>
<td>-</td>
<td>18</td>
<td>3764 (66)</td>
<td>23648 (1754)</td>
<td>15.9</td>
<td>18.1</td>
</tr>
<tr>
<td>+</td>
<td>$^{S}$</td>
<td>606 (98)</td>
<td>2100 (374)</td>
<td>28.9</td>
<td>35.0</td>
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<tr>
<td>+</td>
<td>4</td>
<td>2072 (75)</td>
<td>8220 (913)</td>
<td>25.2</td>
<td>24.4</td>
</tr>
<tr>
<td>+</td>
<td>18</td>
<td>4271 (1191)</td>
<td>26081 (996)</td>
<td>16.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Trypsin detached cells

Undetached cells

a - cells were incubated with 25uCi ml$^{-1}$ $^{51}$Cr.
b - N=5
c - %spon rel= (spont rel/max rel)*100
  %spon rel-T=(spont rel*100)/(max rel at 10h + (spont rel/2))
  spout rel-T is calculated by removing half the supernatent at 6h,
  adding 100ul 1% aqueous Triton X to the same well and assaying
  the supematent after a further 2h. This allows max and spon
  release to be calculated in the same well thereby eliminating
  effects of variable ÿcell growth (Trinchieri and Santoli (1978)).
d - cells labelled in suspension for 2h.
and $10^4$ aliquoted into flat bottomed plates, maximum and spontaneous release studied as described above.

d) $10^6$ HCMV infected and uninfected cells were detached and suspended for 1h in 100uCi $^{51}$Cr for 2h, then washed free of Cr and and $10^4$ cells placed in 96 well plates and studied as above.

e) spontaneous release was also relatively higher at low target cell numbers and a minimum of $5 \times 10^3$ target cells were subsequently used (data not shown).

Cells treated within the same plate had a lower spontaneous release but this was little different from cells incubated overnight and subsequently detached. Both of these treatments was better than the higher spontaneous release levels observed in the cells incubated in suspension.

The effect of HCMV infection on spontaneous release was unexpected in that they had lower spontaneous release than did uninfected fibroblasts (16.4 +/- 4.3 HCMV infected F5000: 33.8 +/- 6.25 uninfected (n=10)). In addition there was considerable variation between different fibroblast lines. Studies were thus performed using both infected and uninfected target cells simultaneously.

3) trypsin/EDTA treatment of HCMV infected cells could have had two major effects on the assay. Firstly, slight damage to the cells could cause increased spontaneous release - this was not a significant effect (Table 2). Secondly, trypsin could remove cell surface structures which were important in target cell recognition. Fibroblasts treated with
trypsin/EDTA and allowed to readhere to slides, had normal surface expression of MHC class I antigens and HCMV antigens recognised by F(ab)\(_2\)-FITC antibody determined by immunofluorescence. Such cells were also susceptible to anti-HCMV and C lysis (Fig 5). Furthermore these target cells were also lysed by a cytotoxic T cell line recognising MHC class I antigens (Fig 20; see Section 3.5.6).

2.8.2 Cold competition assays

The ability of different cells to compete with labelled target cells was examined in cold competition experiments. In these studies effector and \(^{51}\)Cr labelled target cells were mixed at a constant effector:target ratio in the presence of varying numbers of unlabelled competitor cells. The inhibition observed was calculated by:

\[
\% \text{ inhibition} = \left[1 - \frac{(% \text{ sp. rel. with compt. cells})}{(% \text{ specific release})}\right] \times 100
\]

2.8.3 Lectin dependant cytotoxicity

Lectin (PHA) dependant lysis of K562 cells was used to determine the presence of cytotoxic cells independent of antigen specificity and MHC restriction (Bevan and Cohn, 1975). \(5\times10^3\) \(^{51}\)Cr labelled K562 cells/well were cultured with effector cells and PHA, the optimum concentration of lectin being previously determined at 2ug ml\(^{-1}\). \(^{51}\)Cr release was determined after 8-18h and positive cultures were
those with >3SD+spontaneous $^{51}\text{Cr}$ release.

2.8.4 Target cell binding assay

Direct binding of effector cells to K562, HCMV infected and uninfected fibroblasts was examined by a modification of the method described by Roder and Kiessling (1978). $10^6$-$10^7$ effector cells were washed and incubated with 1ml 0.15M saline (pH7.0) followed by 4.0ml 0.15M saline (pH9.0) and then by 1ml FITC (0.7mg ml$^{-1}$ in 0.15M saline pH9.0) on ice for 30min. The cells were washed free of excess FITC and resuspended at 2x10$^6$/ml. 100ul aliquots were incubated with an equal volume containing 2x10$^5$ target cells. The mixture was incubated at 31°C for 10min and centrifuged at 1000g for 5min at room temperature. The cells were then resuspended with a pasteur pipette in RPMI containing 1% paraformaldehyde and examined by phase contrast and fluorescent microscopy to determine the number of FITC positive cells bound to the larger unstained target cells.

2.8.5 Analysis of data

Specific $^{51}\text{Cr}$ release results were calculated and the SD for each triplicate determined. Where appropriate differences were analysed by Students' T test and target cell binding data was analysed by $\chi^2$ test (Spiegel, 1972).

Where four or more effector to target cell ratios from the same population of effector cells had been used, results were expressed in
terms of 'lytic units'. These are defined as:

\[
\text{lytic units} = \frac{10^4}{\text{E:T to produce 50\% lysis of 5000 target cells}}
\]

The 'E:T required for 50\% lysis' was determined by exponential regression analysis, as described by Pross et al (1981). This was performed by linear regression analysis of E:T vs \(\log_e(100-\text{observed \% specific release})\), assuming the maximum release as determined by 1\% Triton X, to be the maximum releasable \(^{51}\text{Cr}\) from the target cells. (Detergent released counts were 85-90\% of the maximum expected release regardless of the target cell used.) In addition the coefficient of correlation (\('r'\) for individual values in the exponential regression calculation was \(>0.85\) in all experiments described.

The use of 'lytic units' calculated by this method allows a more accurate comparison of non-arithmetic curves. The E:T vs specific release is a logarithmic relationship, thus comparison between curves is difficult even in the linear portion of the graph. This technique eliminates attempts to extrapolate to a 50\% specific release point, visually, on a curve rather than a straight line.

Other techniques have also been applied to this question of determining the 50\% point, notably the von Krogh equation, but as shown by Pross et al (1981), results between this and exponential regression analysis did not differ significantly. In view of the ease of
incorporating the latter technique into simple BASIC algorithms this was adopted for routine use.

2.9 TRANSFERRIN BINDING STUDIES

2.9.1 Surface and recycling transferrin binding assays

The expression of surface and recycling transferrin receptors (TfR) was estimated by equilibrium binding of ferric iron saturated transferrin (Tf) to infected and uninfected cells as described by Ward, Kushner and Kaplan (1982a). 10mg ml\(^{-1}\) Tf (Sigma - 95% pure; iron free) in PBS (pH 7.2) (PBS), was incubated with an equal volume of (0.1mg ml\(^{-1}\)) ferric ammonium citrate (Sigma) in 0.01M NaHCO\(_3\), for 4h at room temperature (Larrick and Cresswell, 1979). Excess Fe\(^{3+}\) was removed by dialysis against several changes of PBS for 18h at 4\(^{\circ}\)C. The transferrin was 95% Fe\(^{3+}\) saturated by absorbance at 454nm. Small aliquots of saturated Tf were labelled with \(^{125}\)I using chloramine T to a specific activity between 4949 and 13924cpm ng\(^{-1}\) Tf.

Human MRC-5 or F5000 fibroblasts were grown to confluency in 35mm petri dishes or 24 well plates (Falcon). 1h prior to incubation with \(^{125}\)I-Tf, uninfected or virus infected cells were washed in PBS and incubated in serum free BME for 1h at 37\(^{\circ}\)C, to dissociate any Tf from FCS that was bound to TfR. The plates were then washed in PBS and
incubated with varying concentrations of $^{125}$I-Tf in BME containing 4g l$^{-1}$ BSA (Sigma fraction 4). All assays were performed in duplicate or triplicate with a specificity control at each concentration - $^{125}$I-Tf in the presence of 0.1mg ml$^{-1}$ unlabelled Tf - to determine any non-Tf specific binding.

Plates were allowed to equilibrate for 1h at 37°C or 2h at 4°C. The cells were then washed in 5 changes of ice cold PBS, solubilised in 1 ml 1% aqueous NP40 (BDH) and bound $^{125}$I estimated. The binding results were analysed by Scatchard analysis and linear regression analysis of the bound vs free plot was used to determine the number of Tf molecules bound per cell (Baxter and Fundis, 1979; Frazier et al, 1982; Ward, Kushner and Kaplan, 1982a). Cell number was estimated by detaching comparably treated cells with trypsin/EDTA and counting viable and non viable cells using ethidium bromide and acridine orange (see Section 2.12.1.).

2.9.2 Total cellular transferrin binding

Total TfR in infected and uninfected cells was measured by Tf binding to detergent solubilised cells (Ciechanover et al, 1983). Cells from a petri dish were dissolved in 0.5ml of medium containing 1% NP40. This was incubated with varying concentrations of $^{125}$I-Tf for 2h at 4°C. Specificity controls in the presence of 0.1mg ml$^{-1}$ unlabelled Tf were included at each concentration of 125I-Tf. Following incubation, 0.5ml of 65% saturated ($\text{NH}_4$)$_2\text{SO}_4$, 100mM
Tris-HCl pH 7.6, was added to each tube. The solutions were mixed and allowed to stand at 4°C for a further 10 min, before being filtered through Whatman GPC glass fibre discs. Each disc was washed with a total of 20 ml 32.5% saturated (NH₄)₂SO₄, 100 mM Tris-HCl pH 7.6, containing 1% NP40 and 1 mg/ml BSA. All experiments were performed in duplicate, with specificity controls and ¹²⁵I-Tf incubated in the absence of cell extract to determine the quantity of ¹²⁵I-Tf precipitated by the (NH₄)₂SO₄ solution, at each concentration examined.

2.9.3 Maintenance of F5000 in varying iron concentrations

TfR expression was examined in F5000 cells grown at different concentrations of iron. Iron depleted FCS was prepared by dialysis against 50 volumes of 10 mM sodium acetate-NaOH pH 5.0, 10 mM EDTA and 150 mM NaCl. After 24 h the FCS was dialysed against 20 volumes 10 mM KPO₄ pH 7.5, 150 mM NaCl for a further 24 h. Serum iron estimation (performed by Dept of Chem. Path, RPMS using Autoanalyser II Technicon Method No SF4-0025FL4) confirmed substantial depletion of iron (<0.002 µg ml⁻¹). This serum was added to BME (which contains no added iron) and supplemented as detailed above. Increased concentrations of iron were achieved by adding ferric ammonium citrate (elemental iron = 30%) to BME containing undialysed FCS and supplements (Ward, Kushner and Kaplan, 1982a).
2.9.4 Blocking studies with Tf

NK assays as described were performed in the presence of varying dilutions of added iron saturated Tf (5mg ml\(^{-1}\) to 0.1mg ml\(^{-1}\)).

Indirect immunofluorescence of HCMV infected, PPF treated HCMV infected and uninfected F5000 cells on multispot slides, was performed with OKT9 using a rabbit anti-mouse Ig-FITC (Miles) in the presence of 2% normal rabbit serum to prevent nonspecific HCMV induced Fc receptor binding.

2.9.5 Partial purification of human transferrin receptor

Molt 4 cells were grown to a total number of 4x10\(^9\), washed four times in PBS and incubated in 2.5mg of TPCK treated trypsin (Worthington) for 30min at room temperature either as whole cells or following lysis in extraction buffer at 4\(^\circ\)C (0.01M Tris HCl, 0.15M NaCl, pH8.1, 0.5% NP40) (Schneider et al, 1982; Vondinelich et al, 1983). 10mg soya bean trypsin inhibitor and 67mg bovine haemoglobin (Sigma) were added to the digest and it was centrifuged at 1000g for 20min to remove cell debris. The supernatant was recycled on a 10ml Fe\(^{3+}\) saturated transferrin coupled Sepharose 4B column, the column washed with 90ml 10mM NaCl-Hepes pH7.5 and then eluted with 40ml 50mM glycine/NaOH pH10.0, 1M NaCl and 1% Triton X (if the material was not to be used in the cytotoxicity assays) (Fig 8) (Seligman, Schneider and Allen, 1979). When material from surface labelled cells was passed down the column after treatment with lysis buffer (but not digested with trypsin) material was eluted from the column as indicated. If similar unlabelled material was
Fig 8. Affinity purification of human transferrin receptors. Surface labelled Molt4 cells were lysed as described (see Section 2.9.5.). The lysate was passed down a 10ml Sepharose 4B Fe\(^{2+}\) saturated Tf conjugated column and eluted with glycine/NaOH (pH 10.0). The fractions were counted (•—•) and only 0.012% of initial counts were recovered. Similar results were obtained with \(^{35}\)S labelled cells. However the eluted fractions specifically bound \(^{125}\)I-Tf as described (Section 2.9.5) (△······△), as did the original lysate (△). This assay is not an accurate quantitative estimate of the TfR.
used it was shown to specifically bind to transferrin (Fig 8) (Seligman, Schneider and Allen, 1979). In later experiments the column was eluted with 0.5% aqueous acetic acid. The eluted material when studied on 10% SDS PAGE, had a single band (76kDa) was observed by Coomassie blue staining.

2.10 LOW DENSITY LIPOPROTEIN UPTAKE AND DEGRADATION

$^{125}$I labelled KBr gradient separated low density lipoprotein (LDL) and unlabelled LDL were prepared by Dr B.Knight, MRC Lipid Metabolism Unit. Varying concentrations of $^{125}$I-LDL from 0.25 to 20ug ml$^{-1}$ were added in 1ml of BME (with 4g 1$^{-1}$ BSA) to 48h HCMV infected and uninfected F5000 cells in 35mm petri dishes in duplicate. Specificity controls were performed at 5 and 10ug ml$^{-1}$ $^{125}$I-LDL, by adding 500ug unlabelled LDL. In addition 'blank controls' were studied by incubating $^{125}$I-LDL in medium in the absence of cells. After 4h incubation at $37^\circ$C, 900ul of the medium was removed and stored at $-70^\circ$C until further analysis.

The cell layer was washed with 150mM NaCl, 10mM Tris-HCl (pH 7.4) with 2% BSA at 4°C allowing a 10min incubation between each of three washes. A fourth wash without BSA was applied and the cells were then solubilised in aqueous 1% NP40 solution and samples counted for $^{125}$I as a measure of integrated and bound LDL.

The amount of free $^{125}$I present in the medium was measured as an indication of the rate of LDL degradation. To the harvested 900ul,
225 

224μl 50% TCA was added and incubated on ice for 30min. The samples were centrifuged at 1000g for 30min, and 750μl were aspirated and placed in a fresh tube. To this was added 10μl 4% KI and 30μl 30%H₂O₂ and incubated on ice for 5min. 1.5ml chloroform were added to each tube which was thoroughly vortexed, centrifuged and the bottom chloroform layer aspirated. Another similar chloroform extraction was performed, and 500μl of the aqueous layer then counted for ¹²⁵I.

2.11 INTERFERON ASSAY

IFN was assayed by inhibition of SFV cpe on Vero cells, using an affinity purified human IFNα (Wellcome Laboratories) as a standard. Confluent flat bottomed wells (96 well plate) were incubated overnight with varying dilutions the supernatent in quadruplicate and varying dilutions of the IFN standard. SFV (10⁶pfu) was added to each well and 48h later the plates were fixed in 10% formal saline and stained with 0.1% aqueous crystal violet. The dilution of supernatent producing 50% inhibition of cpe was the end point and IFN (iu ml⁻¹) content related to the standard.

2.12 IMMUNOFLUORESCENT MICROSCOPY AND FLOW CYTOMETRY

2.12.1 Viability staining

Cell viability was determined by diluting the suspension of cells to be examined 1:1 with acridine orange (5μg/ml) and ethidium bromide.
mS(5ug/ml) and examining the cells in a Neubaur chamber under fluorescent and white light (Parks et al, 1979).

2.12.2 Cell morphology

The morphology of HCMV infected and uninfected cells was examined by growing and infecting fibroblasts on 12-spot slides (Harvey-Evans). For nuclear inclusion bodies, the optimum fixative was Bouin's Solution (Albrecht et al, 1980) and stained with Harris's haematoxylin and 1% eosin (Gurr).

2.12.3 Antibodies used and indirect immunofluorescence

The monoclonal and polyclonal antibodies used in these studies are shown in Table 3. HCMV infected cells were fixed by air-drying and acetone (15min at -20°C) or unfixed for surface fluorescence. Monoclonal and polyclonal antibodies were added at the appropriate concentration in the presence of 2% normal human (or rabbit) serum to competitively inhibit Fc receptor binding. Antibody was washed off and goat anti-mouse Ig with 2% serum incubated on the slide for 30min. Excess antibody was washed off and cells mounted in 10% PBS-glycerol. Alternatively, peroxidase-conjugated second antibody was used and the reaction developed with H_2O_2 and diamino benzidine (Sigma) (Pearse, 1981).

Surface staining of mononuclear cells was performed in suspension. 10^6 cells were suspended in 100ul medium with 0.01M sodium azide and mixed with an appropriate dilution of first antibody, in a 96well
### TABLE 3
Monoclonal and polyclonal antibodies used

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<th>Antibody</th>
<th>Antigen recognised</th>
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<td>T3 antigen</td>
<td>BD</td>
<td>Perez-Rojas (1981)</td>
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<td>T3 antigen</td>
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<tr>
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<td>monocyte antigen</td>
<td>Coulter</td>
<td>Todd (1981)</td>
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<td>HLA DR common antigen</td>
<td>BD : Coulter</td>
<td>Nadler (1981)</td>
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<td>HLA A and B</td>
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<td>Tfr</td>
<td>Ortho</td>
<td>Sutherland (1981)</td>
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<td>Stashenko (1980)</td>
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<tr>
<td>anti-HCMV-FITC</td>
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F(ab), anti-HCMV-FITC prepared by immunising rabbit with 4d HCMV inf MRC-5 cells prepared from convalescent human serum
roundbottomed plate. After 30 min at room temperature the cells were washed once in PBS and the second antibody applied. After a further 30 min at room temperature in the dark, the cells were washed once and resuspended in 1% paraformaldehyde PBS. These cells were then examined by EPICS C flow cytometry.

Lymphocytes were usually examined at a flow rate of 1000-5000 cells/sec, using 488nm light from a 5W argon laser at a power of 200mW. The emitted fluorescent light was passed through 525nm long pass filter, 560 short pass beam splitting filter in conjunction with a 560 short pass blocking filter or a 10nm band pass filter (pk pass 530nm). Variations in these settings are described with relevent experiments.

2.12.4 Flow cytometric analysis of isolated nuclei

Flow cytometry is predominantly applied to examine surface labelled cells, as fixation of cells followed by indirect immunofluorescence gives high background non-specific fluorescence. In addition cell yields are very low as fixed cells require numerous washes to reduce this non-specific fluorescence. Unfortunately, those HCMV IEA and EA which are identified by monoclonal antibodies (Table 3), are predominantly nuclear in distribution. Therefore a technique for examining isolated nuclei of HCMV infected cells was developed.

Fibroblast monolayers were infected and grown for 4d to allow expression of LA, or under conditions permitting expression of IEA alone (see Section 2.4.3.). Nuclei were isolated using either hypotonic or
detergent lysis but latterly by scraping the cells off the plates and disrupting them using a Dounce homogeniser in hypotonic buffer (10mM Tris HCl, 0.2mM MgCl₂ pH7.2) (Resh and Erickson, 1985). The isolated whole nuclei were incubated with antibodies E3 or polyclonal F(ab)₂-FITC anti-LA for 45min at 4°C, washed free of antibody by centrifugation and resuspended with goat anti-mouse Ig, if appropriate for 30min at 4°C. Excess antibody was removed and the nuclei counterstained (DNA) with a 0.05mg ml⁻¹ propidium iodide (5min at room temperature).

The nuclei were analysed on an EPICS C using a 200mW, 488nm incident laser beam. Scattered light was detected by forward light scatter detector and at 90° the emitted light was passed through a 525nm long pass filter, to remove scattered incident light and then the beam was split by a 560nm short pass, beam splitting filter. GFL was detected after the shorter wavelength light was passed through a 530nm (10nm width) band pass filter. Red fluorescence (DNA content - propidium iodide) was detected after red light was passed through a 610nm long pass absorbance filter. The signals were collected and 'gated' on red fluorescence so that only particles containing DNA were examined for FAIS and GFL. Fig 9 shows the results obtained in one such experiment; both IEA and LA could be detected in isolated nuclei.

To confirm these results nuclei were sorted and examined by microscopy which showed LA fluorescence in the characteristic inclusions on a red (propidium iodide) background. This technique has obvious
Fig 9. Flow cytometric analysis of nuclei from HCMV infected and uninfected cells. 50,000 nuclei from HCMV infected and uninfected stained with E3 and polyclonal F(ab)₂ and propidium iodide were analysed by EPICS C as described. 2 parameter contoured histograms comparing antibody (GFL) with DNA content. By direct analysis 85% of 46 HCMV infected nuclei expressed LA in all phases of the cell cycle. Cells prepared so as to express IEA, had IEA nuclear staining in 40% of all cells but in over 65% of cell in G₂-M phase.
advantages in that propidium iodide stains DNA content stoichiometrically and G₀-G₁, S and G₂-M phases can be detected (Fried, Perez and Clarkson, 1976; Kruth, 1982). This coupled with quantitative estimation of GFL corresponding to IEA and IA content is currently being applied to the reinvestigation of the question of HCMV replication and the cell cycle.
SECTION 3

T CELL RESPONSES TO HCMV

Most studies of T cell responses to HCMV have investigated lymphocyte proliferation in response to HCMV antigens in vitro (Ten Napel et al, 1977; Moller-Larsen et al, 1977; Gehrz et al, 1977; Reynolds et al, 1979; Meyers, Flournoy and Thomas, 1980; Starr et al, 1980; Schrim, Roenhorst and The, 1980; Wahren, Robert and Nordlund, 1981). In the studies described below, the investigations were performed on PBM of normal persistently infected volunteers to determine:

a) the relationship between serological markers of HCMV infection and proliferative T cell response.

b) the nature and function of responding T cells in proliferation assays.

c) the nature of the virus antigen recognised by the proliferating T cell.

3.1 IDENTIFICATION OF HCMV SEROPOSITIVE SUBJECTS

The standard complement fixation test used in the clinical virology department was used to determine the status of individuals whose PBM were used in the experiments described. However, as the standard definition of a seropositive individual was one who had a cft >1/8 it was possible that
a number of seropositive individuals with low levels of cft antibodies could be included in the seronegative population. Therefore a HCMV specific ELISA was developed, using a modification of the technique described by Booth et al (1979), the technical details of which are described elsewhere (Burroughs, 1984). Using this more sensitive assay a number of individuals defined as being seronegative by cft had anti-HCMV IgG antibodies as determined by the ELISA (40% of a random sample of 45 cft seronegative subjects) (Figs 10 and 11). However, the cft assay was sensitive in detecting seropositive individuals (>95% of subjects with cft >1/8 were positive in the IgG ELISA). Therefore subjects defined as seronegative in these experiments, had a cft titre of <1/8 and serial dilutions of the serum had undetectable levels of HCMV IgG antibody by ELISA (Fig 11).

In addition, this assay proved useful in detecting HCMV specific IgM antibodies in patients with probable primary HCMV infection (Fig 12). The ELISA assay was modified to detect HCMV specific IgM antibodies (Burroughs, 1984) following absorbance of serum with IgG coated latex beads to deplete of possible rheumatoid factor activity. In this patient studied sequentially, appearance of IgM antibodies preceded development of IgG specific antibodies, but both were detected well before a positive cft response was observed. Furthermore, IgM antibodies were present before atypical lymphocytes and no HCMV specific lymphoproliferative response was observed.
Fig 10 Detection of HCMV specific IgG antibodies by ELISA. 93 serum samples were assayed simultaneously, at 1/50 dilution against 'free' HCMV antigen and uninfected fibroblast antigen. The HCMV specific (abs vs. HCMV - abs vs. fibroblast) IgG was compared with serological status as determined by cft. 18 of 46 cft seronegative samples were positive by ELISA when compared with pooled negative control serum (no HCMV IgG by serial dilutions in ELISA assay). Only 2 of 47 cft positive sera were negative by ELISA. This technique was more sensitive in detecting HCMV positive sera than the cft and was used to determine serological status in these experiments.
Fig 11. Serial dilutions of 2 positive (>1/256 cft) (○) and 13 cft negative serum (<1/8 cft) samples (●) were assayed against HCMV and fibroblast antigen by ELISA. Samples negative by cft frequently had low levels of HCMV specific IgG antibody by ELISA.
A 23-year-old presented with general malaise and lethargy (d0) following her return from a holiday in Morocco. 25d later she developed a hepatitis with intranuclear inclusions on the biopsy, an atypical lymphocytosis and a negative heterophile antibody test. Retrospective examination of serum samples showed a raised HCMV IgM titre, and the IgG rose but no increase in cft antibodies was detectable until 55d after onset of symptoms. During the course of this study there was no HCMV or PPD specific proliferative response.

Fig 12. HCMV IgG, IgM and cft antibodies during primary HCMV infection.
3.2 PBM FROM SEROPOSITIVE SUBJECTS PROLIFERATE TO HCMV

Lymphocyte proliferative responses to PHA (2μg ml⁻¹), PPD (20μg ml⁻¹) and HCMV antigen were examined. HCMV antigen was prepared by heat inactivating (56°C for 30 min) sonicated HCMV infected MRC-5 cells diluted to the equivalent of 10⁵ pfu ml⁻¹. Similarly treated and diluted MRC-5 uninfected cell suspension was used as the control antigen. PBM from seropositive subjects proliferated specifically to the HCMV antigen with a peak response after 6d. To investigate the relationship between anti-HCMV cft antibody and the proliferative response, PBM from 53 subjects were examined (31 samples from 25 IS patients and 34 from 28 non-IS and normal subjects) (Table 4).

PBM from seronegative normal, patient or IS subjects showed no proliferation to HCMV as measured either by the difference in incorporated ³H Thymidine between virus stimulated and control cultures or the stimulation index (Table 4). There was increased thymidine uptake by seropositive PBM from normal and IS individuals (Fig 13) but this was insensitive as a measure of previous exposure to HCMV particularly in the IS patients. 10/21 IS seropositive compared with 5/19 non-IS HCMV seropositive subjects had a stimulation index >3.17 (seronegative mean+2SD = 1.54+(1.62)) (Χ²=4.9; P<0.05) even though the geometric mean HCMV cft of these IS patients was greater than the non-IS patients (90.5 and 32.0 respectively). The stimulation index was greater in non-IS
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**Legend**

1. proliferative response to PHA (2 day), PPD & CMV (6 days) as described in Methods
2. diag - clinical diagnosis - CRF - chronic renal failure PAN - polyarteritis nodosa
   ATN - acute tubular necrosis H/U - adult haemolytic uraemic syndrome
   EBV - EBV mononucleosis CMV mono - CMV mononucleosis
   Trpl - renal transplant fib alv - cryptogenic fibrosing alveolitis
   IS - immunosuppressive medication (steroid +/- azathioprine +/- cyclophosphamide
   ^ - unknown
3. diff - difference cpm = (stimulated PBL cpm - control PBL cpm)
4. SI - stimulation index = (stimulated PBL cpm / control PBL cpm)

a) repeat studies performed 14 days after initial estimation
subjects and increased with higher cft titres. This relationship was more evident in the non-IS subjects, whereas IS individuals had either a similar stimulation index in comparison with normal controls or markedly reduced indicies (Fig 13). Among the IS patients there was no difference in the incidence of clinically apparent HCMV infection between patients with high or low stimulation indicies. The test was reproducible between individuals assayed on different occasions (Fig 14) and no direct correlation between a low CMV stimulation index and low PHA stimulation index was detected (Table 4).

3.2.1 Proliferative response required adherent PBM

The requirement for antigen presentation by adherent cells in the proliferative response to HCMV antigen was investigated. PBM were seperated by ficoll hypaque from a normal seropositive subject (cft 1/32) (number 21) and cells further seperated into E⁺ and E⁻ cells with SRBC. The E⁻ cells were then placed in flat-bottomed 96-well plates in serum-free medium and allowed to adhere for 2h. The non-adherent E⁻ cells were washed off and 2x10⁵ E⁺ cells added to the cultures in the presence of optimum concentrations of HCMV and PPD (20ug ml⁻¹) antigens. The cultures were incubated for 6d and pulsed overnight with luCi ³H thymidine. Lymphocyte proliferation was observed in both PPD and HCMV cultures only in the presence of E⁻ adherent cells (Fig 15).
Fig 13. HCMV specific lymphocyte proliferation in response to 'free' HCMV in 30 IS and 28 non-IS, seronegative and seropositive subjects. PBM proliferation was assayed after 6d co-culture with 'free' HCMV or fibroblast antigen (Section 2.7.1). The HCMV specific stimulation index (Table 4) was compared with the level of cft antibody. Although numbers in both groups were small, the proliferative response was more often reduced in IS patients. In these individuals, especially with high cft antibody levels, a high or low proliferative response was observed but this did not correlate with the IS medication or clinical HCMV infection.

Fig 14. Reproducibility of HCMV specific lymphocyte proliferation. The reproducibility of the lymphoproliferative assay was examined by re-assaying PBM from the same 10 subjects 3 or more weeks after the initial examination (Table 4). With one exception (---) a similar response was observed.
Fig 15. HCMV specific lymphocyte proliferation requires the presence of adherent PBM. 2x10^5 PBM/well (subject 21 (cft 1/32)) were cultured, or separated into E^+ (□) and E^- cells (■). The E^- cells were allowed to adhere to a 96-well plate and after 2h non-adherent cells were washed off. 2x10^5 E^- cells were then added back to the adherent E^- cells (□). 20μg ml PPD or 10^6pfu ml heat inactivated HCMV or medium alone was added to the cultures. After 6d the difference in ^3H thymidine uptake between PPD/HCMV stimulated and medium cultures was measured. The HCMV proliferative response was reduced in E^- and E^+ cells cultured alone but reconstituted by adding back the two populations.
3.2.2 HCMV stimulated and unstimulated PBM did not mediate HCMV specific cytotoxicity

Quinann et al (1981; 1982; 1984) described MHC Class I restricted Tc present without secondary in vitro stimulation, in PBM of bone marrow transplant recipients with active HCMV infection. However, using this assay, they have been unable to detect MHC restricted cytotoxicity against HCMV infected targets by PBM of asymptomatic seropositive and seronegative subjects (Quinnan et al, 1981; 1982; 1984; Rook et al, 1983; 1984). PBM from these subjects were able to mediate non-MHC restricted lysis of HCMV infected cells, this is considered in Section 4, but no direct MHC restricted cytotoxicity was found in PBM of 7 seropositive subjects even at E:T of 200:1.

Short term secondary in vitro stimulation was studied. Table 5 shows results for one subject (number 56) (cft >1/256) whose PBM were, maintained in vitro for 5d with or without HCMV (MOI equivalent 5:1; UV inactivated) or co-cultured with 4d HCMV infected autologous fibroblasts. The lymphocytes were harvested and assayed against autologous, partially matched and HLA-mismatched HCMV infected and uninfected target cells in a 6h $^{51}$Cr release assay. No significant MHC-restricted HCMV target cell lysis was observed; although some lysis of HCMV infected target cells sharing 3 HLA A and B antigens was observed with wide variation in this assay (SD >10%) (Table 5).

These results suggested that Tc were not directly present in PBM of asymptomatic HCMV seropositive individuals and that direct stimulation
TABLE 5 Lymphocytes (subject 55) cultured with HCMV (UV inactivated), 4d HCMV infected autologous fibroblasts and medium alone for 6d, do not mediate MHC class I restricted, HCMV specific cytotoxicity.

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<td>5</td>
<td>0(5) 5(5) 17(12) 5(6)</td>
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<sup>a</sup> - lymphocytes were cultured with HCMV (UV) (5:1) or cocultured with HCMV infected F11 for 5d, harvested and used in 6h <sup>51</sup>Cr release assay.

<sup>b</sup> - target cells were 48h HCMV infected primary fibroblast lines (shared haplotypes underlined). Maximum release calculated by Trinchieri method (see Table 3). Results expressed as % specific release (+/-SD).
with HCMV antigen did not activate them as has been described for other human virus specific Tc (McMichael and Askonas, 1978). It was possible that the precursor frequency of Tc was low and thus could not be detected in the assay. This was supported by the findings of Sethi, Stroehman and Brandis (1980) who were only able to detect HCMV specific Tc from 3 patients with recent reactivation after prolonged - 20 days or more - IL2 culture. A further implication of their secondary in vitro stimulation protocol was that HCMV specific Tc could be generated in response to 'free' UV inactivated AD169 virus, and thus did not require a productive infection to be established by the virus in the culture. These observations were investigated by expanding HCMV stimulated PBM (using live, UV irradiated and heat inactivated virus) in IL2 culture.

3.3 'FREE' HCMV STIMULATED T CELL LINES

3.3.1 Maintenance of PBM in IL2 culture

After 5 days culture in vitro with HCMV, PBL were expanded in IL2 culture. Initially, IL2 was prepared from 48h PHA stimulated tonsillar lymphocyte supernatants (affinity column delectinated) and the optimum dose required assayed as described (Section 2.6.3.). $2 \times 10^5\text{ml}^{-1}$ HCMV stimulated PBL, $2 \times 10^6\text{ml}^{-1}$, irradiated autologous PBL with HCMV antigen in the presence of optimal concentrations of IL2, were cultured in RPMI (2ml/well in 24 well plates). After 3d culture the medium
was replaced with fresh IL2 and feeder cells every 7d. The cells were
split depending on cell density as determined by direct microscopy. Sethi,
Stroehman and Brandis (1980) implied that no further addition of antigen
was required in their system, therefore the addition of antigen at weekly
intervals was initially avoided but was added in later cultures. If the
feeder cells were omitted, again as described in the Sethi protocol, the
cell cultures could not be adequately maintained. Using this technique
antigen stimulated IL2 dependant T cell lines could be maintained for up
to 60d (assayed up to 46d) in vitro (Table 6).

3.3.2 Free HCMV stimulated T cell lines were not HCMV specific Tc

HCMV stimulated IL2 dependant T cell lines were assessed for HCMV
specific cytotoxicity in a 6h $^{51}$Cr release assay against autologous
or HLA matched, and HLA mismatched HCMV infected and uninfected target
cells (Table 6). There was no unequivocal MHC restricted, HCMV specific
cytotoxicity using any of these T cell lines even from subjects with
recent HCMV pneumonitis (subjects 64,65,51). Live, UV inactivated (10
min) and heat inactivated HCMV antigen was used, thus absence of Tc cells
was not related to the infectivity of the virus. Significant cytotoxicity
was observed (subjects 20,64,66) but this was neither HCMV specific nor
HLA restricted, and was observed in seronegative as well as seropositive
subjects (Table 6). Therefore these in vitro conditions did not generate
MHC class I restricted, HCMV specific Tc either in normal asymptomatic
HCMV seropositive individuals or patients with recent HCMV reactivation.
TABLE 6 PBM stimulated with HCMV (UV inactivated, heat inactivated or untreated) and maintained in vitro for up to 46d do not mediate MHC restricted, HCMV specific cytotoxicity.

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<td>20</td>
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<td></td>
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<td>6.3</td>
</tr>
<tr>
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<td></td>
<td></td>
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<tr>
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<td>6.5</td>
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<td></td>
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<td>ND</td>
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<tr>
<td>Subject (HCMV cft)</td>
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<td>target cell HLA</td>
<td>max E:T</td>
<td>inf (%sp rel)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
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<td>-----------------</td>
<td>---------</td>
<td>--------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64 (&gt;256)&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A1 Aw23 B8 Bw35  (0)</td>
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<td>4.0</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>K562</td>
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<td>3 (&gt;256) H</td>
<td>12</td>
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<tr>
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<td>A2 A11 Bw35 B40 (1)</td>
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<td>12.6</td>
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<td>L 21</td>
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<tr>
<td></td>
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<td>(4h HCMV)</td>
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<td></td>
<td>(HSV)</td>
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<td>2.8</td>
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<td></td>
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<tr>
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<td></td>
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<tr>
<td>22 (&gt;256) UV</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>5.0</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H 17</td>
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<tr>
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<tr>
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<td></td>
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<tr>
<td>65 (&gt;256)&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>A1 A2 B15 B37   (1)</td>
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<td></td>
<td></td>
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<td>1.4</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>51 (&gt;256)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>H 19</td>
<td>A2 A1 Bw35 B40  (3)</td>
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<td>0.0</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A28 Aw30 B13 B18 (0)</td>
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TABLE 6 contd.

<table>
<thead>
<tr>
<th>Subject (HCMV cft)</th>
<th>HCMV antigen</th>
<th>days in vitro</th>
<th>target cell HLA</th>
<th>max E:T</th>
<th>inf (%)</th>
<th>uninf (%)</th>
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<tr>
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<td></td>
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<td>40</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>40.2</td>
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<td>39.3</td>
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<td></td>
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<td>5.9</td>
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<td></td>
<td></td>
<td>(4h HCMV)(0)</td>
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<td>24.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(HSV) (0)</td>
<td>40</td>
<td>21.8</td>
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</tr>
</tbody>
</table>

UV - UV inactivated
L - untreated 'free' HCMV
H - heat treated (56°C for 30min) 'free' HCMV
- recent (within 3 months) HCMV reactivation
The surface phenotype of the effector cells was determined. PBM were stimulated with free HCMV, maintained in IL2 for 14d and then examined by indirect immunofluorescence using anti-Leu 4a, 3a, 2a and Dr (Table 7). These PBL were predominantly Leu 4a+ and 3a+ similar to the surface phenotype of cells responding by proliferation to other free viral antigens such as VZV (Reinherz et al, 1979). Leu 3a+ Tc have been described in response to different viruses eg. EBV (Meuer et al, 1984), measles (Jacobsen et al, 1984), but these are MHC class II and not class I restricted. The fibroblast target cells used expressed no class II (Dr) antigens on the cell surface by indirect immunofluorescence following HCMV infection at MOI 10:1, thus such effector cells would not be detected using this assay.

To circumvent this problem, PHA stimulated PBM were infected with HCMV, as HCMV antigens might then be expressed in association with class II antigens. Sethi suggested that such cells could be used as target cells in cytotoxicity assays with similar results to fibroblast target cells. However, most authors have failed to detect HCMV antigens, even with IEA monoclonal antibodies, in human PBM infected with AD169 HCMV (Wahren, Robert and Nordlund, 1980; Rice, Schrier and Oldstone, 1984). When PBM were infected at MOI from 100:1 to 1:1, no expression of HCMV IEA, EA or LA was detected using the monoclonal and polyclonal F(ab)2 antibodies (Table 3). Therefore to date, it has not been possible to test effector cells on target cells bearing HCMV and class II antigens and the question as to whether class II restricted Tc specific for HCMV are
TABLE 7. Leu and DR phenotype of short-term T cell lines generated in response to HCMV.

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Antibody to HCMV (CFT titre)</th>
<th>PBM of donor</th>
<th>14-Day T cell lines generated to free HCMV</th>
<th>14-Day T cell lines generated on autologous HCMV-infected fibroblasts</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/2</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>1/8</td>
<td>63</td>
<td>31</td>
<td>44</td>
</tr>
<tr>
<td>67</td>
<td>1/8</td>
<td></td>
<td></td>
<td>73</td>
</tr>
<tr>
<td>13</td>
<td>1/8</td>
<td>62</td>
<td>32</td>
<td>42</td>
</tr>
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<td>68</td>
<td>1/16</td>
<td>54</td>
<td>31</td>
<td>43</td>
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<td>55</td>
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<td>70</td>
<td>1/32</td>
<td>61</td>
<td>21</td>
<td>27</td>
</tr>
<tr>
<td>69</td>
<td>1/128</td>
<td>59</td>
<td>38</td>
<td>30</td>
</tr>
</tbody>
</table>

1 - The numbers in each column indicate the percentage of total cells staining with anti-Leu 4, -Leu 3 and -Leu 2 or -DR.
2 - ND = not done
present remains open.

Furthermore, it was unlikely that cytotoxicity was not observed because class I antigens were not expressed as the fibroblasts were W6/32+ and TCL1 specifically lysed fibroblasts expressing appropriate HLA A and B type (Section 3.4 and Fig 20).

3.3.3 'Free' HCMV stimulated T cell lines proliferated and released IL2 in response to HCMV antigens

These T cell lines established to 'free' HCMV were studied in more detail. L57 (from subject 67), an IL2 dependant, non-cytotoxic T cell line (90% Leu3a+) was established to heat inactivated free HCMV and maintained in vitro for 43d. It was cultured for 10d prior to assay in IL2, but without fresh feeder cells or HCMV antigen, harvested and washed free of any residual IL2. 5x10^3 cells were aliquoted into flat-bottomed 96 well plates with 10^5 irradiated autologous E^-feeder cells, in the presence/absence of IL2 and HCMV antigen. Triplicate cultures were pulsed overnight with ^3H thymidine on 4 consecutive days. In cultures incubated with IL2 similar proliferative responses were observed whether or not HCMV antigen was added (Fig 16a). There was no proliferation in the absence of IL2, however, a proliferative response was observed after 3 days in cultures incubated with HCMV antigen and feeder cells (Fig 16a and b). In later experiments there was no proliferative response of these cells to measles virus antigen suggesting that a HCMV specific proliferative response had been maintained in this T
Fig 16. HCMV specific proliferation of an IL2 dependent T cell line established to 'free' HCMV. 5x10^3 L57 T cells (subject 67), (● ○ ) were cultured in the presence (a) or absence (b) of 10^5 irradiated (3000rads), autologous E PBM (♦ ◊ ). To these cultures HCMV antigen (-----) or medium (------) was added, with (solid symbols) or without (open symbols) IL2. The proliferative response was measured by a 6h ^3H thymidine pulse at daily intervals. The T cells proliferated in response to IL2 or HCMV antigen in the presence of E PBM; a greater proliferative response to IL2 was observed in the the presence of E PBM.
cell line. Furthermore, the results show that the presence of feeder cells augments growth of the line, independent of possible IL2 secretion by the feeder cells, even in the presence of optimum IL2 concentrations (Fig 16a and b).

The proliferation induced by HCMV was associated with release of a factor into the supernatent which had IL2-like activity. L57 was seeded into round bottomed wells at $10^4$ cells/well in the presence of doubling dilutions of 'free' heat inactivated HCMV, together with $10^5$ E$^-$ irradiated feeder cells. Control wells containing only feeder cells, virus antigen and feeder cells, and L57 cells alone were included. After 48h culture, half (100ul) the supernatent was removed and stored for 48h at $4^\circ$C to assay for IL2 activity. 100ul of RPMI was added back to the culture which was then pulsed with 1uCi $^3$H Thy for 6h and harvested after a total of 72h. The IL2 activity was measured using 10d PHA blasts, as described in methods, in a 24h assay and the stimulation index (cells + supn/cells + medium) determined. The degree of proliferation observed to HCMV antigen after 72h, was related to the relative amount of IL2 activity present in the supernatent after 48h of culture (results similar to those for EA proliferation Section 3.6.2. and Fig 37).

3.3.4 HCMV specific proliferative T cell clones

T cell lines proliferating in response to HCMV were cloned in limiting dilution culture. 0.5 to 100 cultured T cells from 4 lines (3
were 14d after stimulation with heat inactivated HCMV and maintained in IL2 and an MLR line as control) were added to 0.5-1.0x10^5 irradiated autologous PBM, and optimum concentrations of HCMV and IL2, in 96 well round-bottomed microtitre plates. The cultures were refed with fresh medium and IL2 every 3d and examined for cell growth. At weekly intervals fresh irradiated feeder cells and HCMV antigen were added. After 3 weeks, maximum numbers of wells showed growth (Fig 17a and b and Table 8) and no new 'growth+' wells were detected after that time.

The cloning efficiency under these conditions was low (precursor frequency in Table 8) and when cell cultures were moved from round-bottomed to flat bottomed 96-well plates 60% of the wells failed to grow. Ultimately only small numbers of cloned T cells grew to sufficient numbers to allow further analysis of effector function. The ability of these T cells to respond to gradient purified HCMV virions and IL2 was examined. 5x10^3 cloned cells were added in triplicate to 5x10^4 E^- irradiated feeder cells in the presence of 2.5ug ml^-1 gradient purified HCMV virions. One clone from each of the three cultured cell lines responded to this antigen although all responded to exogenous IL2. Unfortunately all these cloned T cell lines failed to grow sufficiently to allow further studies.

Class I MHC restricted cytotoxicity was not detected in T cells from bulk 'free' HCMV stimulated cultures, but this does not exclude the possibility that MHC class II restricted T cells could be present (Section 3.3.2). As growth conditions were suboptimal to permit transfer of T
Fig 17. Cloning of d14 HCMV stimulated T cell lines by limiting dilution.

a) 100-1cpw of 14d T cell lines from subjects 13 and 68, and d8 MLR cells were cultured for 3 weeks in the presence of 10^7 autologous irradiated PBM, IL2 and HCMV antigen. Wells were scored visually for growth and then placed on autologous irradiated PBM in flat bottomed plates. There was a low 'cloning efficiency' as only 1/151 and 1/31 of the T cells grew, as compared with 1/8 MLR cells.

b) 14d HCMV stimulated T cells were grown as above and examined after 2 (○), 3 (●) and 4 (■) weeks culture. There was no further outgrowth in previously negative wells after 3 weeks incubation.
TABLE 8. 3 proliferative T cell clones generated to 'free' HCMV antigen proliferated to purified HCMV virion (2.5ug ml⁻¹) and IL2.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Precursor freq</th>
<th>HCMV virion</th>
<th>medium</th>
<th>IL2</th>
</tr>
</thead>
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<td>1/78</td>
<td>clone 1</td>
<td>4326 (129)</td>
<td>2664 (37)</td>
</tr>
<tr>
<td></td>
<td>clone 2</td>
<td>2782 (343)</td>
<td>2662 (-)</td>
<td>3540 (238)</td>
</tr>
<tr>
<td>68</td>
<td>1/151</td>
<td>clone 1</td>
<td>4956 (615)</td>
<td>1596 (158)</td>
</tr>
<tr>
<td>13</td>
<td>1/31</td>
<td>clone 1</td>
<td>4332 (317)</td>
<td>3177 (-)</td>
</tr>
<tr>
<td></td>
<td>clone 2</td>
<td>2584 (410)</td>
<td>2671 (-)</td>
<td>3830 (-)</td>
</tr>
</tbody>
</table>

a - precursor frequency as determined for 14d 'free' HCMV stimulated PBM in limiting dilution culture (see Section 3.3.5)
b - 5x10⁶ cloned T cells (as determined by limiting dilution) were cultured in the presence of IL2 or 2.5ug ml⁻¹ HCMV virion with 5x10⁴ E autologous feeder cells for 66h followed by a 6h ³H thymidine pulse - 1µCi/well. The cells of clone 67/1 were 88% T4⁺.
clones to flat bottomed plates, their cytotoxic activity was assessed directly after 3 weeks culture in the initial plates. Total cytotoxicity was assayed against K562 cells in the presence of 2ug ml\(^{-1}\) PHA to bypass the requirement for antigen specificity (Bevan and Cohn, 1975) (Fig 18). These results suggested that a number of cytotoxic precursor cells were present in such 14d HCMV stimulated cells (frequency 1/275 cells with 1/35 cells growing under these conditions). To again test whether class I restricted Tc were present, PBM were grown under limiting dilution conditions with heat inactivated HCMV (10\(^5\)pfu) for 6d and then in IL2, changed at 3d intervals, for a total of 21d. All wells at an initial input of 5000cpw had visible cell growth, and were divided in two and assayed against autologous and HLA mismatched HCMV infected target cells (Fig 19). Similar, simultaneous but separate cultures from the same individual were assayed against K562 cells in the presence of PHA. There was no MHC class I restricted Tc activity detected but some cytotoxic cells were present in the cultures (a precursor frequency from this experiment cannot be established as only 3 input cell concentrations were examined). These observations, although preliminary, suggest that a few cytotoxic cells were generated by stimulating with 'free' HCMV particles but their specificity has not been established, although they were not MHC class I restricted HCMV specific Tc.
**Fig 18.** Growth of 14d HCMV (heat inactivated) stimulated T cells in limiting dilution culture (as described) on 5x10⁴ (●), and 10⁵ (○) feeder cells. The frequency of all cytotoxic cells, regardless of specificity (■: 10⁻⁴ [68]) was determined against K562 cells in the presence of PHA. 1/275 of the input cells were cytotoxic.

**Fig 19.** Limiting dilution analysis of HCMV specific Tc precursor cells using heat inactivated 'free' HCMV antigen. 5,000-40,000 PBM (subject 68) were grown in 96 well plates with 10⁵ irradiated PBM and HCMV for 6d. IL2 was then added for a total of 21d. All wells exhibited cell growth and cytotoxicity was assessed against autologous (●) and HLA mismatched (○) HCMV infected, and K562 (+PHA) (Fig 18). Although some cytotoxicity was detected (K562), there were no HCMV specific MHC class I restricted Tc generated.
3.4 EFFECT OF HCMV INFECTION ON DIFFERENTIATED Tc

MCMV inhibited the generation of ectranelia specific Tc in vivo and in vitro (Ho, 1980) and HCMV has been reported to reduce the proliferative response to ConA (Carney and Hirsch, 1981; Rogers et al., 1985). Casali, Rice and Oldstone (1984) found that in contrast to influenza and measles virus, AD169 strain HCMV infection in vitro had no effect on NK activity against fibroblasts or K562 cells, PHA induced proliferation and in vitro Ig synthesis. However HCMV infection of activated IL2 dependant cells, may reduce the ability of such cells to grow in vitro or affect their cytotoxic function. The ability of HCMV to affect differentiated cytotoxic T cell function in vitro was therefore investigated.

TCLl an IL-2 dependent cytotoxic T cell line established in a one way MLR and maintained in vitro for 45d prior to these experiments. Its surface phenotype was 95% Leu4a+, 98% Leu2a+, 2% Leu3a+ and variably 70-80% DR+. The lytic specificity was established against a panel of HLA defined PHA blasts as being against class I HLA antigens A7, A28, B7 and B44 - the phenotype of the irradiated stimulator PBM (Fig 20). There was no cytotoxic activity against K562 cells (Borysiewicz et al., 1985a).

TCLl were incubated for 1h in suspension with HCMV (MOI=20:1; volume<1ml) at 48h, 24h and 2h prior to the assay. The cells were then washed free of HCMV and cultured in RPMI supplemented with IL2 until assay. TCLl cells infected for 24 and 48h continued to grow in IL2 (Fig
Fig 20. TCL1 cells were studied in a 6h $^{51}$Cr release assay against 48h PHA blasts and fibroblasts (passage 12+) mismatched, and sharing identity (underlined) at MHC class I loci with the original PBM stimulator cells used to generate TCL1. Only those cells with shared identity were lysed. Primary fibroblast lines expressed HLA class I antigens that could be recognised by allospecific Tc.
The cells were harvested (viability >95%), washed and cytotoxic activity studied against PHA-blasts in a 4h $^{51}$Cr release assay (Fig 22). There was no reduction in specific Tc activity in this cell line following HCMV infection. Some of the harvested cells were washed and resuspended for a further 24h in optimum concentrations of IL2. These cultures were pulsed for 6h with $^3$H thymidine and harvested. Following virus infection, there was a reduction in the IL2 response up to 48h following infection, but cells infected for 72h had a similar proliferative response to uninfected cells (Fig 23). HCMV did not affect expression of HLA-DR on TCL1 cells (Fig 24). It remains possible that HCMV might interfere with release of IL2 or inhibit IL2 responsiveness in the initial stages of the proliferative response.

3.5 GENERATION OF HCMV SPECIFIC Tc LINES

3.5.1 Co-culture of PBM with HCMV infected fibroblasts

Generation of HCMV specific Tc could not be achieved by stimulation with 'free' HCMV antigen. Various possible explanations for this have already been discussed but a further explanation could be that the antigens recognised by Tc were not present under the in vitro conditions used e.g. non-structural antigens, some of which may be expressed on the surface of virus infected cells (Stinski et al, 1979), would not be expressed in the absence of a cell permissive for the virus. Thus
Fig 21. TCL1 cells infected for 48 and 24h with HCMV (MOI 5:1) and placed back in IL2 culture until they were harvested and counted with acridine orange/ethidium bromide. TCL1 cells continued to grow following HCMV infection.

Fig 22. HCMV infected TCL1 cells continued to mediate antigen specific cytotoxicity. Susceptible target cells (closed symbols) were lysed by TCL1 cell infected with HCMV for 0 (●), 2 (▲), 24 (■) and 48h (♦), in a 6h 51Cr release assay. There was no lysis of previously insusceptible targets - 0 (○) and 48h (◇).
Fig 23. HCMV infected TCL1 cells proliferated to IL2, although initially the proliferative response was reduced. Uninfected or HCMV infected TCL1 cells (48, 24 and 2h previously) were maintained in optimum concentrations of IL2. The cells were then harvested, washed and 2x10^5 viable cells placed in medium alone or with fresh IL2 for a further 20h. ^H thymidine incorporation after a 4h pulse (lucI/well) was estimated and results expressed as a stimulation index (cpm in IL2/cpm in medium).

Fig 24. HLA-DR expression by TCL1 cells is not affected by HCMV infection (O).
the culture conditions were ammended and PBM were co-cultured with HCMV infected autologous fibroblasts allowing expression of such antigens. The protocol adopted was as in Section 2.7.2. In initial experiments $2 \times 10^6$ PBM from a seropositive individual (cft $= 1/16-32$; subject number 34) were co-cultured with $6 \times 10^4$ autologous fibroblasts, infected for 3h with HCMV (MOI 10:1). Wells were harvested from the plate on days 1, 4, 6 and 8 and the number of viable fibroblasts as determined by acridine orange/ethidium bromide staining was estimated. In addition the lymphocytes were harvested and cytotoxicity assayed against autologous HCMV infected fibroblasts and K562 cells. The number of fibroblasts in the HCMV infected lymphocyte co-cultures did not increase during the experiment whereas uninfected and HCMV infected cells cultured in the absence of PBM increased initially (Fig 25a). The lymphocytes from such co-cultures had some NK activity against K562 cells, but after 6 days co-culture an increased cytotoxicity against HCMV infected autologous target cells infected for 24h, but not 6d, was observed (Fig 25b). This cytotoxicity did not correlate with NK activity against K562 cells which decreased during the same period. The impairment of growth in the HCMV infected monolayer was however unlikely to be secondary to this cytotoxic activity which was only demonstrable after 6d.

These experiments were then extended to determine if MHC class I restricted Tc were generated using this protocol. The protocol was modified by harvesting cells 6d following stimulation and incubating them for a further 6d on fresh HCMV infected autologous fibroblasts in the
Fig 25. Outgrowth of HCMV infected fibroblasts is inhibited by co-culture with autologous PBM. 2x10^6 PBM (subject 34) were cultured with HCMV infected (●; ) and uninfected (▲; ) autologous fibroblasts. The number of fibroblasts was counted after 1, 4 and 6d culture and compared with HCMV infected (○) and uninfected (△) fibroblasts cultured alone. The cytotoxic activity of co-cultured PBM was examined (E:T=20:1), after 1, 4 and 6d, against autologous HCMV infected fibroblasts infected at the same time as the original co-cultures (except for 6d when a 24h target cell was also used) and K562 cells. Outgrowth of HCMV infected cells was inhibited after 24h. PBL co-cultured with HCMV infected cells lysed 24h but not 6d HCMV infected autologous fibroblasts after 6d.
presence of IL2, for a further 7-8d. In initial experiments, PBM from subject 59 (cft 1/32) were co-cultured as described on autologous HCMV infected, autologous uninfected fibroblasts (ratio of lymphocytes to fibroblasts 40:1) and with live HCMV (MOI 10:1). The cultures were harvested after 14d in vitro and assayed against autologous HCMV infected and uninfected target cells. In this experiment yields of lymphocytes after 14d culture was about 8% of the original input cell number and although it was increased in later experiments to between 10-15%, the number of effector cells frequently limited the number of investigations that could be performed in each individual case. These cells lysed HCMV infected target cells only if lymphocytes were cultured in the presence of HCMV infected autologous fibroblasts (Fig 26). If E+ alone or adherent cell depleted PBM were used in the initial co-culture only small numbers (<10^4) of viable lymphocytes were present after 14d, precluding further cytotoxicity assays. Live virus stimulated cells once again did not produce any significant lysis (results for subject 59 in Table 6).

These cytotoxicity assays were performed at the maximum E:T possible with the number of available effector cells. To determine the minimum E:T required and whether the response was MHC restricted, prior to a study of this response in a larger number of seropositive individuals, MHC restricted cytotoxic activity was estimated at varying E:T (subject 3) following the protocol as before (Fig 27); subsequently an E:T of 20:1 was used. These effector cells did not lyse autologous fibroblasts infected
Fig 26. PBM (subject 59) co-cultured with autologous HCMV infected and uninfected fibroblasts (6d). The lymphocytes were then cultured in IL2 for a further 10d with fresh HCMV infected (□) and uninfected (■) fibroblasts added after 8d. Cytotoxic activity against HCMV infected and uninfected autologous fibroblasts was assayed in a 6h ⁵¹Cr release assay.

Fig 27. Cytotoxicity (+/- SD) expressed by one short term T cell line (subject 3), generated by co-culture of PBM with autologous HCMV infected fibroblasts and maintained in IL2 (Section 2.7.2), at varying effector to target ratios against HCMV infected (●) and uninfected (○) HLA matched targets. Specific ⁵¹Cr release from HLA mismatched HCMV infected (♦) and uninfected (◇) is only shown at E:T=20:1.
with UV irradiated virus.

8 seronegative and 10 seropositive normal volunteers with no clinical evidence of recent HCMV reactivation were then studied. PBM were co-cultured with autologous HCMV infected fibroblasts, and expanded in vitro in the presence of fresh HCMV infected cells and IL2 as described. Only PBM from 3 of 8 seronegative (subjects 9, 11, 71), as compared with all the seropositive subjects, established T cell lines using this technique. There was no virus specific lysis by T cells from seronegative subjects, but 9 of 10 seropositive individuals’ T cell lines lysed virus infected cells - the exception being subject 34 (cft 1/16). 8 (subjects 40, 72, 68, 59, 73, 69, 52 and 3) of these 9 lines lysed only HCMV infected autologous fibroblasts but not HCMV infected HLA mismatched cells (Fig 28). There was no relationship between the HCMV cft and the level of cytotoxicity.

The results obtained in 5 of the subjects (9, 72, 69, 40 and 70) are shown in detail in Fig 29 to show the pattern of cytotoxicity observed. In all cases where K562 cells were used to assess NK activity, some lysis of these cells was observed (Fig 29a, b and d). Lymphocytes from seronegative subjects had low levels of cytotoxicity against all the target cells except K562 cells. Most (7/9 seropositive individuals with MHC restricted lysis as shown in Fig 28) had low levels of cytotoxicity against HLA mismatched HCMV infected and uninfected target cells (as shown for subjects 72 and 69) and low lysis of uninfected autologous cells (Fig 29b and c). The two exceptions to this are shown in detail (Fig 29d and
Fig 28. Cytotoxicity expressed by 13 short term (14d) T cell lines, generated as described (Section 3.5.1), against autologous and HLA mismatched HCMV infected and uninfected target cells. The E:T was 20:1 and the % specific $^{51}$Cr release from uninfected target cells has been subtracted from lysis of HCMV infected cells. Cytotoxicity was restricted to lysis of HCMV infected autologous fibroblasts in 8 of 10 seropositive subjects.
Fig 29. Cytotoxicity expressed by 5 of the 13 subjects from Fig 28 shown in more detail. All lines were assayed in a 6h $^{51}$Cr release assay at E:T=20:1, against HCMV infected (▲) and uninfected (□) autologous and HLA mismatched target cells and when possible against K562 cells (■).

a) subject 9 (seronegative) - no significant lysis except for K562 cells.
b) subject 72 (cft 1/16) and c) 69 (cft 1/128) both show preferential lysis of autologous HCMV infected cells, although significant lysis of K562 cells was observed.
d) subject 40 (cft 1/16) had very high levels of lysis of the mismatched infected, uninfected cells and K562 cells.
e) subject 70 (cft 1/32) preferentially lysed both autologous and HLA mismatched HCMV infected cells.
subject 40 had high levels of $^{51}$Cr release from HCMV infected autologous fibroblasts and correspondingly low levels of lysis of the autologous uninfected cells. However, there were unexpectedly high levels of release from mismatched infected, uninfected and K562 cells. The other subject (70) who had HLA unrestricted lysis of HCMV infected cells was unfortunately not assayed against K562 targets; the lysis observed in this case would be compatible with preferential lysis of HCMV infected cells by NK cells (Section 4).

The surface phenotype of three of the T cell lines was determined and in contrast to the predominance of Leu3a$^+$ cells in T cell lines stimulated with free HCMV, two of the cell lines were mostly Leu2a$^+$ (Table 7).

3.5.2 HLA restriction and virus specificity

The preferential lysis of autologous compared with HLA mismatched HCMV infected target cells suggested that the effector cells were HLA restricted in their lytic activity. In confirmation of this, 14d and 21d T cell lines from 2 subjects (72 and 3), for whom non-autologous but HLA A- and B-matched, fibroblast lines were available as target cells, produced equivalent virus-specific lysis of the matched HCMV infected cells (with little or no lysis of mismatched infected target cells) (Fig 30 & 31). Evidence of virus specificity is shown in Fig 31 and 34, where HLA matched HSV infected target cells were not lysed by these HCMV specific Tc.
Fig 30. HLA restriction of cytotoxicity expressed against HCMV infected targets by 2 T cell lines (subjects 72 and 3) generated as described (Section 3.5.1), one maintained in vitro for 14d (72) and the other for 21d (3). Target cells were autologous (\(\text{\textbullet}\) aut) or HLA matched (\(\text{\textcircled{A}}\)) and HLA mismatched (\(\text{\textcircled{B}}\)) HCMV infected and uninfected fibroblasts (E:T, 20:1).

Fig 31. Virus specificity of cytotoxicity expressed by a T cell line (subject 3) against HCMV infected (\(\text{\textcircled{A}}\) ), HSV infected (\(\text{\textcircled{B}}\)), and uninfected (\(\text{\textbullet}\)) targets, showing specificity for HCMV (E:T, 20:1). There was equivalent lysis of targets infected for 6 and 48h with HCMV prior to the 6h \(^{51}\)Cr release assay.
3.5.3 HCMV specific Tc lysed cells expressing IEA and EA

To determine whether the virus antigens being recognised by Tc appeared early or late in the virus cycle, HCMV infected fibroblasts were used as target cells only 6h post infection at the start of the cytotoxicity assay. As shown in Fig 31, HLA-matched fibroblasts infected with HCMV for 6h were lysed as effectively as fibroblasts infected for 48h. Fibroblasts infected for this length of time express IEA and EA but not LA as detected by monoclonal and polyclonal antibodies.

Further studies were performed using HLA-matched HCMV infected PPF blocked target cells which again expressed only IEA and EA but no LA. When these target cells were studied in a 6h assay in the continued presence of PPF, using effector cells generated by the standard protocol (T11⁺-90%, T8⁺-50%, T4⁺-31%) (subject 74) there was lysis of the HCMV infected HLA-matched target cells but lower levels of lysis of mismatched cells (Fig 32).

The further delineation of whether the antigen recognised by HCMV specific Tc lines was an IEA or EA product has proved difficult. Attempts at inhibiting Tc activity with polyclonal and monoclonal antibodies against LA and EA (Table 3) have been unsuccessful, although in view of the restricted number of epitopes recognised by such a small panel of antibodies, make direct interpretation of this data impossible. As in the case of MCMV (Reddehase, Kiel and Koszinowski, 1984; Reddehase and Koszinowski, 1984), it should be possible to produce target cells infected with HCMV in the presence of CHX, after 3h removing the block to
Fig 32. 21d T cell line (50% T8⁺), generated by co-culture with partially matched HCMV infected stimulator cells, lysed 4d HCMV infected, PPF blocked target cells ( □ ) matched at HLA Aw24, but not HLA mismatched or uninfected ( □ ) cells. 6h ⁵¹Cr release assay, E:T=10:1.
translation but incubating with AD to prevent further transcription. Such an approach may enhance expression of the major IEA in the target cells (Jeang and Gibson, 1980). However, infection with HCMV and CHX for 3h followed by culture in AD for a further 6h, when IEA could be detected in the fibroblast monolayer (Fig 3), followed by detachment and a 5h cytotoxicity assay resulted in a spontaneous 51Cr release of >50% in most experiments, making interpretation of results difficult (also Section 4). Modifications using different incubation times, increased MOI and other blocking agents are in progress.

An alternative approach, has been to transfect human fibroblast cultures with the gene encoding the major 72kDa IEA. Boom et al (1984) were able to stably co-transfect TK- rat fibroblasts with the EcoRI-Sal fragment of the Hind III E fragment of HCMV AD169 (Fig 1), and the HSV TK gene. Cloned lines expressing the HCMV IEA as determined by immunofluorescence with E3 monoclonal antibody were obtained. In collaboration with this group, the PES plasmid containing this segment of HCMV DNA was grown and plasmid DNA isolated (Maniatis, Fritsch and Sambrook, 1982). The plasmid was transfected into human fibroblasts using the standard Ca2+ precipitation transfection procedure (Graham et al, 1974) followed by 10% glycerol shock for 15 min. 24-48h following transfection up to 20% of fibroblast in a spot slide culture expressed the major 72kDa IEA detected with E3 (Fig 33). Unfortunately expression was only transient; it was reduced by 72h and absent after 96h. In addition, subsequent attempts resulted in only 5% or fewer cells expressing IEA. The
Fig 33. Transfection and expression of the HCMV major 72kDa IEA in F5000 cells. F5000 cells were transfected with PES DNA as described (Section 3.5.3) and examined by indirect immunofluorescence using the E3 monoclonal antibody and goat anti-mouse Ig-FITC, after 48h. 20% of cells exhibited nuclear fluorescence (x400))
transfection protocol was varied to use different quantities of plasmid DNA and other human HLA defined tissue culture lines such as HeLa cells, all unsuccessfully. This approach would offer the best target cells as further study of the gene products required for Tc susceptibility would be possible. However, the variation observed together with the temporary expression of this antigen using standard transfection with the PES plasmid limits its usefulness in producing suitable target cells.

3.5.4 Delayed expression of HCMV specific Tc activity

The reproducibility of the Tc response generated using this method of co-culture was studied in 3 subjects (72 - 3 occasions; 3 - 3 occasions and 59 - 2 occasions) at 6 monthly intervals. In each subject restricted lysis of autologous HCMV infected cells was observed, although the level of cytotoxicity varied between assays.

The use of 6h as opposed to 18h cytotoxicity assays, as frequently used with fibroblast target cells (Timonen, Saksela and Hayry, 1979; Quinnan et al, 1981) was intended to reduce the possible augmentation of NK activity in vitro following co-culture with virus infected cells, thereby reducing the non-specific lysis in the assays. Furthermore all effector cells had been refed with IL2 containing medium at least 3d previously, again intending to reduce possible enhancement of non-specific lysis by either IFN or IL2 (Brooks, 1983). However, in two instances when 14d lines, established against HCMV infected autologous fibroblasts in the usual way, were used as effector cells, data obtained at 6h showed no
virus-specific $^{51}$Cr release. After the 6h assay 100ul of fresh medium was added to the wells and $^{51}$Cr release measured again after a further 12h (total incubation time - 18h), using an adjusted formula for calculating specific release (Trinchieri and Santoli, 1978). The cytotoxicity observed after 18h was still restricted to autologous HCMV infected target cells which would not be expected with NK target cell lysis. Furthermore such cytotoxicity would be difficult to explain by the requirement for target cells to express IA, as in the case of subject 72, 48h infected cells were not lysed after 6h whereas initially 4h infected cells were lysed at 18h (Fig 34).

3.5.5 Presence of HCMV infected cells is required for maintenance of Tc

To determine whether re-exposure to HCMV-infected cells after transfer to IL2 was a necessary requirement for the detection of HCMV-specific Tc in these short-term T cell cultures, PBM from 4 subjects, from 3 of whom, a HCMV specific Tc response had been previously generated, were studied as follows. After 5d initial co-culture with HCMV-infected fibroblasts, mononuclear cells were separated on Ficoll gradients and placed in IL2 containing medium with feeder cells but no further HCMV-infected fibroblasts were added. The surface phenotype at 14d was predominantly Leu3a$^+$, and no virus-specific cytotoxicity was detected (Table 9).
Fig 34. Delayed expression of HCMV specific cytotoxicity. Short term T cell lines (A - subject 3; B - 72) generated as described (Section 3.5.1) were assayed against fibroblast targets infected with HCMV for 4 or 48h (□□□□), with HSV for 12h (□□□□□), or uninfected (□□□□). No HCMV specific cytotoxicity was seen after 6h, but there was lysis of HLA matched HCMV infected cells after 18h.
TABLE 9. Requirement for HCMV infected autologous fibroblasts during culture to generate HCMV specific Tc.

<table>
<thead>
<tr>
<th>Subject&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Leu4</th>
<th>Leu2</th>
<th>Leu3</th>
<th>% +ve cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% virus sp&lt;sup&gt;b&lt;/sup&gt; &lt;sup&gt;51&lt;/sup&gt;Cr release&lt;sup&gt;h&lt;/sup&gt;</th>
<th>HLA mismatch</th>
</tr>
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<tr>
<td>68</td>
<td>85&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
<td>47</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>72</td>
<td>69</td>
<td>6</td>
<td>84</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>67</td>
<td>92</td>
<td>5</td>
<td>72</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*<sup>a</sup> - determined by indirect immunofluorescence with anti-Leu4, Leu2 and Leu3.

*<sup>b</sup> - 6h assay against 48h HCMV infected cells - E:T 20:1.

*<sup>c</sup> - PBM co-cultured with HCMV infected autologous fibroblasts for 5d. Viable lymphocytes removed and maintained in IL2 without antigen for a total of 14d prior to assay.

*<sup>d</sup> - ND = not done
3.5.6 Requirement for autologous/non-autologous stimulator cells

The use of stimulator fibroblasts to generate HCMV specific Tc, particularly whether autologous or non-autologous, was investigated. The first experiments were performed to determine whether co-culture with autologous or non-autologous uninfected fibroblasts induced the generation of cytotoxic T cells with non-specific or in the latter case specific allogeneic Tc. It had been found that fibroblasts do not stimulate a MLR, unlike irradiated PBM cells, and this has been interpreted to be the result of absent expression of class II MHC antigens (Schellekens and Eijsvogel, 1970; Sorensson, 1972). However, IFN may be released following co-culture of PBM with a number of different cell lines with induction of DR expression (Pober et al 1984). Furthermore, cytotoxicity of uninfected mismatched fibroblasts as opposed to autologous uninfected fibroblasts was increased following co-culture (Fig 28d). Cytotoxicity of uninfected fibroblasts was also increased following co-culture with uninfected fibroblasts (Fig 25).

T cells from a MLR were grown and could be expanded into a T cell line which killed allogeneic 48h PHA blasts (probably via recognition of class I determinants) and uninfected fibroblast targets of susceptible HLA type (Fig 20). PBM were then co-cultured for 6d in the presence of mitomycin C treated PBM or uninfected autologous or syngeneic fibroblasts. PBM from these co-cultures did not selectively lyse uninfected target cells although there was increased non-specific lysis (Fig 35). The numbers of such fibroblast stimulated cells was small and short term T
Fig 35. Co-culture of PBM with HLA mismatched uninfected fibroblasts does not generate allospecific Tc activity. PBM (subject 11) were cultured with autologous (F48) ( ● ), HLA mismatched fibroblasts ( ■ ) and irradiated PBM from the same HLA mismatched donor ( ♦ ). After 6d culture cytotoxicity was assessed against uninfected fibroblasts in a 6h assay. There was no allospecific cytotoxicity although non-specific lysis of fibroblasts, including autologous cells was generated.

<table>
<thead>
<tr>
<th>subj (HCMV cft)</th>
<th>antigen (LA / EA)</th>
<th>stimulation index</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 (&lt;8)</td>
<td>LA</td>
<td></td>
</tr>
<tr>
<td>66 (32)</td>
<td>LA</td>
<td></td>
</tr>
<tr>
<td>20 (&lt;8)</td>
<td>EA</td>
<td></td>
</tr>
<tr>
<td>68 (32)</td>
<td>EA</td>
<td></td>
</tr>
</tbody>
</table>

Fig 36. Proliferative response of PBM to HCMV EA. PBM from a seropositive (subject 68) and seronegative (subject 20) were cultured with HCMV LA, HCMV EA and fibroblast antigen prepared as described (Section 2.4.3). After 5d, a 18h H thymidine pulse was used to measure proliferation. Proliferative response was expressed as a stimulation index (cpm + HCMV antigen/cpm + fibroblast antigen). Low levels of stimulation by both LA and EA preparations were observed with seropositive PBM.
cell lines were not established. Thus the lysis of mismatched fibroblasts in cocultures is probably non-specific and these cultures do not generate specific alloreactive Tc.

These experiments have been extended to observe the effects of stimulating PBM using the standard protocol, but with autologous, partially matched, mismatched and foetal fibroblasts infected with HCMV, as stimulator cells. T cell lines were established from most such cultures and in the case of most autologous and 2/6 partially matched co-cultures, HCMV specific Tc were generated. Using mismatched or foetal fibroblasts (F5000) no HCMV specific Tc have so far been generated (Table 10). However, the number of these experiments is limited and before these results can be fully interpreted further studies are required.

3.5.7 HCMV specific Tc clones

In spite of generating HCMV specific Tc lines, it has proved difficult to establish Tc clones. The low frequency of outgrowth of clones (Table 8; Figs 17,18 and 19) suggested that in vitro conditions were suboptimal and these have been investigated. The overall conclusions of these experiments can be summarised as:

1) although clones of K562 cells were established using soft agar cloning (Fathman and Fitch, 1982) clones of IL2 dependant cells were not established. These assays were performed with tonsillar supernatent, and thus the IL2 was probably suboptimal. Although this technique may establish clones with soluble antigen, it would be difficult
TABLE 10. Generation of HCMV specific Tc using autologous, partially matched and HLA mismatched HCMV infected fibroblasts as stimulator cells and PBM from seropositive subjects.

<table>
<thead>
<tr>
<th>subject</th>
<th>autologous</th>
<th>partially matched</th>
<th>HLA mismatch</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>34</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>73</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>72</td>
<td>+(x3)</td>
<td>no growth (x2)</td>
<td>-</td>
</tr>
<tr>
<td>70</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>69</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>68</td>
<td>+(x2)</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>67</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>52</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>40</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>21</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+(x3)</td>
<td>no growth</td>
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</tr>
<tr>
<td>59</td>
<td>+(x2)</td>
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<td>51</td>
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<td>-</td>
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</tr>
<tr>
<td>78</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

Total Tc - 16/25 2/7 0/6

+ - MHC restricted Tc generated assayed against 1 HLA matched and mismatched target cells with >7% excess lysis of HCMV infected HLA matched cells.
- - no MHC restricted cytotoxicity by above criteria.
ND - not done
no growth - PBM cocultured but insufficient cells to perform full assay.
to maintain the Tc in the presence of HCMV infected fibroblasts. With higher activity IL2, growth in the absence of antigen may be used to select clones after 7d, and then these cultured with HCMV infected cells (experiments in progress).

2) the IL2 although adequate for the establishment and support of T cell lines was inadequate for efficient cloning. This has led to the use of commercial and MLA IL2.

3) three weeks culture in the original limiting dilution plate was required for visible clones to be established (Fig 17). After this time there were no new wells growing out and some wells showing initial outgrowth regressed. Outgrowth was also more efficient in round-bottomed as opposed to flat bottomed 96-well plates. No growth was observed when similar attempts at cloning were carried out using Terasaki plates, but the reasons for this are almost certainly due to technical difficulties in handling the small volumes used.

4) feeder cells - $10^5$, 2500rad irradiated, fresh autologous PBM were optimal for cloning (Fig 18).

5) the optimum number of stimulator cells required is still unknown but their growth has to be restricted by irradiation or mitomycin C and more frequent changes of medium employed to overcome loss of cultures.

The application of these modifications together with the use of more specific antigens (Section 3.6.3), may permit the establishment of Tc clones and these experiments are in progress.
3.6 STIMULATION OF PBM WITH HCMV EA

3.6.1 Proliferation of PBM to HCMV EA

The proliferative responses described (Sections 3.2 and 3.3) were in response to structural proteins of the virus. Antibodies against HCMV IEA and EA have been described (The, Klein and Langhuysen, 1974; Middledorp, Jongsma and The, 1985) and as such antigens are probably T dependant a population of EA specific T helper cells may be present in PBM of seropositive subjects. This was investigated by preparing 4d HCMV infected or 4d HCMV infected F5000 fibroblasts in the presence of PPF. Both cells expressed HCMV IEA and EA in 80% of cells by immunofluorescence but only the unblocked cells had HCMV LA (Fig 3). Fibroblasts were harvested, washed, suspended at $10^6$ ml$^{-1}$ and sonicated. Equal dilutions of these cells were used as antigens in a 6d proliferation assay. PBM from a seropositive individual (subject 68) proliferated at a low level in response to both LA and EA antigens (Fig 36), although the proliferative response was much lower than that observed with 'free' HCMV (Table 4; Fig 13).

3.6.2 Generation of EA specific proliferative T cell lines

When PBM were cocultured in the presence of autologous HCMV infected fibroblasts and the lymphocytes separated from fibroblasts after 5-6d, a series of T4$^+$ T cell lines were established which were not.
cytotoxic for autologous or HLA mismatched HCMV infected target cells (Table 9). As Tc in other systems require Th cell for their generation, the specificity of non-cytotoxic T cells generated following co-culture with HCMV infected autologous fibroblasts was examined. A T4+ non-cytotoxic T cell line was generated by co-culture with HCMV infected autologous fibroblasts and maintained in vitro for 35d prior to assay, in the absence of further addition of HCMV antigen. 4d after the last addition of IL2, cells were harvested and washed free of excess IL2. 10^4 T cells were then cultured for 48h in flat bottomed 96 well plates in the presence of 2x10^5 autologous PBL (not E rosette separated) in the presence or absence of HCMV antigens prepared as described in methods - equivalent to 10^4 sonicated infected, or uninfected fibroblasts per well. After 48h 100ul of the supernatent from each well (total volume 200ul) was removed and stored at 4°C for IL2 activity assay, and the medium replaced with fresh RPMI. After 66h culture 1lCi 3H Thy was added and the lymphocytes harvested 6h later. There was a HCMV specific proliferation but only in cultures grown in the presence of antigen prepared in the presence of PPF i.e. expressing IEA and EA, and a corresponding increase in IL2 activity in these cultures (Fig 37). The increase in IL2 release in feeder cell cultures following incubation with antigen may reflect IL2 release from the unseperated feeder cells in response to the antigen, but no proliferative response was observed. It is of interest that LA expressing cells which might be expected to express at least some EA did not induce a proliferative
Fig 37. HCMV EA specific proliferation and IL2 release from a non-cytotoxic T4+ cell line (35d in vitro). The lymphocyte line was established as described (Section 3.6.2) and 10^4 cells were co-cultured in the presence of 2x10^3 irradiated autologous PBM in the presence of HCMV IA ( ), HCMV EA ( ), HCMV IEA ( ) and fibroblast antigen ( ) in the absence of exogenous IL2. After 48h supernatent was removed and assayed for IL2 activity (Section 2.6.3), and proliferation of the T cells measured at 72h. There was a proliferative response to HCMV EA which was accompanied by increased IL2 release to this antigen.
response. As IEA and EA cells had been grown in the presence of PPF for 4d it is possible that the amount of some of the EA had been relatively increased compared with cells grown without inhibitor which might account for the apparent discrepancy.

3.6.3 Proliferative and cytotoxic T cell responses to a purified 72kDa EA

In collaboration with S.Graham and B.Rodgers proliferative and Tc responses were examined to a purified HCMV EA. The 72kDa HCMV EA was purified from sonicated 4d HCMV infected cells, by affinity chromatography using a column of H11 monoclonal antibody conjugated to Sepharose 4B (Rodgers, Borysiewicz and Sissons, 1985). Antibodies against this antigen were detected in the majority of HCMV seropositive (11/16) but not seronegative individuals. PBMs from 10 of these 16 seropositive subjects proliferated in response to the antigen (Fig 38). When the proliferating T cells were expanded in IL2 culture for 14d, in contrast to the results with free HCMV antigen only 8/13 short term lines were predominantly T4+. The cytotoxic activity of 2 such lines was examined against 48h HCMV infected target cells matched at HLA A and B loci and HLA mismatched target cells. In these two instances there was preferential lysis of the HLA matched HCMV infected cells (Fig 39). These results are preliminary and are not unequivocal evidence of Tc lysis, as no uninfected target cells were used in the assay and the same HCMV infected fibroblasts were the matched target cells in both cases, therefore not excluding the possibility that these cells may have been intrinsically more susceptible.
Fig 38. PBM from HCMV seropositive subjects proliferate to a purified 72kDa HCMV EA. PBM from HCMV seropositive and seronegative subjects were studied in a 6d proliferation assay against 2.5ug ml\(^{-1}\) 72kDa EA. There was no proliferative response in seronegative subjects but 10/16 seropositive PBM had a stimulation index >3.

Fig 39. Cytotoxic activity in T cell lines generated to the 72kDa EA. T cell lines were established with 2.5ug ml\(^{-1}\) 72kDa EA and maintained in the continued presence of antigen and IL2 for 20d. They were then assayed against HLA matched (-----) and HLA mismatched (------), HCMV infected target cells. In both instances there was preferential lysis of the HLA matched HCMV infected target cells.
to non-specific lysis. However, further studies are in progress.
SECTION 4

NATURAL KILLER CELL LYSIS OF HCMV INFECTED CELLS

HCMV infected cells, like many virus infected cells are susceptible to lysis by NK cells. These investigations were performed to determine:

a) the nature of the effector cells.

b) which virus gene products are required for susceptibility to NK lysis.

c) the nature of the target structure recognised on the HCMV infected cell.

4.1 NK LYSIS OF HCMV INFECTED CELLS

4.1.1 Lysis of HCMV infected fibroblasts by unstimulated PBL

To determine whether HCMV infected cells were lysed by PBL from normal seronegative subjects, PBM seperated by ficoll/hypaque were adhered to plastic petri dishes, passed down 2x10cm nylon wool columns to remove adherent cells and then assayed at varying effector:target ratios against HCMV infected, uninfected and K562 targets in a $^{51}$Cr release assay (Table 11). In 6 of 9 subjects, lysis by PBL of HCMV infected fibroblasts was greater than lysis of uninfected cells in a 20h assay at E:T = 100:1.
<table>
<thead>
<tr>
<th>Subject</th>
<th>Age and Sex</th>
<th>Untreated PBL</th>
<th>IFN-pretreated PBL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uninfected</td>
<td>IEA (SD)</td>
</tr>
<tr>
<td>1.</td>
<td>36 M</td>
<td>13.2 (2.1)</td>
<td>47.5 (5.0)</td>
</tr>
<tr>
<td>2.</td>
<td>35 M</td>
<td>3.9 (3.1)</td>
<td>40.9 (3.9)</td>
</tr>
<tr>
<td>3.</td>
<td>24 M</td>
<td>4.4 (5.5)</td>
<td>14.0 (2.9)</td>
</tr>
<tr>
<td>4.</td>
<td>33 M</td>
<td>34.6 (5.7)</td>
<td>23.7 (—)</td>
</tr>
<tr>
<td>5.</td>
<td>27 M</td>
<td>35.7 (2.9)</td>
<td>—</td>
</tr>
<tr>
<td>6.</td>
<td>38 M</td>
<td>0.0 (3.6)</td>
<td>—</td>
</tr>
<tr>
<td>7.</td>
<td>37 M</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8.</td>
<td>31 M</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9.</td>
<td>24 F</td>
<td>25.3 (1.6)</td>
<td>10.9 (6.0)</td>
</tr>
<tr>
<td>10.</td>
<td>27 F</td>
<td>31.3 (2.1)</td>
<td>27.6 (3.0)</td>
</tr>
<tr>
<td>11.</td>
<td>23 F</td>
<td>31.9 (4.4)</td>
<td>41.7 (4.4)</td>
</tr>
</tbody>
</table>

* Untreated PBL were assayed against target cells prepared as described in Materials and Methods and were incubated at 100:1 (E:T) for 20 hr (± 1 SD).

* Eighteen hour IFN-α-pretreated PBL were incubated at 50:1 (E:T) for 6 hr (± 1 SD).

* - not done.
4.1.2 Lysis of HCMV infected cells by IFN treated PBL

When PBM are treated with IFN there is increased NK lysis of susceptible target cells (Trinchieri and Santoli, 1979). NK lytic activity was studied against HCMV infected fibroblasts following incubation of adherent cell depleted PBM (PBL) (Section 2.5.2), for 18h with 4 - 10,000iu ml⁻¹ human αIFN (Fig 40). Lytic activity was enhanced in a 6h ⁵¹Cr release assay in a dose dependant manner reaching a plateau between 100 - 1000iu ml⁻¹. In time course studies, enhanced cytotoxicity against HCMV infected fibroblasts was observed in a 6h assay when PBL were preincubated for as little as 4h (total time = 10h) although maximal stimulation occurred after 6h preincubation (Fig 41). Overnight incubation with 1000iu ml⁻¹αIFN was thus routinely employed to pretreat lymphocytes to obtain maximum enhancement of NK activity. Longer term studies, incubating PBM with 1000iu ml⁻¹αIFN for between 1 to 5d and assaying against K562 cells, showed that the enhanced NK activity was lost by 48h. However, when PBL were cultured in 20% IL2 conditioned medium (<20iu ml⁻¹ IFN by plaque reduction) the NK activity was maintained at higher levels throughout the experiment (Fig 42).

When PBL from 11 subjects were pretreated with 1000iuαIFN, PBL from all but 1 subject lysed HCMV infected more readily than uninfected fibroblasts, although lysis of both uninfected and HCMV infected cells was increased. The difference in lytic activity was now observed in a 6h assay ⁵¹Cr release assay at E:T = 50:1 (Table 11). PBL from the three subjects whose untreated PBL produced no excess lysis of HCMV infected
Fig 40. NK activity against HCMV infected cells is enhanced by pretreatment with human αIFN. 4x10⁶ ml⁻¹ PBM were depleted of adherent cells and incubated for 18h with varying concentrations of affinity purified human αIFN from 4-10,000iu ml⁻¹. PBM were then assayed against HCMV EA target cells in a 6h ⁵¹Cr release assay at varying E:T (100:1-6.25:1). NK activity was enhanced up to 1,000iu ml⁻¹ but very high levels suppressed activity.

Fig 41. Enhancement of NK activity against HCMV-EA target cells is evident 4h after incubation with 1,000iu ml⁻¹. PBM depleted of adherent cells were incubated in the presence (●) or absence (○) of 1,000iu ml⁻¹ for varying lengths of time, prior to a 6h ⁵¹Cr release assay (results shown at E:T=100:1).
Fig 42. IFN enhanced cytotoxicity is lost within 48h of treatment but IL2 enhanced cytotoxicity is retained for 6d. PBM depleted of adherent cells were incubated with 1,000iu ml⁻¹ IFN or 20% conditioned medium with IL2 (<20iu ml⁻¹ IFN) and then assayed against K562 at E:T=50:1.

Fig 43. HCMV infected target cells expressing IEA are no more susceptible to NK lysis than CHX and AD treated uninfected fibroblasts. PBM from 3 subjects depleted of adherent cells were incubated with 1,000iu ml⁻¹ for 18h prior to a 6h Cr release assay against HCMV infected CHX and AD treated cells (●——●) and similarly treated uninfected cells (●——●). There was no excess lysis of the HCMV IEA expressing cells.
200

cells, had enhanced cytotoxicity following IFN pretreatment.

4.1.3 NK lysis of HCMV infected fibroblasts was not MHC restricted.

It has been suggested that autologous fibroblasts were less susceptible to NK lysis than non-autologous cells (Takkanen, Timonen and Saksela, 1980). This was investigated using both uninfected and HCMV infected fibroblasts autologous, partially HLA matched and mismatched to PBL in a 18h $^{51}$Cr release assay. Again, HCMV infected cells were more readily lysed than uninfected cells but there was no difference in the lysis of autologous partially HLA matched or mismatched target cells (Table 12).

4.1.4 Susceptibility to NK lysis is coincident with HCMV-EA expression

F5000 cells were firstly infected with HCMV for 4d (LA), infected for 4d in the presence of PPF (EA) or with CHX and AD to express IEA alone, then with $^{51}$Cr as described and used as target cells in a 6 (IFN treated) or 18h (untreated) release assay with IFN treated (E:T,50:1) or untreated (E:T,100:1) PBL. Cells expressing HCMV EA and IEA were lysed as readily as (4d) HCMV infected cells expressing LA (Table 11). PPF pretreatment alone did not inhibit or enhance NK mediated lysis of uninfected MRC-5 cells (Fig 46). Target cells expressing IEA alone were used only in the 6h assay with IFN treated effector cells as spontaneous release was >40% in 18h assays. Lysis of HCMV-IEA cells was similar to lysis of uninfected fibroblasts, identically treated with CHX and AD (Fig
TABLE 12 Lysis of autologous, partially matched and HLA mismatched, HCMV infected and uninfected primary fibroblasts by NK cells.

<table>
<thead>
<tr>
<th>Fibr.</th>
<th>PBL (subject number)</th>
<th>%sp 51Cr release (E:T,100:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>HCMV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+/-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 -</td>
<td>13.8 (16)(^A)</td>
<td>7.1 (7)(^1)</td>
</tr>
<tr>
<td></td>
<td>13.3 (1)</td>
<td>28.8 (10)</td>
</tr>
<tr>
<td>2 -</td>
<td>15.6 (2)(^1)</td>
<td>14.1 (4)(^A)</td>
</tr>
<tr>
<td></td>
<td>40.2 (2)</td>
<td>25.9 (6)</td>
</tr>
<tr>
<td>3 -</td>
<td>16.3 (12)(^0)</td>
<td>16.4 (2)(^1)</td>
</tr>
<tr>
<td></td>
<td>49.4 (3)</td>
<td>78.5 (5)</td>
</tr>
<tr>
<td>4 -</td>
<td>-3.4 (14)(^0)</td>
<td>16.6 (4)(^1)</td>
</tr>
<tr>
<td></td>
<td>35.2 (8)</td>
<td>34.4 (3)</td>
</tr>
<tr>
<td>5 -</td>
<td>33.7 (18)(^0)</td>
<td>32.2 (8)(^0)</td>
</tr>
<tr>
<td></td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

\(^a\) superscript figures in table indicate the number of shared HLA A and B determinants between PBL and fibroblasts (A=autologous).

\(^b\) 18h 51Cr release assay (+/- SD); nd = not done.
Fibroblasts sham infected with UV inactivated virus even at MOI >20:1 were not more susceptible to NK lysis than uninfected fibroblasts.

The requirement for expression of HCMV-EA was studied in a time course experiment to determine when increased susceptibility to lysis was observed. MRC-5 fibroblasts were infected with HCMV (MOI=5:1) at 48,16,10,6 and 2h prior to the assay. 1h prior to the assay the cell layer was washed and medium containing 2ug ml⁻¹ AD added to infected and uninfected cells to block further DNA transcription. Enhanced susceptibility to lysis was observed at 16h when only HCMV-IEA and -EA were expressed (Fig 44). Cells infected for 6 to 10h expressing predominantly IEA and 2h infected cells which might have virus antigens on their surface by direct virus-cell attachment/fusion showed no enhanced susceptibility to lysis compared with uninfected cells.

4.1.5 Susceptibility of HCMV infected cells to NK lysis is independent of IFN

IFN is released following HCMV infection of fibroblasts and co-culture of PBL and NK targets (Starr et al, 1980). The effect of this IFN on HCMV infected target cell susceptibility to NK lysis was investigated. Pretreatment of uninfected fibroblasts with IFN reduces their susceptibility to NK lysis (Fig 45) (Trinchieri and Santoli, 1979). However this "protective" effect of IFN in HCMV infected cells is lost, but there was no further enhancement of target cell susceptibility (Fig 46).

During the cytotoxicity assay IFN was released but this was
Fig 44. MRC-5 fibroblasts were infected with HCMV (MOI 5:1) for varying periods and then treated with AD (20ug ml⁻¹) to block additional viral DNA transcription. These target cells were incubated with IFN-pretreated PBL at varying E:T in a 6h ⁵¹Cr release assay. 16h infected cells, expressing predominantly HCMV EA, were lysed as readily as 48h infected cells expressing HCMV LA (+/- SD; * indicates values significantly (p<0.05 by Student's t-Test) different from lysis of uninfected fibroblasts).
Fig 45. Uninfected fibroblasts are 'protected' from NK mediated lysis by pretreatment with 1,000iu ml$^{-1}$ (in a 25ml T25 flask on a confluent monolayer of MRC-5 cells). Fibroblasts were then used as target cells in a 6h $^{51}$Cr release assay with IFN treated PBM at varying E:T. The protective effect was evident between 6-12h following treatment with IFN.

Fig 46. HCMV-infected, HCMV-infected treated with PPF (expressing only IEA and EA), uninfected and uninfected treated with PPF alone MRC-5 fibroblasts were used as target cells in a 6h $^{51}$Cr release assay with IFN-pretreated PBL (E:T, 50:1). All targets were either IFN treated (1,000iu ml$^{-1}$ for 18h) (□□) or untreated (□□). This IFN pretreatment of the target cells reduced susceptibility to lysis of uninfected fibroblasts. HCMV-infected fibroblasts were more susceptible to lysis than uninfected cells, and this susceptibility was not significantly affected by IFN pretreatment.
iu ml\(^{-1}\) IFN in the first 6h of the assay. The possibility that this intra-assay IFN might enhance target cell susceptibility was studied by adding excess exogenous \(\alpha\) IFN (8,000 - 1,000 iu ml\(^{-1}\)) to the cytotoxicity assay, using IFN pretreated PBL as effector cells. No increase in \(^{51}\)Cr release was observed. Thus although NK activity of PBL was increased following IFN treatment, the enhanced target cell susceptibility of HCMV infected cells was probably independent of IFN.

4.2 IDENTIFICATION OF NK CELLS LYSING HCMV INFECTED CELLS

4.2.1 Separation of NK cells lysing HCMV infected cells on Percoll

Timonen, Ortaldo and Herberman (1981) showed that NK cells characteristically are LGL and can be separated from B and T cells by Percoll gradient centrifugation (Timonen and Saksela, 1980). This technique was applied to study the distribution of NK cells mediating lysis of HCMV infected cells in Percoll gradients (Section 2.5.4). PBL depleted of plastic and nylon wool adherent cells were separated on a 42.5% to 62.5% discontinuous Percoll gradient. NK lytic activity against HCMV infected and K562 cells was present in the same 47.5-52.5% fractions (Fig 47a). The cells from these fractions were morphologically typical large granular lymphocytes with azurophilic granules on 10% Giemsa staining and represented 10% of PBL recovered from the gradient (Fig
Fig 47a and b. PBL depleted of adherent cells were incubated with IFN for 18h and separated on a discontinuous Percoll-PBS gradient (42.5-62.5%) at 550g for 30min. Lymphocytes were harvested from the different fractions:

a) they were assayed against K562 ( ▲ ) and HCMV EA ( ● ) target cells at varying E:T (50:1-6.25:1). Lytic activity against both HCMV EA and K562 cells is present predominantly among lymphocytes in the lowest percoll densities.

b) cells were counted ( ● ) and analysed with anti-Leu 7 ( ▲ ) and anti T11 ( ○ ). The fractions mediating NK lysis had increased Leu 7a but the majority of Leu 7a cells were in inactive fractions.
47b). When the surface phenotype of the lymphocytes in the various fractions was analysed, 20% of all Leu7a+ cells were present in fractions with NK activity (Fig 47b).

4.2.2 NK cells lysing HCMV infected cells express T11

NK lysis of HCMV infected cells by lymphocyte subsets, as defined by the monoclonal T cell antibodies anti-T11, -T4 and -T8, was studied. PBM depleted of adherent cells by plastic adherence and nylon wool columns had slightly increased NK activity against K562, HCMV infected and uninfected cells. NK activity was not detected in E+ rosetted cells (>95% T11+ cells), when a 2h incubation period for rosetting was used (Section 2.5.3). NK activity was present in the 'E-' population although reanalysis of these cells, using the anti-T11 monoclonal antibody, showed that although reduced, 37% T11+ cells were still present in this population.

The question as to whether the NK cells that lysed HCMV infected fibroblasts expressed low levels of the E receptor on their surface, as do NK cells lysing K562 (Herbermann, 1982), was studied by sorting T11+ cells according to intensity of fluorescence (Fig 48). Nylon wool passed PBM were stained with anti-T11, goat anti-mouse Ig-FITC, and analysed on the EPICS C. The PBM were sorted by intensity of fluorescence into 'high', 'intermediate' and 'absent' fluorescent populations. Sorted samples were reanalysed to determine the purity of the sort. These cells were then used as effector cells in a 12h 51Cr release assay against K562,
Fig 48. NK cells mediating lysis of HCMV EA and K562 cells express low levels of T11. Adherent cell depleted PBM were stained with anti-T11 and sorted as described (Section 2.5.7 and 4.2.2) into high, intermediate and low intensity fluorescent populations, and purity of sort re-analysed. The cells were then studied in a 12h ^51Cr release assay against HCMV EA, uninfected and K562 target cells. PBM expressing intermediate amounts of T11 were enriched for NK activity against all three target cells.
4d HCMV infected PPF blocked and uninfected F5000 cells. Cells with intermediate staining were enriched for NK activity against all three target cells, whereas no NK activity was present in the strongly T11+ cells. Similar experiments with anti-T4 and -T8 showed NK activity in the T4-, but in both T8+ and T8- cells against all 3 targets.

4.2.3 Leu 7a+ and Leu 7a- PBL lysed HCMV infected fibroblasts

Abo and Balch (1981) produced a monoclonal antibody (Leu 7a) that identified human LGL. The number of Leu7a+ (and Leu11a+) PBL, correlated with the NK activity of PBL against K562 cells (Fig 49). Most anti-K562 NK activity was present in the Leu 7a+ subset of PBL although some activity was present in the Leu 7a- fraction (Abo, Cooper and Balch, 1982). The relative contribution of these two populations to NK mediated lysis of HCMV infected cells was therefore examined. Addition of anti-Leu 7a to the cytotoxicity assay did not inhibit lysis, therefore adherent cell depleted PBM were further depleted of Leu 7a+ cells (Section 2.5.6). The number of Leu 7a+ cells as assessed by indirect immunofluorescence corresponded to the number of non-viable cells present after incubation with adsorbed rabbit serum (Table 13). The cells were then cultured overnight in the presence or absence of γIFN (1000iu ml⁻¹). Non viable cells were removed by Ficoll/Hypaque centrifugation and an aliquot of the PBL was examined by indirect immunofluorescence with Leu 7a (Table 13). The remaining cells were studied in a 6h and 18h ⁵¹Cr release assay against K562, Molt-4,
Fig 49. The number of Leu 7a+ and Leu 11a+ cells in PBM was associated with NK activity against K562 cells. Adherent cell depleted PBM from 8 subjects, were analysed to determine the number of Leu 7a+ (○) and Leu 11a+ (▲) cells and these were compared with NK activity against K562 in a 6h assay (E:T, 100:1-6.25:1). Higher numbers of PBM of both phenotypes were associated with increased cytotoxicity of K562 cells.

Fig 50. IFN pre-treated and untreated, adherent cell depleted PBM were depleted of Leu 7a+ cells (■) or C alone (□) and studied in a 6 and 18h 51Cr release assay against HCMV LA, HCMV EA, uninfected F5000, Molt4 and K562 cells (E:T, 50:1-6.25:1). Lysis of uninfected cells was negligible with both sets of effector cells in this experiment and therefore these results are not shown. Lysis of HCMV infected cells was mediated by Leu 7a+ cells but also by Leu 7a+ cells particularly after 18h incubation.
Enhanced NK activity following IFN treatment of Leu 7a depleted PBL is not associated with reappearance of Leu 7a+ cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells (10^6)</th>
<th>Leu 7a (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/H PBM</td>
<td>1.16x10^8</td>
<td>1% n-v; 8% Leu 7a+</td>
</tr>
<tr>
<td>Nylon wool x1</td>
<td>1.04x10^8</td>
<td>1% n-v</td>
</tr>
<tr>
<td>Nylon wool x2</td>
<td>1.01x10^8</td>
<td>1% n-v; 9% Leu 7a+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pre F/H (%)</th>
<th>post F/H (%)</th>
<th>Leu 7a %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+IFN</td>
<td>13.2</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
<td>-IFN</td>
<td>15.0</td>
<td>0.7</td>
<td>1.6</td>
</tr>
<tr>
<td>+IFN</td>
<td>0.9</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>-IFN</td>
<td>1.7</td>
<td>10.6</td>
<td>11.5</td>
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<td>+IFN</td>
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</tr>
<tr>
<td>-IFN</td>
<td>0.6</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

n-v = non-viable
F/H = ficoll/hypaque separation
HCMV-LA, HCMV-EA and uninfected fibroblast target cells (Fig 50).

PBL depleted of Leu 7a+ cells lysed both HCMV and K562 cells less readily than total PBL. However this reduction was less marked against HCMV infected fibroblasts than tumour cells. IFN treatment of both PBL and Leu 7a+ depleted PBL, enhanced NK lysis. This enhanced lysis was not associated with the re-appearance or increased numbers of Leu 7a+ cells in the depleted populations (Table 13).

4.2.4 Leu 11a+ PBM lysed HCMV infected cells

Negative selection studies were supplemented by FACS selected Leu7a+ and Leu11a+ lymphocyte subpopulations. Previous studies (Abo, Cooper and Balch, 1982; Lanier et al, 1983) have shown that NK activity against K562 cells is present in both Leu7a+ and Leu11a+ subpopulations. The results of negative selection with Leu7a were confirmed by positive selection. PBL were pretreated with IFN or incubated in medium alone for 18h at 37°C and then stained with Leu 7a. Cells were sorted at 2000 cells/sec using forward angle light scatter (FALS) - cell size - and log. green fluorescence as sorting parameters. Leu 7a+ and Leu 7a− cells were collected simultaneously into medium. The cells were kept on ice (approx 6h) then washed and used in the cytotoxicity assay as described, against HCMV-EA targets. Leu 7a+ cells (>97% Leu 7a+ on reanalysis) lysed the targets more readily than did the Leu 7a− cells (<1% Leu 7a+), but IFN pretreatment enhanced NK lysis in both subpopulations (Fig 51).
Fig 51. Lysis of HCMV EA expressing cells by Leu 7a \(^+\) and Leu 7a \(^-\) cells separated by FACS. PBM were treated for 18h with IFN \(\bullet \) or maintained in medium alone \(\circ\), and then stained with anti-Leu 7a and goat anti-mouse Ig-FITC. Cells were sorted using the EPICS C (2,000 cells/sec) into Leu 7a \(^+\) \(\circ\) and 7a \(^-\) \(\bullet\) populations. NK activity (12h assay) in both populations was enhanced by IFN but NK activity against HCMV EA predominated in the \(7a^+\) cells.
Although NK activity against K562 and HCMV infected cells is found predominantly in the Leu 7a+ subpopulation, the number of 'active' Leu7a+ NK cells within that subset is probably small. Percoll gradient fractionated PBL mediating NK activity were only slightly enriched for Leu7a+ cells (20% compared with 11% in PBL) and 20% of all Leu7a+ were present in the low density fractions (Fig 47b).

Anti-Leu 11a is a murine IgM monoclonal antibody that recognises the Fc receptor present on the majority of human NK cells and has been used to separate NK cells lysing tumour targets (Lanier et al, 1983). PBL pretreated with IFN were sorted into Leu 11a+ (>95% Leu 11a+ on reanalysis) and Leu 11a- (<1% Leu 11a+) subpopulations. Again cells mediating lysis of HCMV infected cells were present predominantly in the Leu 11a+ population (Fig 52).

Thus in studies examining the surface phenotype of effector cells mediating lysis of K562 and HCMV infected cells there was little discernable difference. Both NK populations were LGL which could be separated into the same percoll fractions, had low levels of surface T11, variable expression of T8 and were predominantly Leu7a+ and Leu11a+.

4.2.5 Adherence and depletion studies of NK cells

To determine whether NK cells lysing HCMV infected cells were the same cells that lysed K562 and uninfected fibroblasts, PBL were examined in adherence depletion experiments. PBL binding to K562 cells were
Fig 52. PBL pretreated with IFN were sorted by EPICS C (Section 2.5.7) into Leu 7a, 7a, 11a and 11a subpopulations and were assayed against HCMV EA target cells (4 different E:T ratios at each assay). NK lytic activity was predominantly mediated by 7a and 11a PBL.

<table>
<thead>
<tr>
<th>Effector Cell</th>
<th>Target</th>
<th>E : T</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562 adh PBL</td>
<td>K562</td>
<td>20</td>
</tr>
<tr>
<td>K562 non-adh PBL</td>
<td>K652</td>
<td>20</td>
</tr>
<tr>
<td>K562 adh PBL</td>
<td>HCMV</td>
<td>20</td>
</tr>
<tr>
<td>K562 non-adh PBL</td>
<td>HCMV</td>
<td>20</td>
</tr>
</tbody>
</table>

Fig 53. PBL were separated into K562 binding and K562 non-adherent cells as described (Section 2.5.5). Cells eluted from conjugates lysed both K562 and HCMV EA target more readily than K562 non-adherent cells, although some lysis was observed in the latter population. (12h 51Cr release assay; E:T 20:1).
separated by the method described by Timonen et al (1979b) (Section 2.5.5). NK cells lysing HCMV infected cells predominantly bound to K562 cells, although in this experiment K562 lysis was not fully depleted in the K562 non-adherent cells (Fig 53).

NK cells lysing HCMV infected cells are probably the same cells that lysed uninfected fibroblasts. When PBL, pretreated with IFN and depleted of adherent cells, were incubated on uninfected fibroblasts 10% of the PBL bound to the monolayer. When the unbound PBL were assayed against K562, HCMV infected and uninfected fibroblasts there was a reduction in the lysis of tumour lines (K562 and Molt4) but complete abrogation of NK mediated lysis of fibroblast and HCMV infected cells was observed (Table 14). The surface phenotype of the PBL adherent to the fibroblasts could not be determined as the number of cells recovered was small. Flow cytometric analysis of the non-adherent cells did not reveal any significant reduction in the T11, T4, T8, DR, Bl, Leu 7a or Leu 11a subsets.

To determine whether PBL could be 'recruited' into the subset that lysed HCMV infected fibroblasts, PBL depleted of adherent cells were incubated on fibroblast monolayers for 2h and the non-adherent cells harvested, washed and incubated in fresh medium with IFN for 18h. Half the previously adhered cells were once again incubated on uninfected fibroblast monolayers for 2h, and the non adhered cells harvested. The lymphocytes were then assayed against K562 and HCMV EA and uninfected fibroblasts (Fig 54). The lytic activity of cells incubated on
TABLE 14 Depletion of NK lytic activity by adherence to uninfected fibroblasts

<table>
<thead>
<tr>
<th>hours adhered</th>
<th>K562</th>
<th>Molt4</th>
<th>HCMV EA</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>207 (100)</td>
<td>ND</td>
<td>117 (100)</td>
<td>64 (100)</td>
</tr>
<tr>
<td>1</td>
<td>127 (61)</td>
<td>ND</td>
<td>10 (9)</td>
<td>17 (26)</td>
</tr>
<tr>
<td>2</td>
<td>41 (20)</td>
<td>ND</td>
<td>&lt;1 (&lt;1)</td>
<td>&lt;1 (&lt;1)</td>
</tr>
<tr>
<td>3</td>
<td>45 (22)</td>
<td>ND</td>
<td>&lt;1 (&lt;1)</td>
<td>&lt;1 (&lt;1)</td>
</tr>
<tr>
<td>Expt 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>191 (100)</td>
<td>ND</td>
<td>120 (100)</td>
<td>44 (100)</td>
</tr>
<tr>
<td>1</td>
<td>75 (39)</td>
<td>ND</td>
<td>18 (15)</td>
<td>22 (51)</td>
</tr>
<tr>
<td>2</td>
<td>20 (10)</td>
<td>ND</td>
<td>&lt;1 (&lt;1)</td>
<td>&lt;1 (&lt;1)</td>
</tr>
<tr>
<td>3</td>
<td>26 (14)</td>
<td>ND</td>
<td>&lt;1 (&lt;1)</td>
<td>&lt;1 (&lt;1)</td>
</tr>
<tr>
<td>Expt 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>325 (100)</td>
<td>200 (100)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
<td>227 (70)</td>
<td>44 (22)</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>93 (29)</td>
<td>10 (&lt;5)</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>104 (32)</td>
<td>10 (&lt;5)</td>
<td>ND</td>
</tr>
</tbody>
</table>

- lysis of target cells expressed as LU (Section 2.8.5). Figures in parentheses indicate the percent lytic activity remaining after adherence.
Fig 54. Adherent cell depleted PBM were incubated for 2h on a confluent monolayer of uninfected F5000 cells (lymphocyte:fibroblast 10:1) (▲) or in a plastic flask (●). The non-adherent cells were harvested and placed in culture with IFN for 18h (4x10⁵ PBM/ml; 1000iu/ml IFN). The previously adhered cells were then either studied directly or incubated for a further 2h on uninfected fibroblasts as described (♦). Lysis of HCMV and uninfected F5000 cells in particular was not affected by incubating on uninfected cells prior to the addition of IFN, but markedly reduced by incubating on uninfected F5000 after IFN activation.
fibroblasts prior to IFN treatment was similar to that of IFN treated PBL that had not been adhered. However, adherence following IFN treatment again depleted the PBL of effector cells, particularly those lysing HCMV infected and uninfected fibroblasts. These observations suggest that NK cells mediating lysis of HCMV infected cells also lyse uninfected fibroblasts, and they may be recruited into this subpopulation of cells from an 'inactive' population.

4.3 TARGET CELL BINDING ASSAYS

4.3.1 Increased binding of PBL to HCMV infected target cells

A possible explanation for the increased susceptibility to lysis of HCMV infected target cells might be increased fragility of and consequent $^{51}$Cr release by, HCMV infected target cells. To determine whether the increased susceptibility to NK lysis was associated with increased binding of NK cells, a modification of the target cell binding assay described by Roder and Kiessling (1978) was used (Section 2.8.4). Effector cells, depleted of adherent cells, pretreated with IFN and labelled with FITC, were mixed with target cells and incubated to allow conjugates to form. The cells were examined by UV/phase contrast microscopy and the number of effector cells binding to target cells, as a proportion of the total number of lymphocytes was determined (Table 15). Target cell binding was greater to HCMV infected than uninfected
### TABLE 15

Target cell binding of IFN pretreated PBL to HCMV infected fibroblasts

<table>
<thead>
<tr>
<th>Target cell</th>
<th>Cells bnd/ total PBL counted</th>
<th>%PBL bnd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected fibroblasts</td>
<td>95/1090</td>
<td>8.72</td>
</tr>
<tr>
<td>HCMV infected fibroblasts</td>
<td>144/1118</td>
<td>12.88</td>
</tr>
<tr>
<td>K562</td>
<td>93/470</td>
<td>19.8</td>
</tr>
</tbody>
</table>

a E : T 1:1 assayed as described in Methods

b p< 0.0001 between values by X²
fibroblasts.

4.3.2 A flow cytometric method to facilitate target cell binding estimations

The target cell binding assay as described by Röder and Kiessling (1978) had numerous difficulties: 1) a large numbers of cells had to be examined, 2) it was difficult to assess whether a lymphocyte was bound to the target cell particularly if it was above or below the target cell in the plane of the slide, 3) operator error and bias. These problems were overcome by single blind reading of slides and reading multiple slides of the same assay by one or more observers, but the number of assays that could be performed was limited. The protocol was therefore modified to determine if the target cell binding assay could be performed using flow cytometry.

K562 and the other target cells used in these studies were generally larger than PBL and could be separated from the bulk of PBL by flow cytometry using the FALS (Fig 55). This was insufficient to distinguish conjugates as the lymphocytes when bound were too small to significantly affect the FALS impulse. PBL were incubated with FITC as described and could then be separated from K562 cells both by FALS and log green fluorescence (LGFL) intensity. The PBL and target cells were mixed together (E:T=1:1), incubated at 31°C, to block target cell lysis, for 10min, and centrifuged (1000g for 5m at room temperature). The medium was aspirated and cells were resuspended in 1% paraformaldehyde in PBS using a pasteur pipette. The cells were analysed with 488nm incident light.
Fig 55. Flow cytometric analysis of target cell/PBL binding. K562 and PBL labelled with FITC (Section 2.8.4 and 4.3.2) were analysed on an EPICS C (laser - 200mW 488nm; LGFL hv-1025mV; FALS gain 1) and results expressed as a 2 parameter contour histogram analysing 20,000 cells. When the two cells were mixed under the conditions of the target cell binding assay (panel 3) then a third population of 'cells' was identified, which when sorted revealed predominantly conjugates of K562 and PBL. The results were analysed by quadrant analysis, using cursors as indicated in the figure, to determine the number of 'conjugates' as compared with the number of PBL in the lower right-hand quadrant. See also Table 16.
at 200mW from a 5W argon laser, measuring FALS (gain 1) and LGFL (high voltage 900mV) between 530-560nm at 250 cells/sec flow rate.

By quadrant and projection analysis an estimate of the number of conjugates could be obtained (Fig 55). The number of conjugates measured expressed as a percentage of PBL correlated directly both with estimates of target cell binding by direct microscopy and $^{51}$Cr release when PBL from 9 individuals were assessed and compared with simultaneous 4h $^{51}$Cr release assays using K562 targets (Table 16).

To ensure that only true effector:target conjugates were enumerated the following controls were employed. Both target cells and effector cells were incubated separately and examined to estimate the number of effector or target cell clusters that entered the 'conjugate' area on analysis. Secondly, cells were diluted and run at a rate 250 cells/sec which reduced the possibility of non-bound effector and target cells being analysed simultaneously particularly as the differential pressure between the sheath fluid and sample stream was low to allow conjugates to be analysed. Only >95% viable target cells were used, as non-viable target cells were smaller and any conjugates with such cells would not register. Non specific binding was checked by using two controls - mixing paraformaldehyde fixed K562 and PBL-FITC and performing assays with EDTA to inhibit conjugate formation.
TABLE 16 Comparison of target cell binding to Molt4 cells and NK mediated lysis by 9 different individuals.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Cell numbers</th>
<th>% bound</th>
<th>51Cr release assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(3)</td>
<td>(4)</td>
<td>50:1 (+/−SD)</td>
</tr>
<tr>
<td>A</td>
<td>6885</td>
<td>499</td>
<td>7.2</td>
</tr>
<tr>
<td>B</td>
<td>8007</td>
<td>494</td>
<td>6.2</td>
</tr>
<tr>
<td>C</td>
<td>7235</td>
<td>233</td>
<td>3.2</td>
</tr>
<tr>
<td>Dd</td>
<td>8168</td>
<td>335</td>
<td>4.1</td>
</tr>
<tr>
<td>E</td>
<td>17225</td>
<td>1165</td>
<td>6.8</td>
</tr>
<tr>
<td>F</td>
<td>6530</td>
<td>710</td>
<td>10.9</td>
</tr>
<tr>
<td>G</td>
<td>13271</td>
<td>701</td>
<td>5.2</td>
</tr>
<tr>
<td>H</td>
<td>16376</td>
<td>893</td>
<td>5.4</td>
</tr>
<tr>
<td>K</td>
<td>978</td>
<td>56</td>
<td>5.7</td>
</tr>
</tbody>
</table>

a - cell numbers determined by quadrant analysis (3)-PBL; 
(4)-conjugates
b - % 'conjugates' corrected for cross-over of Molt4 cells
c - IU calculated on 5 E:T (100:1-6:1)
d - sample heavily contaminated with RBC.
As NK cells lysing HCMV infected cells were a subset of those lysing K562 cells and could also be depleted on monolayers of uninfected F5000 cells, cold-competitor studies were performed to determine if the target structure(s) recognised by the effector cells on HCMV infected cells were also present on K562 and uninfected cells. PBL pretreated with IFN and depleted of adherent cells were incubated with $^{51}$Cr labelled HCMV infected PFP treated, uninfected F5000 and K562 cells (E:T=50:1) with similar unlabelled cells were added to cultures in varying numbers as cold competitors. The assays were performed in the presence of 2ug ml$^{-1}$ AD to prevent possible cross infection. Although NK lysis of HCMV infected cells was inhibited by uninfected F5000 cells addition of unlabelled K562 cells did not produce significant inhibition (Fig 56). In addition, the inhibition of lysis produced by HCMV-EA cells was greater than the inhibition produced by uninfected cells even when labelled uninfected cells were used as targets.

These results suggested that the target structure(s) recognised by NK cells on HCMV-EA cells was also present on uninfected fibroblasts and was recognised by the same subset of NK cells. However, the target structure(s) must either be bound with greater affinity by NK cells or be expressed in increased amounts in the HCMV infected target cell. Although the increased susceptibility of HCMV infected cells to NK lysis is coincident with HCMV-EA expression, the target structure recognised by the
Fig 56. Cold target competition between K562, uninfected and HCMV EA fibroblasts: 5x10^5 Cr-labelled target cells were incubated with varying numbers of unlabelled K562 (●), uninfected (■), or HCMV EA (▲) fibroblasts in the presence of AD (10μg ml^-1) to prevent possible cross infection. PBL pretreated with IFN were added at an E:T of 50:1 in two separate experiments (a and b) and ^51Cr release measured at 6h. Figures in parentheses indicate percent specific ^51Cr release in the absence of competitor cells. There was reciprocal cold target competition between HCMV EA and uninfected fibroblasts.
NK cell was probably not encoded by HCMV. An alternative explanation, although not entirely consistent with the adherence depletion results, was that two or more populations of NK cells, one recognising 'host' target structure(s) the other recognising 'viral' structure(s) might be interacting to result in the increased lysis observed.

4.5 HCMV INFECTED GUINEA PIG FIBROBLASTS ARE NOT LYSED BY HUMAN NK CELLS

HCMV is species specific and only human fibroblasts can be productively infected in vitro, although HCMV can abortively infect fibroblasts of other species particularly guinea pig fibroblasts (Stinski, 1983). In these cells, HCMV antigen expression is blocked during the 'early' phase and no viral DNA replication occurs. HCMV-IEA (Fig 57) (Michelson et al, 1979; Stinski, 1978) and some of the EA (Stinski, 1978), expressed in the permissively infected cell were also expressed in guinea pig fibroblasts. As HCMV infected guinea pig fibroblasts have been more extensively investigated than other non-permissive cells, they were used to test whether at least those HCMV-EA and IEA that are expressed in such cells could themselves be target structures recognised by human NK cells. Guinea pig fibroblasts were grown from primary explants of adult heart tissue cultured in MEM supplemented with 15% FCS. HCMV infected (MOI=10:1) in the presence or absence of PPF, and uninfected human F5000 and guinea pig fibroblasts were
Fig 57. Guinea pig fibroblast (passage 4) as used in the cytotoxicity assays (Fig 58 and 59) were infected with HCMV for 24h, fixed in acetone and examined by indirect immunofluorescence with E3 and goat anti-mouse Ig-FITC. 30-40% of the cell layer expressed HCMV IEA (x250).

Fig 58. Human NK cells lyse human but not guinea pig fibroblasts expressing IEA and some EA. F5000 cells and guinea pig fibroblasts were infected with HCMV (MOI 20:1) in the presence of PPF and expression of IEA was confirmed by indirect immunofluorescence (Fig 57). Infected and uninfected cells were used as target cells in a 6h $^{51}$Cr release assay with IFN treated PBL from 5 different HCMV seronegative donors. F5000 fibroblast expressing HCMV IEA and EA were lysed but not the infected or uninfected guinea pig cells.
used as target cells in a 6h $^{51}$Cr release assay using PBL from 5 seronegative subjects, depleted of adherent cells and preincubated with IFN in two separate experiments. There was no increased lysis of HCMV infected guinea pig fibroblasts as compared with uninfected cells whereas HCMV infected F5000 cells were again more susceptible to NK lysis (Fig 58).

NK activity has also been identified in guinea pig spleen cells (Ermin et al, 1980). To determine if any HCMV induced changes in guinea pig fibroblasts could be recognised by guinea pig as opposed to human NK cells, the above experiment was repeated using guinea pig lymphocytes isolated by Ficoll/Hypaque (d 1.078) centrifugation of adult spleen cells. These cells were used as effector cells (IFN unstimulated) in an 8h $^{51}$Cr release assay at varying E:T against F5000 and guinea pig fibroblasts infected with HCMV or uninfected. There was no increased susceptibility to lysis of HCMV infected F5000 cells but HCMV infected guinea pig fibroblasts were more readily lysed (Fig 59).

These results, although not conclusive, supported the possibility that the NK target structure(s) in HCMV infected cells was not a virally encoded product but a host cell determinant, or a virus induced modification of the host cell, whose expression is increased coincident with HCMV EA expression.
Fig 59. HCMV infected and uninfected guinea pig and F5000 (PPF treated) cells were studied in a 6h cytotoxicity assay with human PBL (●) and guinea pig spleen cells (depleted of adherent cells) (♦). Guinea pig spleen cells lysed HCMV infected guinea pig fibroblasts more readily than uninfected cells, but there was no excess lysis by these effector cells of human HCMV infected cells.
A number of putative target structures for NK cells on virus infected and tumour cells have been proposed. The studies described suggested that the target structure(s) on HCMV infected cells is probably present on uninfected fibroblasts but its expression may be increased in HCMV infected cells. Vondinelich et al (1983) suggested that the TfR may be a target structure for NK cells on tumour cell lines. As Tf is essential for cell growth in vitro (Hutchings and Sato, 1978; Trowbridge and Domingo, 1981) and TfR are expressed on cultured cells including fibroblasts (Hamilton, Wada and Sussman, 1979) the effect of HCMV and other virus infection on TfR expression was studied to determine whether the TfR could also be a NK target structure on virus infected and uninfected human fibroblasts.

4.6.1 Transferrin binding to HCMV infected cells

To determine whether the TfR may be a NK target structure on fibroblast and HCMV infected cells TfR expression was measured. It was possible that a virus encoded protein lacking the epitope for anti-T9 monoclonal antibody could bind human transferrin, therefore TfR expression was assayed by binding of Fe³⁺ saturated 125I-Tf (Ward, Kushner and Kaplan, 1982a; Frazier et al, 1982; Ward, Kushner and Kaplan, 1982b). F5000 and MRC-5 oells uninfected, at various times following HCMV infection and PPF blocked HCMV infected cells were studied. MRC-5 cells
both HCMV infected and uninfected had lower TfR levels than similarly treated F5000 cells but the relative differences between infected and uninfected cells were the same for the two lines.

There was increased Tf binding to HCMV infected cells as compared with uninfected cells at 37°C (Fig 60, 61 and 67; Table 17). This binding was Tf specific as it was competitively inhibited by unlabelled human Tf. Scatchard analysis of free vs bound Tf at varying concentrations, was used to determine whether there were differences in the $1/K_D$ (dissociation constant) for the Tf-TfR interaction. (from the gradient of the regression line) and the number of Tf binding sites expressed in HCMV infected and uninfected cells by linear regression analysis to determine the 'X' axis intercept (Ward, Kushner and Kaplan, 1982a; Baxter and Funder, 1979; Lefkowitz and Michael, 1983). $1/K_D$ was the same for both HCMV infected and uninfected cells (Fig 60); a difference in the gradient was observed at 6h in Fig 60, but this was not a consistent finding. The relationship between bound and free Tf was linear, suggesting a single receptor ligand interaction occurring both in the HCMV infected and uninfected cells with the same $1/K_D$, implying that it was probably the cellular TfR and not a virus encoded protein binding labelled Tf.

Tf binding sites/cell were increased 16h following HCMV infection and in PPF treated HCMV infected cells, expressing only HCMV IEA and EA, at a time when the infected cells were more susceptible to NK lysis (Table 11; Fig 44; Table 16). HCMV infected cells expressing IEA alone (2-6h
Fig 60. TfR expression is enhanced in HCMV infected cells when HCMV IEA
and EA are expressed, and is coincident with increased susceptibility to
NK lysis. Binding of $\text{Fe}^{2+}$ saturated $^3\text{I-Tf}$ was assayed (Section
2.9.1), in uninfected (●), and HCMV infected (MOI, 20:1) confluent F5000
cells at 18h (■) and 6h (▲) following infection. From the intercept of
the Scatchard plot, the Tf binding sites/cell (+/− 1SE) was: uninfected
F5000 = $4.6 \times 10^5$ (+/−0.3); 6h infected = $3.4 \times 10^5$ (+/−0.1); and
18h infected = $8.8 \times 10^5$ (+/−0.4) molecules/cell.
Fig 61. Total and recycling Tf binding is increased in HCMV infected cells. Confluent F5000 fibroblasts were infected with HCMV 48h prior to assay (MOI=20:1). Tf binding to infected and uninfected fibroblasts at 37°C (recycling) and 4°C (surface), and binding to solubilised cells (total) was studied as described (Section 2.9.1 and 2.9.2) and binding/cell determined by linear regression analysis of Scatchard plots. There was increased total and recycling TfR in HCMV infected compared with uninfected F5000 fibroblasts (+/- 1SE).
following infection) did not show increased Tf binding and were not more susceptible to lysis (Table 11; Figs 43 and 44).

TfR are rapidly endocytosed following Tf-TfR interaction and after Fe$^{3+}$ release from the receptor-ligand complex with the reduced pH in receptosomes, TfR are recycled to the cell surface (Hopkins, 1983; Klausner et al, 1983; Lamb et al, 1983; Brown, Anderson and Goldstein, 1983). Studies of Tf binding at 37°C assayed recycling Tf binding sites. To determine whether increased Tf binding at 37°C was a result of an increase in the number of binding sites in infected cells, or greater availability of sites already present but not actively cycling, and if they were expressed on the surface of the fibroblast, Tf binding was performed at 4°C and in supernatents of cells solubilised in 1% NP40, to determine surface and total binding respectively (Ciechanover et al, 1983). Total and surface Tf binding were increased in HCMV infected cells and these receptors were able to recycle at 37°C (Fig 61). However the number of sites on the surface of HCMV infected cells was only marginally increased. The surface binding of OKT9 monoclonal antibody was also examined using $^{125}$I-goat anti-mouse Ig second antibody (all performed at 4°C). There was a small increase in bound counts in the infected cells similar to the results of direct Tf binding. By indirect immunofluorescence of HCMV infected, PPF treated HCMV infected and uninfected cells, OKT9 staining was faint but uniformly distributed.

The ability of the TfR to recycle in the HCMV infected cell was also examined using the Na$^+$ ionophore monensin. At a concentration of
20μg ml⁻¹ monensin has been shown to inhibit recycling of the LDL receptor and Tf binding by permitting receptor-ligand complexes to enter the cell but preventing uncoupling and hence receptor recycling (Basu et al, 1981). When HCMV infected or uninfected cells were treated with monensin alone at 37°C for varying periods of time and then Tf binding measured at 4°C to estimate the TfR remaining on the cell surface, there was only a slight reduction of surface TfR. However, when HCMV infected and uninfected cells were incubated at 37°C with 20μg ml⁻¹ monensin in the presence of saturating concentrations of unlabelled Tf, there was a rapid loss of surface TfR both in HCMV infected and uninfected cells (Fig 62) implying that receptor ligand interaction and endocytosis of the complex occurs normally in the HCMV infected cell. These cells could not be used directly in NK assays as monensin treatment was reversible and cytotoxicity assays could not be performed in the continued presence of monensin as it inhibited NK lysis probably by blocking release of granules.

4.6.2 NK lysis of virus infected cells correlates with Tf binding

It has been shown that there is marked variation in the susceptibility of different virus infected cells to NK mediated lysis (Santoli, Trinchieri and Lief, 1978; Rager-Zisman and Bloom, 1982). Tf binding at 37°C and susceptibility to lysis of F5000 cells infected with a number of viruses was examined (Table 17). The incubation times following infection with each virus varied but were selected so as to
Fig 62. Monensin blocks TfR recycling in HCMV infected and uninfected fibroblasts. The recycling of surface TfR was studied by incubating fibroblasts in the presence of monensin (20ug ml^-1) alone (-----) or monensin with Fe^{3+} saturated Tf (--------) for varying periods. The cells were then placed on ice and the medium removed and washed with ice cold PBS. The surface binding of {sup}125I-Tf was then estimated. Both in uninfected and HCMV infected, cells lost more than 50% of surface receptor within 3 minutes of incubating cells in the presence of Tf. This indicates that the TfR in HCMV infected cells can recycle as rapidly as in uninfected cells.
Table 17 - Transferrin binding and NK lysis of virus infected fibroblasts.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Time post inf(h)</th>
<th>Tf binding sites/cell x 10^-5</th>
<th>NK lysis (LU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFV</td>
<td>6-12</td>
<td>2.8</td>
<td>136</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>6-12</td>
<td>6.0</td>
<td>-</td>
</tr>
<tr>
<td>Polio</td>
<td>6-12</td>
<td>5.4</td>
<td>198</td>
</tr>
<tr>
<td>Uninfected</td>
<td>-</td>
<td>5.6</td>
<td>181</td>
</tr>
<tr>
<td>HSV</td>
<td>24-30</td>
<td>5.4</td>
<td>161</td>
</tr>
<tr>
<td>HCMV</td>
<td>18-24</td>
<td>10.0</td>
<td>356</td>
</tr>
<tr>
<td>HCMV</td>
<td>48+</td>
<td>10.8</td>
<td>418</td>
</tr>
</tbody>
</table>

a) MOI = 10:1

b) NK lysis of virus infected target cells by IFN treated PBL from 3 separate subjects studied in a 6h 51Cr release assay.
recover >90% viable cells with a spontaneous release of <40% in 6h from an infected monolayer to act as target cells. Tf binding expression was reduced in SFV infected cells 12h following infection and they were less susceptible to NK lysis.

Virus infected and uninfected F5000 cells were also studied in reciprocal cold competition assays (Fig 63a). HCMV infected cells readily competed with other virus infected and uninfected cells, whereas SFV infected cells, with reduced Tf binding competed poorly. This suggested that Tf binding measured at 37°C was related to the ability of a virus infected cell to act as a competitor. The efficiency of a virus infected cell to act as a competitor, was estimated by the cold competitor:target ratio required for 50% inhibition of lysis as calculated by exponential regression analysis of 4-5 different cold competitor:target ratios for each combination. This was compared with the relative Tf binding between the competitor and target cell, irrespective of the infecting virus, in the 32 reciprocal combinations studied (Fig 63b). There was a relationship between the relative Tf binding at 37°C and the inhibition observed which was non-linear, but it suggests that virus infected cells with increased Tf binding compete more efficiently, relative to the Tf binding of the target cell.

4.6.3 Distribution of TfR in vaccinia infected cells

The distribution of TfR in vaccinia infected cells was also studied at 4°C, 37°C and in disrupted cells (Fig 64). These
Fig 63. Cold competition by virus infected F5000 cells is related to TfR expression and was independent of the infecting virus. F5000 fibroblasts infected as in Table 17, with SFV (▲), Polio (■), HSV (◇), HCMV (◆) (24h and 48h (●) infected) or uninfected (○) were studied in reciprocal cold competition assays at competitor/target ratios of 10:1, 5:1, 2.5:1 and 1.25:1. Simultaneously, TfR expression was determined by Tf binding at 37°C as described (Section 2.9.1). Each cold competition assay against an individual labelled target cell produced a series of inhibition curves (as shown against uninfected target cells in A). The efficiency of competition was determined by the cold competitor to target ratio required to produce 50% inhibition when the curves were examined by exponential linear regression analysis (r>0.9 in all cases). The 50% inhibition cold competitor/target ratio was then plotted against the relative TfR expression in each cold competitor/target cell combination (Panel B). As the relative TfR expression was reduced (i.e. greater TfR expression in the target cell than cold competitor) there was a reduction in the efficiency of the competition observed.
cells are relatively insusceptible target cells (Santoli, Trinchieri and Lief, 1978). In contrast to HCMV infected cells, the total TfR in vaccinia infected cells was unchanged 8h following infection (MOI=100:1). However recycling TfR measured at 37°C was reduced.

4.6.4 NK lysis of uninfected fibroblasts correlated with Tf binding

Tf binding at 37°C by uninfected F5000 cells was dependant on the iron content of the medium in which the cells were cultured (Fig 65, 66 and 67) (Ward, Kushner and Kaplan, 1982a). To determine whether such cells were more or less susceptible to NK lysis, F5000 cells of the same passage were grown to confluency in 50mm petri dishes and the medium replaced with medium containing 10% iron depleted FCS, or 10% normal FCS supplemented with increased concentrations of ferric ammonium citrate for a further 4 days. TfR expression and susceptibility to NK lysis were then studied as previously described. F5000 cultured in iron depleted serum were more susceptible to lysis with increased TfR expression whereas cells cultured in iron supplemented medium had reduced TfR expression and were less susceptible to lysis (Fig 65, 66 and 67).

4.6.5 Effect of HCMV on fibroblasts grown in excess and reduced Fe^{3+}

The effect of HCMV infection on TfR expression and susceptibility to NK lysis of fibroblasts maintained in reduced or excess Fe^{3+} was examined. F5000 cells were allowed to grow to confluency in 35mm PD and the medium changed to BME with 10% normal FCS, 10% dialysed FCS (no
Fig 64. TfR expression in vaccinia infected and uninfected cells. 

\[ ^{125}I\text{-Tf} \] binding was studied in uninfected and 8h vaccinia infected cells (MOI 100:1), at 37°C ( ), 4°C ( ) in solubilised cells for total receptor expression ( ). Unlike HCMV infected cells there was no difference in the number of TfR in the vaccinia infected and uninfected cells, but the number of recycling TfR was significantly reduced.

<table>
<thead>
<tr>
<th>Tf binding/cell</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td></td>
<td>7.2 x 10^5</td>
<td>3.3 x 10^5</td>
<td>1.6 x 10^5</td>
</tr>
<tr>
<td></td>
<td>(0.4)</td>
<td>(0.4)</td>
<td>(0.2)</td>
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Fig 65. Susceptibility to NK lysis of uninfected F5000 cells is related to their TfR expression. Uninfected F5000 cells were grown to confluency and then maintained in 10% Fe^3+ depleted FCS (A), 10% FCS (B) and 10% PCS supplemented with 20μg ml^-1 Fe^3+ in the form of ferric ammonium citrate (C), for 4 days. Tf binding in identically treated cultures was determined at 37°C (Section 2.9.1), and was A = 7.2x10^5 (+/-0.2) mol/cell; B = 3.3x10^5 (+/-0.4) mol/cell; C = 1.6x10^5 (+/-0.2) mol/cell. Susceptibility to NK lysis was studied in a 6h ^{51}Cr release assay by IFN treated PBL from two different subjects (--- ,---- +/- 1SD).
Fig 66. Uninfected fibroblasts cultured in excess Fe$^{3+}$ had reduced TfR expression and were less susceptible to NK lysis. Confluent F5000 cells maintained in the presence of BME and 10% FCS supplemented with varying amounts of Fe$^{3+}$ in the form of ferric ammonium citrate showed reduced Tf binding (■) and were less susceptible to NK lysis by IFN treated PBL in a 6h Cr release assay (●—● lysis at E:T, 50:1 +/- LSD; or lytic units ▲—▲).
measurable Fe$^{3+}$) or 10% normal FCS supplemented with 20ug ml$^{-1}$ ferric ammonium citrate for a further 4 days. The medium was then aspirated, half the petri dishes in each treatment group were infected with HCMV (MOI=20:1) and medium supplemented as before, added back to the cells for a further 48h. The cells were labelled with $^{51}$Cr and used as target cells in a 6h assay with IFN treated PBL from 2 HCMV seronegative subjects. Simultaneously, Tf binding at 37°C was estimated by Tf binding in similarly treated F5000 cells, expression of IA and virus released after 6d were examined.

There was increased Tf binding in the uninfected cells grown in reduced Fe$^{3+}$ and reduced expression in cells cultured with increased Fe$^{3+}$ compared with cells grown in normal serum (Fig 67). This again correlated with the susceptibility to NK lysis of these uninfected cells. Following HCMV infection which progressed to HCMV IA expression in all 3 target cells, there was a further increase in Tf binding in fibroblasts irrespective of Fe$^{3+}$ content of medium. This was associated with increased susceptibility to NK lysis, although this enhancement was most marked in those cells where the initial TfR expression was lowest, it did not strictly correlate with TfR expression alone (Fig 67). Virus release was also reduced in cells maintained at low Fe$^{3+}$ concentrations.
Fig 67. Effect of HCMV on fibroblasts grown in reduced or excess Fe³⁺. Uninfected F5000 cells were grown in dialysed FCS, Fe³⁺ supplemented FCS or untreated FCS as described (Section 2.9.3). After 4d culture these cells were left uninfected or infected with HCMV (10:1) for 48h prior to assay. Tfr expression at 37°C was estimated as before and the cells were used as target cells in a 6h ⁵¹Cr release assay with IFN treated PBL from two subjects (●, ▲). There was no inhibition to HCMV LA expression, and when virus yields were examined after 6d culture (only in normal and reduced FCS) 5.28x10⁶ pfu ml⁻¹ (untreated) and 2.25x10⁵ pfu ml⁻¹ (reduced Fe³⁺) HCMV were released. As before uninfected cells had reduced and increased TfR expression depending on the Fe³⁺ content of the medium, and if TfR were reduced these cells were less susceptible to NK lysis. HCMV infection resulted in an increase in TfR expression in all three treated cells but although this was associated with an increase in susceptibility to NK lysis it was not entirely proportional to the number of TfR expressed.
4.6.6 Tf binding in IFN treated fibroblasts

Uninfected fibroblasts cultured in the presence of IFN are less susceptible to NK mediated lysis (Fig 45 and 46). This reduced susceptibility to lysis of F5000 cells pretreated with 1000iu ml\(^{-1}\) IFN was also associated with reduced Tf binding (Fig 68).

4.6.7 NK lysis is not inhibited by excess Tf

To determine whether saturating the TfR with excess Tf could inhibit NK mediated lysis of HCMV infected cells, NK assays were performed in the presence of up to 5mg ml\(^{-1}\) Fe\(^{3+}\)-Tf. No inhibition of NK lysis was observed.

4.6.8 Isolated TfR failed to inhibit NK lysis

Vondinelich et al (1983) showed that OKT9 affinity purified TfR obtained from Molt 4 cells could inhibit lysis of K562 cells. TfR has also been isolated by affinity chromatography on Fe\(^{3+}\) saturated Tf conjugated to agarose (Light, 1977). This protocol was used to partially purify TfR, to determine if such preparations could inhibit NK lysis. The presence of active TfR in the column eluates was initially monitored by Tf binding followed by 32% saturated (NH\(_4\))\(_2\)SO\(_4\) precipitation (Fig 8). Fractions were assessed for inhibitory activity against K562 cells as these were the standard target cell used by Vondinelich et al (1983). Although the initial surface digest was inhibitory (Fig 69), no inhibition was observed in fractions with Tf.
Fig. 68. Incubation of uninfected F5000 cells (x) with 1,000iu ml⁻¹ IFN (○) for 18h, resulted in reduced Tfr expression when studied by equilibrium binding at 37°C with ¹²⁵I-Tf.

Fig 69. NK lysis of K562 cells was inhibited by a trypsin digest of Molt4 cells. A surface trypsin digest of Molt4 cells was prepared as described (Section 2.9.5) and a similarly prepared 'digestion mixture' in the absence of Molt4 cells was used as control. Trypsin inhibitor and bovine haemoglobin was added to both mixtures to block the activity of the enzyme. The solutions were then added at varying dilutions to K562 cells with IFN treated PBL (4h assay; E:T 50:1). There was a more marked inhibitory effect with the Molt4 surface digest. Further purification of such digests on Tf-Sepharose columns failed to detect this inhibition in the Tfr containing fractions.
Improved yields were obtained of a single 76kDa protein, up to 30μg ml\(^{-1}\) (Abs\(_{280}\)) using acid elution from the Tf column. However, this material also failed to inhibit K562 lysis.

4.7 EFFECT OF HCMV INFECTION ON OTHER CELL SURFACE RECEPTORS

In addition to TfR, cultured fibroblasts express a number of other cell surface receptors including receptors for LDL (Brown, Anderson and Goldstein, 1983) \(\alpha_2\) macroglobulin (Dickson, Willingham and Pastan, 1981), epidermal growth factor, insulin etc. The TfR is expressed over the total surface of the cell and only migrates to the coated pit region following receptor-ligand interaction. Other cell surface receptors, such as possibly those for \(\alpha_2\) macroglobulin and LDL are found in greater numbers in the coated pit region (Brown, Anderson and Goldstein, 1983; Bretscher and Pearse, 1984). Expression of these receptors which are not distributed evenly over the cell surface was studied to determine if expression of other receptors in the HCMV infected cell was also increased, or whether this was a restricted or selective phenomenon, for TfR.

4.7.1 LDL binding and degradation

Confluent F5000 cells were infected with HCMV (MOI=10:1) for 48h. The cells were washed and incubated with serum-free medium for
Fig 70. LDL binding was increased in HCMV infected cells but there was no increase in the rate of LDL degradation. $^{125}$I-LDL at varying concentrations was added to 48h HCMV infected (●) and uninfected (▲) fibroblasts in the presence (---) or absence (-----) of 0.1mg ml$^{-1}$ unlabelled LDL as specificity control. There was increased binding/uptake of LDL in HCMV infected cells (panel A). However, when the rate of degradation of LDL was measured (Section 2.10) there was no significant difference between the two cells (panel B).
2h. Varying concentrations of $^{125}$I-LDL were added to the cell layers, with specificity controls (excess unlabelled LDL) and petri dishes without cells incubated with labelled LDL. After 4h incubation at 37°C the supernatent was harvested and free $^{125}$I extracted to measure LDL degradation, in addition to cell bound $^{125}$I. There was an increase in the $^{125}$I-LDL bound to HCMV infected compared with uninfected cells but no increase in LDL degradation was observed (Fig 70). The increased binding may reflect an increase in recycling LDL receptor number in HCMV infected cells.

4.7.2 $\alpha_2$ macroglobulin receptor

$\alpha_2$ macroglobulin receptor expression was examined by equilibrium binding analysis as with LDL and Tf using the method described by Dickson, Willingham and Pastan (1981). There was no significant increase either in recycling (measured at 37°C) or surface (measured at 4°C) $\alpha_2$ macroglobulin receptor in HCMV infected cells.
SECTION 5

DISCUSSION

These investigations were undertaken to examine the nature and specificity of human lymphocyte responses of normal persistently infected individuals to HCMV. In summary the observations described in the preceding sections were:

a) T cells

1) T cells from seropositive individuals proliferated in response to 'free' HCMV antigens. These cells were predominantly T4+ and non-cytotoxic; such T cell lines and clones were established. These lines continued to proliferate specifically to HCMV antigens and this was associated with release of IL2 activity. Under the conditions used in the lymphocyte proliferation assay, T cells responded predominantly to HCMV LA, although EA specific responses, to both a mixture of EA and to a single 72kD EA were also present.

2) MHC class I restricted, HCMV specific Tc were generated from PBM of normal seropositive individuals without clinical or serological evidence of HCMV reactivation. These cells were predominantly T8+ and could only be generated in response to HCMV infected fibroblasts but not to 'free' HCMV antigens. These cell lines were maintained in the presence of HCMV infected autologous primary fibroblast lines and at least a proportion of the Tc recognised HCMV IEA/EA which are expressed prior to
HCMV DNA replication.

b) **NK cells**

1) NK cells mediating lysis of HCMV infected fibroblasts were non-adherent LGL, and predominantly surface T4⁻, low levels of T11, T8⁺ or T8⁻, but also Leu 7⁺ and Leu 11⁺. Their lytic activity was enhanced by human IFN and IL2. NK cells mediating lysis of HCMV infected cells were probably of the same subset of cells that lysed uninfected fibroblasts which were in turn, a subset of the NK cells mediating lysis of K562 tumour cells.

2) HCMV infected cells were more susceptible to human NK lysis. This susceptibility was evident before viral DNA replication had occurred, when only HCMV IEA and EA were expressed. The susceptibility to NK lysis was independent of IFN; uninfected and fibroblasts infected with other viruses competitively inhibited HCMV NK lysis. Furthermore, guinea pig fibroblasts expressing some EA were not lysed. These observations suggested that the target structure(s) recognised by NK cells on HCMV infected cells were probably not virally encoded, but expressed on uninfected fibroblasts, although their expression might be increased coincident with HCMV-EA following infection.

3) Vondinelich et al (1983) proposed the TfR as an NK target structure on tumour cells. The expression of TfR in HCMV and other virus infected cells was examined to determine whether the same might apply to virus infected cells. HCMV infected cells expressed an increased number of
TfR when HCMV EA were expressed and this correlated with increased susceptibility to NK lysis. SFV infected cells were relatively insusceptible to NK lysis and had reduced TfR expression. Moreover, susceptibility of uninfected fibroblasts to NK lysis was also related to TfR expression. However, the increase in susceptibility of HCMV infected cells to NK lysis was not associated with increased surface TfR and NK lysis was not inhibited by excess Tf or partially purified TfR.

5.1 T CELL RESPONSES

The immune response protects the host against acute virus infection and in the case of persistent viruses, may play an important role in limiting reactivation, thereby helping to maintain an equilibrium between the virus and host. Clinically, this is exemplified by the increased frequency of severe complications associated with these viruses in the context of immunosuppression e.g. in the case of HCMV, by the morbidity and mortality associated with HCMV infection in IS transplant recipients.

HCMV specific antibody is probably important in preventing primary infection or reducing the morbidity associated with such infections (Meyers et al, 1983). In addition, maternal antibody may partly protect the foetus against reactivation of the virus during pregnancy (Stagno et al, 1982). The protective role of antibody during established HCMV
infection (primary and secondary) is even less well established, although hyperimmune globulin has been used in the treatment of patients with HCMV infection (Blacklock et al, 1985). As the antibody response is predominantly directed against the virion, it is possible that these antibodies may be important in inactivating virus once it has been released. However, it is also well recognised that many patients with severe secondary HCMV disease have high titres of different types of anti-HCMV antibodies.

Thus cell mediated immune responses may be particularly important in such infections, in addition to their role in the recovery from primary infection. Studies of specific cell mediated immunity to HCMV have however been often limited to the investigation of proliferative responses. There was no reduction of lymphocyte proliferation to HCMV antigen in IS bone marrow transplant recipients with severe HCMV infection (Meyers, Flournoy and Thomas, 1980). However, when MHC restricted cytotoxicity of HCMV infected cells by PBM from patients with active disease, was examined (Quinnan et al, 1981; 1982; 1984), failure to develop this response was associated with a poor prognosis of HCMV infection. By analogy, MCMV specific Tc protect mice against MCMV challenge (Quinnan et al, 1977; Ho, 1980) even when disease is established (Reddehase et al, 1985).

In the case of another human herpesvirus, EBV, Tc develop after primary IM and remain at a high precursor frequency in PBM of normal seropositive individuals (Moss et al, 1981; Rickinson et al, 1981;
Yao et al, 1985). This has led to the suggestion that such cells may be important in limiting the reactivation of EBV infection in the normal persistently infected individual thereby helping maintain the virus-host equilibrium. Similar Tc may be important in determining the recovery from HCMV infection and yet these Tc were not directly measurable in PBM of normal persistently infected subjects, their role in these circumstances was unknown. These studies were undertaken to determine whether HCMV specific MHC restricted Tc were present in normal persistently infected individuals as with EBV specific Tc, the conditions required to generate Tc in vitro and the specificity of the response with respect to the virus cycle in the infected cell.

HCMV infection is widely prevalent in the population, thus it was important to define from the outset which individuals had been infected with the virus. The ideal situation would be to demonstrate HCMV infection by direct isolation or at least identifying the virus genome in cells from an individual. However, these techniques are currently insensitive and as with the other herpesviruses, serological status is equated with previous exposure. The identification of seronegative individuals is therefore dependent on the sensitivity of the antibody detection assay used. Subjects identified as seropositive by the cft assay (Krech, 1973) proved to be seropositive by the more sensitive IgG ELISA assay (Booth et al, 1979; Burroughs, 1984). However 40% of cft 'seronegative' individuals had HCMV IgG antibodies by ELISA at 1/50 serum dilution. It was therefore decided, albeit arbitrarily, to define a
seronegative subject as one with undetectable serum HCMV IgG in serial serum dilutions from 1/25.

Most studies of HCMV specific T cell immunity have used HCMV specific lymphocyte proliferation as a measure of 'cell mediated immunity' against the virus (Ten Napel et al, 1977; Moller-Larsen et al, 1976; Gerhz et al, 1977; Reynolds et al, 1979; Levin et al, 1979; Meyers, Flournoy and Thomas, 1980; Schrin, Roenhorst and The, 1981). As with other antigen specific proliferation assays, PBM were incubated with the antigen and proliferation measured by \(^3\)H thymidine uptake, estimated 5-7 days later depending on the antigen. A proliferative response to HCMV was observed in seropositive but not in seronegative subjects. Numbers in each group were small but some increase in the magnitude of the proliferative response was seen in those with cft titres >128. Although this assay is reproducible it is insensitive in determining previous exposure to the virus especially in IS individuals. In this group of patients it is interesting (although patient numbers were small) that the proliferative response, was reduced in a subgroup of the strongly seropositive patients. This did not however correlate with the development of clinical HCMV infection. In the one patient with primary HCMV infection who was sequentially studied, the HCMV proliferative response was delayed, as has been observed by Levin et al (1979).

The use of this simple assay has obvious advantages in attempting to assess 'cell mediated immunity' in large numbers of individuals. However, as the nature and function of the T cells responding in these
cultures is unknown, a restricted aspect of the T cell response to HCMV may be being examined. Reinherz et al (1979) showed that under similar conditions VZV antigen induced proliferation of T4+ cells, whereas in the MLR predominantly T8+ responded (Reinherz et al, 1980). Cultures established to inactivated influenza virus resulted in the generation of non-cytotoxic T4+ T cell lines and clones that augmented HA specific antibody production in vitro (Lamb et al, 1982). However, Sethi, Stroehman and Brandis (1980) reported that secondary in vitro stimulation of PBM from patients who recovered from HCMV infection, with UV inactivated HCMV resulted in the establishment of HCMV specific Tc. Thus the nature of the T cells from normal seropositive subjects, responding in these cultures was examined.

The HCMV specific proliferative response, was dependent on the presence of adherent cells and E+ cells in the culture as with other such responses. Cells cultured in the presence of HCMV infected MRC-5 cells or UV inactivated HCMV did not mediate MHC restricted cytotoxicity. Furthermore when T cell lines were established in IL2, they were T4+, did not mediate HCMV specific MHC restricted cytotoxicity but even after 47 days culture continued to proliferate in response to HCMV antigen. This proliferation was associated with release of IL2 in response to antigen challenge. Thus these cells shared many of the characteristics of Th cell lines and clones established to other antigens. Direct help in antibody production was not demonstrated because of difficulties in generating specific HCMV antibody in vitro; only low
titres of specific IgG (1/6 experiments) or no specific antibody (5/6) was produced by the method described by Callard and Smith (1981).

The antigenic specificity of these T cell lines was also investigated. T cell clones from such lines proliferated in response to gradient purified HCMV suggesting that predominantly HCMV LA were recognised. This is not surprising as mostly HCMV LA was present in the 'free' HCMV used to stimulate PBM (Waner, 1975). As the majority of the HCMV specific antibodies are directed against HCMV LA, these proliferating T cells may be representative of HCMV specific Th although direct evidence for this is lacking as yet.

However, antibodies against HCMV IEA and EA have also been detected (The, Klein and Langenhuysen, 1974; Michelson-Fiske, Horodinceanu and Guillon, 1977; Michelson et al, 1979; Blanton and Tevethia, 1981; Middeldorp, Jongsma and The, 1985). PBM from seropositive subjects proliferated in response to HCMV EA and a non-cytotoxic T cell line established to HCMV infected cells proliferated and released IL2 in response to HCMV EA. In addition PBM proliferated in response to an isolated 72kDa HCMV EA. Thus HCMV specific proliferative T cell responses are generated both to LA and EA.

In contrast to the findings of Sethi, Stroehman and Brandis (1980) no HCMV specific Tc could be generated against 'free' HCMV even using PBM from patients following recent HCMV infection, as originally described. Several possibilities could have explained this discrepancy. Firstly, it was possible that target cells might not express the relevant...
determinants for Tc recognition. Target cells expressed HLA class I antigens as detected by W6/32 monoclonal antibody and HLA class I specific Tc, and also expressed HCMV antigens as detected by an anti-HCMV polyclonal antibody and C. The possibility that trypsinisation of cells had removed HCMV antigens recognized by Tc was excluded by performing assays having infected and labelled cells in situ. Retrospectively, these same target cells were later lysed by Tc generated against autologous HCMV infected fibroblasts.

Secondly, it was possible that the presence of live HCMV could have inhibited the development of Tc responses. Ho (1980) noted that MCMV inhibited the development of anti-LCMV Tc and Carney and Hirsch (1981) reported that HCMV infection of monocytes from seronegative individuals or monocytes from patients with primary HCMV mononucleosis, inhibited Con A induced proliferation of PBM. However, HCMV specific Tc were not generated in the presence of UV (as originally described by Sethi et al) or heat inactivated HCMV. Furthermore, an allospecific Tc line (recognising MHC class I determinants) was not inhibited in its Tc activity, maintained expression of the DR antigens and continued to proliferate in response to IL2. However, it is interesting that between 24 to 48h following infection, the proliferative response to IL2 was reduced. Rodgers et al (1985) have described the release of an inhibitor of IL1 activity as measured by the mouse thymocyte assay, following HCMV infection of monocytes and it remains possible that such an inhibitor could have affected generation of the Tc response against live virus, as
TCLl was a cell line with stable differentiated Tc activity. The release of such an inhibitor could not however account for the failure to develop Tc against the UV or heat inactivated virus preparations as these treatments abrogated release of the HCMV induced inhibitor (Rodgers et al, 1985).

Thirdly, it was possible that the precursor frequency of HCMV Tc was so low that in normal seropositive individuals they would not be detected even after prolonged in vitro culture. No significant MHC restricted cytotoxicity was detected even by limiting dilution analysis of such cultures. Lectin mediated cytotoxicity was detected in such experiments, so it remains possible that class II restricted Tc, as has been described to other viruses including HSV and EBV (Meuer et al, 1983; Yasukawa and Zarling, 1984; Jacobson et al, 1984), were generated. These would not have been detected in the fibroblast assay as class II antigens were not expressed.

The protocol described by Sethi did not allow expression of all HCMV antigens and it was therefore modified to co-culture PBM with HCMV infected autologous fibroblasts. Under these conditions inhibition of growth of HCMV infected cells was observed. It is interesting that the number of fibroblasts in control cultures continued to increase, which may be indicative of incomplete infection of the cell layer. However, on co-culture of PBM with HCMV infected cells, inhibition of fibroblast growth was observed. This was not merely the result of coculture of fibroblast monolayers with mononuclear cells, observed by Moller and
Moller (1965), and cytotoxic activity was detected in PBM after 6 days which was distinct from NK activity against K562 cells. Interestingly this cytotoxicity was detected only against 24h HCMV infected but not against 6 day HCMV infected target cells.

Using this protocol HCMV specific, MHC class I restricted Tc were detected in PBM of normal seropositive individuals. At least two possibilities, not mutually exclusive, might explain the requirement for infected fibroblasts in generating HCMV specific Tc. First, fibroblasts express non-structural HCMV IEA and EA not present in 'free' virus preparations (Stinski, 1977, 1983). In the absence of fibroblasts no cell fully permissive for HCMV replication (with expression of IEA, EA and LA) is present during secondary in vitro stimulation. Although it has been suggested (Rice, Schrier and Oldstone, 1984), that a small number of monocytes may be infected with HCMV, this infection did not proceed beyond expression of IEA. Moreover, it was only observed with recent clinical isolates of HCMV and not laboratory adapted virus strains like AD169. T cell lines generated in this way recognised HCMV infected target cells expressing HCMV IEA and EA suggesting that at least a proportion of the effector cells in these bulk cultures recognised the non-structural antigens of the virus.

Another possible reason for the requirement of fibroblasts is that the HCMV antigens had to be 'presented' in association with autologous class I MHC determinants which may only be possible in the permissively infected cell. This is being investigated by stimulating PBM with
partially matched and HLA mismatched infected cells. PBM cocultured with HLA A and B mismatched uninfected fibroblasts did not proliferate as did PBM stimulated with irradiated PBM from the same donor in a one-way MLR. This had also been previously observed by Schellekens and Eijsvogel (1970) and Sorensson (1972), but in addition direct cytotoxicity assays failed to detect any allospecific Tc activity. Preliminary experiments suggest that HCMV specific Tc are more readily generated with autologous or partially matched HCMV infected fibroblasts and they have so far not been generated using HCMV infected mismatched or F5000 fibroblasts in the six subjects studied.

Measurable HCMV-specific cytotoxicity was only observed when the population of responding cells was expanded in IL2; this presumably reflects a relatively low frequency of HCMV specific Tc in the cultures after the initial stimulation period. In order to allow such Tc to grow, irradiated feeder cells and the continued presence of HCMV infected fibroblasts was required. This may reflect a requirement for antigen either to maintain antigen specificity, as also seen in murine influenza specific Tc clones (Andrew and Braciale, 1981) or a requirement for antigen to prevent downregulation of IL2 receptors and thus restrict growth of these cells in IL2 (Cantrell and Smith, 1982).

The inability to generate Tc using 'free' HCMV in seropositive individuals or recently convalescent patients and yet HCMV specific Tc could be generated from some of the same individuals by co-culture with HCMV infected fibroblasts may be explained by differences in the HCMV
antigens or their presentation in vitro. However, there is no readily available explanation for the inability to reproduce the results of Sethi, Stroehman and Brandis (1980), except for possible minor differences in other reagents used.

The recognition of HCMV IEA or EA by Tc lines is particularly interesting in the context of the Tc response to other herpesviruses. The antigen recognised by EBV specific Tc, termed LYDMA, remains unidentified (Moss et al, 1981; Rickinson, 1985). However, it is present in EBV transformed B cell lines and is thought to be expressed in non-productively infected cells or early in the productive cycle of EBV replication. Reddehase and Koszinowski (1984a; 1984b) have also demonstrated that the majority of directly activated MCMV specific Tc isolated from regional lymph nodes following MCMV footpad inoculation, recognized target cells expressing MCMV IEA. Thus at least in the case of 3 herpesvirus infections, 1 in the acute phase or 2 during persistent infection, virus specific Tc recognise antigens expressed prior to viral DNA replication. Although speculative, it may be argued that recognition of such antigens would permit more rapid limitation of dissemination of locally reactivated virus. Furthermore, pharmacological or direct IS may inhibit the generation of such responses in vivo, thereby allowing dissemination and secondary HCMV disease to occur in such patients.

The recognition of either HCMV IEA or EA as possible target antigens for Tc responses, requires the identification of which of the 15
or more IEA and EA proteins are recognized (Stinski, 1977; Stinski, 1983). Several approaches are being applied to answer this question:

1) the generation of HCMV specific Tc clones from the T cell lines and maintenance of these cells in vitro to allow studies with effector cells of single specificity. Initial cloning studies presented here have failed to generate such Tc clones even though Tc activity was evident in the parent line. In many of the experiments, the culture conditions were inadequate and therefore modified. One method being investigated is to bypass the requirement for the continued presence of antigen, to maintain IL2 receptor expression in activated T cells by culturing and cloning in the presence of anti-T3 antibody or anti-T3 coupled to sepharose. However, it must also be recognised that whereas virus specific and other Tc can be readily generated and cloned in murine systems, only a small number of human virus specific Tc clones have been described. There has been little difficulty in generating Tc specific for alloantigens including minor histocompatibility antigens (Pathman and Fitch, 1982). Virus specific Th clones are well documented (Lamb et al, 1982a; 1982b) and Tc clones restricted by class II MHC antigens are described against HSV (Yasukawa and Zarling, 1984), EBV (Meuer et al, 1983) and measles (Jacobsen et al, 1984). However, class I restricted Tc clones are less well documented e.g. EBV specific (Wallace et al, 1982) and influenza specific (Fleischer, 1982). This may be related to the nature of the stimulator cells that are required to generate and maintain
these responses. Class II virus specific Tc were all generated in response to inactivated virus infected PBM as stimulator cells and even in the case of EBV class I restricted Tc, B lymphoblastoid lines were used. Again if class I Tc are activated in vivo eg. as found in CSF of mumps meningitis patients (Kreth et al, 1983), such Tc may be more readily established in vitro.

It is thus possible that secondary in vitro stimulation is an inefficient method for generating these responses particularly if a non-lymphoid stimulator cell is used. The reason for this is unclear. Activation of T cell responses from in vitro studies requires the close association of antigen with MHC determinants not only for Tc but particularly Th responses (Burakoff et al, 1984). As the latter are essential to the development of Tc and other responses, failure to stimulate both cell populations or in contrast activation of T suppressor cells may result in failure to develop the antigen specific Tc.

2) In the absence of HCMV specific Tc clones, further identification of the antigens recognised by Tc lines is being studied. The initial question is whether one or more of the 5 or so HCMV IEA may be an antigen recognised by Tc (Stinski, 1983). Many MCMV specific Tc recognised IEA following primary infection and thus it is important to determine whether the same applies in man. Unfortunately, the preparation of fibroblasts expressing IEA alone using inhibitors such as CHX and AD increased spontaneous $^{51}$Cr release, making them unsuitable as targets
in cytotoxicity assays. An alternative approach adopted was to transfect primary human fibroblasts with HCMV IE genes. This technique has the advantage that defined antigens are expressed which can be modified and the structures recognised by Tc identified. Such techniques have been adopted successfully with alloantigens (Mellor et al, 1982) and more recently with the influenza NP gene (Townsend et al, 1984).

A small part of the Hind IIIE fragment encoding the major 72kDa HCMV IEA was transfected into human fibroblasts but results have been disappointing, in that expression of IEA was transient and variable, precluding cytotoxicity studies. This technique offers great advatages in determining the virus antigen specificity of Tc clones, thus future experiments will be performed using larger segments of the IE region - possibly the Hind IIIE fragment itself. Different vectors such as vaccinia (Moss, 1985) or murine retrovirus (Williams et al, 1984), may be used to transfect established HLA defined human lines so that even a very few stably transfected cells could be cloned and expanded in vitro without the finite time span of diploid human fibroblast lines.

3) An alternative or supplementary approach to the use of transfected cell lines is the generation of T cell lines to purified IEA and EA antigens. From the evidence of influenza, where T cell lines were established to HA peptide fragments, it would be anticipated that a predominantly Th response might be generated. Stimulation with the 72kDa HCMV EA identified by the monoclonal antibody H11 (Rodgers, Mundin and Sissons, 1985), produced a predominantly proliferative T cell response but
preliminary studies in two subjects identified MHC restricted lysis of HCMV infected cells. This response is of particular interest as most cytotoxic responses against isolated proteins have been class II restricted. Secondary class I restricted murine Tc responses have been generated to isolated influenza HA (Zweerink et al., 1977), sendai virus glycoproteins (Hale, Lyles and Fan, 1980) and HSV glycoproteins (Lawman et al., 1981) particularly if the glycoproteins have been inserted into liposomes containing MHC class I determinants. This raises the question as to exactly how the 72kDa EA antigen is being presented - is it passively adsorbed on to the surface of cells in the culture or processed by APC in the system. These questions are currently being investigated.

Most HCMV IEA and EA have a nuclear distribution, including the H11 antigen at least in the early (<72h) stages of infection, and has not been detected on the cell surface (Rodgers, Mundin and Sissons, 1985). There are glycoproteins expressed on the surface of HCMV infected cells - one report identifies a surface protein 6h post infection (Amadei et al., 1983) and Stinski (1977) identified two glycoproteins (145kD and 100kD) on the surface of HCMV infected cells 20h p.i. The determinants recognised by MCMV specific Tc are probably predominantly nuclear in distribution in the case of IEA specific Tc; LA specific Tc, however, recognise cell surface LA, as evidenced by the observation that cells into which MCMV LA have been inserted by fusion are lysed (Reddehase, Kiel and Koszinowski, 1984a; 1984b). The LYMMA antigen of EBV has not been definitely identified although at least one candidate surface structure is
Reovirus specific Tc recognise the structural sigma-1 protein expressed on the surface of infected cells. Townsend et al (1984) has shown that NP expressed intracellularly, is often the antigen identified by influenza specific Tc. Therefore in the current state of knowledge the anatomical location of a virus protein does not preclude it being a target structure thus any of the non-structural IEA/IE of HCMV could be potential antigens for HCMV specific Tc.

4) The identification of HCMV specific Tc in normal seropositive individuals suggests that precursor Tc are present in PBM but does not necessarily imply that such cells are important in limiting reactivation in vivo. In man, as opposed to animal models this question can only be approached indirectly - initially by demonstrating a quantitative relationship between the number of Tc and virus shedding/clinical evidence of reactivation. Using a direct assay for HCMV cytotoxicity, Quinnan et al (1981,1982,1984) suggested that a quantitative difference in $^{51}$Cr release is related to improved survival following HCMV infection associated with BMT. However, this assay is insufficiently sensitive to detect HCMV specific Tc activity in PBM of seropositive subjects directly or more likely, the cells are in an inactive precursor state requiring activation. It also does not allow the determination of Tc stage specificity and is hampered by the presence of high background non-specific lysis at the E:T (100:1) that is used. Furthermore although grouped data as presented by Quinnan and co-workers supported the
hypothesis proposed, others noted large variations between results particularly when individual patients were studied sequentially (Breinig et al, 1984).

Thus limiting dilution studies are being used to examine the role of HCMV specific Tc and the numbers of stage specific Tc in different clinical situations. These enable a quantitative approach to be adopted rather than the qualitative presence or absence of Tc activity as detected by protocols described in this report. Preliminary results suggest a HCMV specific Tc precursor frequency of $\frac{1}{20,000}$ peripheral blood T cells in normal seropositive adults and higher ($\frac{1}{6000}$) in a recently convalescent patient following HCMV reactivation. No HCMV specific Tc have been detected in seronegative subjects. The EBV regression assay was used to measure EBV specific Tc precursors; a minimum frequency of $\approx 1/10,000$ peripheral blood T cells has been detected (Rickinson et al, 1981; Yao et al, 1985). In situations where the EBV specific Tc frequency is reduced e.g. renal transplant recipients (Gaston, Rickinson and Epstein, 1982; Yao et al, 1985) there was increased shedding of EBV and an increased incidence of EBV lymphomas (Crawford et al, 1981).

Until these studies are completed the role of Tc in limiting reactivation of HCMV in vivo must be speculative. However, the similarities between these observations and those relating to EBV are striking, and would support the suggestion that these cells may be important in limiting HCMV reactivation. The recognition of IEA/EA by such cells would equip them for such a role, as destruction of cells expressing
HCMV antigens prior to virus release would decrease the number of virus producing cells in the focus of reactivation, whereas other responses such as anti-viral neutralising antibodies may effectively inhibit metastatic dissemination in tissue fluids.

5.2 NATURAL KILLER CELLS

Tc are not the only cytotoxic effector cells that can mediate lysis of HCMV infected cells. NK cells kill both tumour and virus infected cells in vitro and this cytotoxicity is independent of MHC restriction and prior exposure to HCMV. Previous studies have shown that HCMV infected cells are susceptible to NK mediated lysis, and recognised that the effector cells were E−, non-adherent lymphocytes, but expressed Fc receptors for IgG; their cytotoxic activity was enhanced by IFN (Kirmani et al, 1981; Starr and Garrabrant, 1981).

5.2.1 Effector cells mediating NK lysis of HCMV infected cells

These studies confirmed that HCMV infected cells were more susceptible to lysis by NK cells. The NK cell activity was enhanced by human IFN in a dose dependent manner and a period of between 4-10h following addition of IFN, was required for enhanced NK activity to be evident. This activity was not retained for longer than 24h in vitro but if NK cells were cultured in IL2 containing medium, enhancement of NK
activity was observed after 24h and was retained for the 5 days of the study. This effect may be very important in vivo for two reasons. Firstly LGL may themselves be an important source of IFN and thus autoregulate NK activity to HCMV as well as influenza and HSV as suggested by Djeu et al (1983). Secondly, Shellam et al (1983) have shown that in vivo susceptibility to MCMV infection is related to NK activity against MCMV but this is also dependent on the ability of different mouse strains to release IFN following MCMV infection.

In these NK studies, only PBMC from seronegative individuals (as defined above) were used, to prevent possible effects of virus specific activation of effector cells or direct effects of HCMV Tc. Effector cells were depleted of adherent cells, in order to reduce the possibility that monocyte mediated cytotoxicity rather than NK mediated lysis was being detected in the assay. Furthermore, IFN rather than IL2 was used to obtain maximally in vitro activated NK cells, as such a protocol would be less likely to activate specific Tc responses. In addition, cytotoxicity mediated by NK cells was non-MHC restricted.

Initial studies confirmed the findings of Kirmani et al (1981) that the effector cells were non-adherent and present in the E~ fraction. NK cells that mediated lysis of K562 cells (K562-NK) expressed a number of different surface antigens including Tll, T8, Leu 7a and Leu 11a. As part of the initial characterization of NK cells mediating lysis of HCMV (HCMV-NK) the expression of these antigens was investigated by both positive (using FACS isolated fractions) and negative (by C mediated
lysis) selection. HCMV-NK expressed low levels of T11, variable amounts of T8 and were predominantly Leu 7a+ and Leu 11a+; morphologically they were LGL.

Thus by surface phenotype these cells were identical to K562-NK (Herberman, 1982), and similar cells were identified by Lopez and co-workers to mediate lysis of HSV infected cells (Fitzgerald, von Wussow and Lopez, 1982). Although HCMV-NK were predominantly Leu 7a+ there was a significant number of Leu 7a- cells with similar activity. IFN increased the cytotoxic activity of both Leu 7a fractions studied. The enhancement of NK activity in Leu 7a- cells was not associated with reappearance of Leu 7a+ cells in the culture. Thus whereas recruitment from a pool of inactive cells may occur following IFN incubation (see below), this is not associated with expression of Leu 7a.

Heterogeneity with respect to tumour target cell recognition and lysis has been recognised within the human NK system by binding depletion studies (Ortaldo and Herberman, 1983), cold competition studies and in 'NK' clones (Hercend et al, 1983; Allavena and Ortaldo, 1984) but has not been extensively investigated with virus infected cells. Although this question is best approached by examining 'NK' clones (experiments in progress) a broader approach is to examine selectivity within a population of PBM, depleting effector cells by binding to specific target cells. Using this approach Timonen and co-workers separated NK cells lysing fibroblast and K562 target cells (Timonen and Saksela, 1978; Timonen et al, 1979a and b; Saksela et al, 1979).
HCMV-NK bound and could be eluted from K562 cells although not all NK activity was separated in this assay. In assessing NK activity of eluted cells, it must be remembered that NK activity is reduced following interaction of the NK cell with its target cell (Perussia and Trinchieri, 1981). HCMV-NK activity is decreased in unbound cells following incubation on uninfected or infected fibroblast monolayers to a greater extent than K562-NK or Molt4-NK. These investigations together with cold competition studies suggested that HCMV infected cells were lysed by the same subset of NK cells that lysed uninfected fibroblasts, which in turn were a subset of the K562-NK cells.

Timonen et al (1979a and b) and Saksela et al (1979) separated NK cells into those capable of lysing fibroblasts which they equated with a 'mature' population and NK cells which only lysed tumour target cells such as K562. This separation may serve to explain the inability of K562 cells to compete for lysis of uninfected and infected fibroblasts but not the failure of reciprocal inhibition which may be explained by differences in affinity for target structure(s). The effect of IFN in recruiting NK cells from an inactive pool, at least against uninfected and HCMV infected fibroblasts was demonstrated by adsorbing PBL to uninfected fibroblasts before and after IFN treatment. Adherence before IFN treatment only marginally depleted NK activity whereas after IFN treatment marked reduction was observed.

Thus HCMV-NK are probably a subpopulation of NK-K562 cells which share surface phenotype, density, morphology and are augmented in their
activity by preincubation with IFN.

5.2.2 Increased susceptibility to NK lysis of HCMV infected cells

The increased susceptibility of HCMV infected fibroblasts to NK lysis required the expression of HCMV EA. Cells expressing both HCMV IEA and EA, were more susceptible to lysis, but not cells expressing HCMV-IEA alone either in time course studies or even with increased expression of IEA following CHX and AD treatment (Stinski, 1983). The subsequent expression of IA did not additionally enhance susceptibility. HCMV induced susceptibility to NK mediated lysis therefore required virus entry and IEA and EA expression, but did not require viral DNA replication or expression of viral structural proteins.

It was possible that the increased susceptibility to lysis was a result of IFN release or might be an expression of increased cell fragility after virus infection. However, only low levels of IFN were released on co-culture of HCMV-infected cells with lymphocytes. Moreover, when IFN was added in excess to an 18h cytotoxicity assay, no additional enhancement of lysis was observed. Fitzgerald et al (1983) using HSV infected target cells observed low levels of IFN release during cytotoxicity assays and these levels did not correlate with lysis of infected cells.

This implied that once NK activity had been maximally stimulated by pretreatment of PBL with IFN, no additional augmentation of lytic activity is produced by intra-assay IFN release. Pretreatment of HCMV infected
cells with IFN did not affect their susceptibility to lysis, although the 'protective' effect of IFN on uninfected fibroblasts (Trinchieri and Santoli, 1978) was lost. Bukowski and Welsh (1985) reported that MCMV infected cells similarly lost this protective effect of IFN whereas LCMV infected cells did not and were less susceptible to NK lysis. This has led to the suggestion that loss of 'protective' effect may be important in vivo, in determining whether NK cells were protective. There was no enhancement of susceptibility to lysis as has been reported with some viruses e.g HSV in HeLa cells (Munoz, Carrasco and Fresno, 1983). These observations together with the direct effects of IFN on NK cells suggest that the major effect of IFN is to augment NK activity but it does not determine the increased susceptibility of HCMV infected cells to NK lysis.

The increased target cell susceptibility was not due to increased fragility of virus infected cells: HCMV LA and PPF treated cells have lower levels of spontaneous $^{51}$Cr release than do uninfected fibroblasts. Lytic susceptibility was associated with increased effector-target cell binding. Although not all conjugates formed in target cell binding assays resulted in lysis of the target cell (Bonavida, Bradley and Grimm, 1983), binding did correlate with $^{51}$Cr release (Roder and Kiessling, 1978).

These assays were time consuming and difficult to control for objectivity, therefore the assay was modified to detect target cell binding by the FACS with a single laser. Similar studies have been reported by Berke (1985). This may have a wider application than similar
previous studies which required 2 colour fluorescence with dual laser systems. The increased NK binding to HCMV infected cells suggested that increased recognition of an NK target structure(s) on HCMV infected fibroblasts may at least in part explain the increased lysis of HCMV infected cells.

The reciprocal cold competition observed between uninfected, HCMV EA, HCMV LA, HSV, SFV, vaccinia and poliovirus infected cells in addition to indicating that a similar subset of NK cells mediated lysis, would also be consistent with the possibility that a similar target structure(s) was recognised. The combination of increased target cell binding, increased $^{51}$Cr release and partial inhibition by uninfected cells may have two explanations. Firstly there may be interaction of two or more subsets of NK cells: one group recognising 'fibroblast' target structure(s), the others HCMV specific structure(s). The evidence that virus antigens may themselves be target structure(s) is limited, although in the case of HSV, Bishop, Glorioso and Schwartz (1983) have proposed that HSV glycoproteins may be directly involved. However, these workers did not investigate the possibility that expression of viral determinants might be associated with altered expression of host cell structures, which may suggest an alternative interpretation of their observations. Secondly, the results of reciprocal inhibition experiments - particularly as HCMV infected cells were more effective at inhibiting lysis of uninfected fibroblasts than uninfected fibroblasts themselves - might indicate that there was recognition of a 'fibroblast' target structure(s) which was
enhanced coincident with HCMV EA expression.

To examine the role of HCMV IEA and EA as target structure(s) for NK cells, advantage was taken of the observation that guinea pig fibroblasts express HCMV IEA and some EA antigens (Stinski, 1983) with no viral DNA replication and no expression of LA. These IEA/EA had the same size by SDS PAGE as HCMV antigens in human cells and were recognised by anti-IEA monoclonal antibodies. There was no increased susceptibility of HCMV infected guinea pig fibroblasts to human NK mediated lysis although guinea pig lymphocytes were able to lyse guinea pig HCMV infected cells more readily than uninfected cells, but not human HCMV infected fibroblasts. These results suggested that viral products expressed at a time when the cell was more susceptible to NK lysis were probably not the target structure(s) recognised by NK cells. However, it may still be argued that not all EA were expressed in these cells and that post-translational modification of the proteins may be different in the guinea pig fibroblasts.

Thus the results presented would favour the concept that HCMV infected cells were more susceptible to lysis when IEA and EA were expressed and this susceptibility did not require viral DNA replication. The evidence presented would favour the hypothesis that the target structure recognised is not virally encoded but a host cell structure(s) whose expression is increased coincident with IEA/EA expression. HCMV increases host cell nucleic acid and protein synthesis and thus increased expression of a host cell protein is possible following infection. Again,
the ability of NK cells to lyse cells prior to virus replication may enable them to function effectively in eliminating either HCMV infected cells either following exogenous or endogenous infections. Other virus infected cells were also susceptible to NK lysis prior to virus replication. Only certain SV40 transformed cells were lysed by NK cells when the non-structural T antigen was expressed (Lewis and Cook, 1985). Again whether such susceptibility is due to virus induced changes in the host cell or direct recognition of viral determinants is unknown, but it suggests that the recognition of cells during early stages of virus infection by NK cells may be an important host defence mechanism.

5.2.3 NK lysis of virus infected cells and Tfr expression

Various NK target structure(s) have been proposed in different NK-target cell systems. The lysis of target cells by both Tc and NK cells, is probably similar (Podack, 1985), but the target is probably different. The ‘receptor’ of NK cells is unknown although it is clear that it probably is not the same complete T3-Ti complex proposed for T cells (Reynolds et al, 1985; Ritz et al, 1985; Robertson, 1985). Identification of NK target structure(s) has been hampered by lack of a homogenous population of cells and even when these were available, both NK clones with a single target cell specificity and NK clones which lysed different target cells were found. Both types of clone may also mediate ADCC (Alvera and Ortaldo, 1984). The proposed target structures have included virus structures - retroviral (Roder, Karre and Kiessling, 1981).
and HSV (Bishop, Glorioso and Schwartz, 1983), membrane glycoproteins of YAC-1 cells (Roder, Ahrlung-Richter and Jondal, 1979), the glycolipid asialo GM2 (Young et al, 1981), lectin-sugar interactions (Stutman et al, 1980) and more recently the TfR (Vondinelich et al, 1983).

The TfR was proposed as an NK target structure, as increased TfR expression was associated with greater ability to competitively inhibit NK lysis of K562 cells and anti-T9 affinity isolated TfR blocked NK lysis of K562 cells (Vondinelich et al, 1983). This was supported by the observation that transfected murine L cells which stably expressed the human TfR acquired susceptibility to human NK lysis (Newman, Warner and Dennert, 1984). The relationship between the changes in the number and/or physiological recycling of the TfR and the differential susceptibility to NK lysis of virus infected cells (Rager-Zisman and Bloom, 1982; Trinchieri and Santoli, 1978) was investigated.

The TfR is present on the surface of most if not all cells growing in vitro (Ward, Kushner and Kaplan, 1982a and b; Frazier et al, 1982) and blocking its binding site by a monoclonal antibody inhibited cell growth (Trowbridge and Domingo, 1981). The receptor itself is a glycoprotein (MW = 180kDa) composed of two equal subunits each of 800 amino acids linked by a disulphide bond and it binds to a palmitate residue in the cell membrane. The gene for human TfR has been sequenced and a primary amino acid structure obtained (McClelland, Kuhn and Ruddle, 1984). Each receptor binds two Fe\(^{3+}\)-Tf molecules with high affinity and the complex is rapidly internalised (Schneider et al, 1982; Dautry-Varsat,
Ciechanover and Lodish, 1983); iron dissociates from the complex following a reduction in the pH in endosomes and the resultant apo-Tf-TfR complex is then recycled to the cell surface where it dissociates and more Fe$^{3+}$-Tf bound (Dautry-Varat, Ciechanover and Lodish, 1983).

Tumour cell lines in vitro are rapidly dividing cells and frequently express a high level of TfR (Vondinelich et al, 1983). However, its role as an NK target structure has been challenged (Dokhelar et al, 1984) as firstly Fe$^{3+}$-Tf did not inhibit NK lysis (although others dispute this (Baines, Lafleur and Holbein, 1983)), secondly monoclonal anti-TfR antibodies did not inhibit NK lysis and thirdly the loss of TfR from the surface of K562 target cells following maturation with sodium butyrate did not correlate with the kinetics of the loss of their susceptibility to NK lysis (Dokhelar et al, 1984).

However, some virus infections, particularly HCMV, are associated with altered host cell protein and nucleic acid synthesis; viral glycoproteins (such as VSV-G) (Strous et al, 1983) or virus particles (Smith and De Harven, 1974; 1978; Roizman and Batterson, 1985) are transported with host cell proteins via the golgi complex. It is thus possible that virus induced alterations in the rate or nature of this transport mechanism could affect expression of a NK target structure such as the TfR, thereby explaining the different susceptibilities to NK lysis of virus infected cells.

The susceptibility to NK lysis of virus infected cells and their
ability to compete as cold targets varied with TfR expression, as measured by Tf binding at 37°C. This was also the case with uninfected fibroblasts grown in the presence of different Fe^{3+} concentrations.

The distribution of TfR in HCMV infected cells was further investigated. Total and recycling receptor expression was doubled in infected cells. This increase coincided with expression of HCMV EA and increased susceptibility to NK lysis. The similarity between the 1/K_D of Tf binding at 18h post infection compared with uninfected cells suggested that the increased binding was unlikely to be due to a virus encoded protein but rather enhanced expression of the host TfR.

However, the increased total and recycling TfR is not associated with a proportional increase in the binding of Tf to the surface of HCMV infected cells (measured at 4°C). The slight increase observed would suggest that, as a surface binding event was required for NK lysis of target cells, the correlation observed between susceptibility to NK lysis and increased TfR expression did not necessarily support the TfR as a direct NK target structure on virus infected cells.

Furthermore even lysis of K562 cells has not been inhibited with excess Fe^{3+}-Tf or partially purified TfR. The inability to inhibit lysis with excess Tf was similar to the observations of Dokhelar et al (1984), but at variance with other studies (Baines, Lafleur and Holbein, 1983). These observations do not in themselves negate the TfR being a NK target structure: firstly, culture of fibroblasts in the presence of excess Tf does not downregulate the number of TfR (Ward,
Kushner and Kaplan, 1982a) and secondly, there could be differences in the affinity of NK cells and Tf for the TfR. The failure of partially purified TfR to inhibit NK lysis does make it more difficult to support its direct role as a target structure. This failure is at variance with the observations of Vondinelich et al (1983), although it is conceivable that this could be explained by differences in the method of affinity purification and elution of TfR from the solid phase.

There was therefore a close correlation between recycling TfR expression in these cells and their susceptibility to, and ability to competitively inhibit NK lysis which was independent of the infecting virus. In addition there was a good temporal association between the development of enhanced susceptibility to NK lysis of HCMV infected cells and TfR expression; susceptibility to lysis of uninfected fibroblasts was also related to TfR expression. However, these close correlations have to be balanced with the minor differences in surface TfR expression in HCMV infected cells and the failure of Tf and isolated TfR to inhibit NK lysis. Whilst it remains possible that virus induced changes in expression or cycling of the TfR could be indirectly associated with changes in the target structure(s) recognised by NK cells, these observations suggest that the TfR itself is unlikely to be the NK target structure on virus infected fibroblasts.

In view of the increase in the number of TfR in HCMV infected cells other cell surface receptors were investigated to determine whether similar increases occur. There was increased binding of LDL to HCMV.
infected as opposed to uninfected cells but the number of $\alpha_2$-macroglobulin receptors was unchanged. Both these receptors are predominantly distributed in coated pits (Bretschner and Pearse, 1984) although some LDL receptors are situated outside these regions especially in unstimulated cells. The effect of HCMV infection is not restricted by the anatomical distribution of the receptor. Two alternative mechanisms may explain this increase in TfR and LDL binding. Firstly, it is possible that the increase in TfR and LDL may represent a response to host cell metabolic changes, in this instance induced by HCMV e.g. cell division is preceded by increased TfR expression in lymphocytes (Neckers and Cossman, 1983) and as DNA and RNA synthesis is induced by the virus, secondary increased TfR expression may have preceded this. Alternatively, HCMV induces host cell RNA synthesis with increased expression of a number of other functional host cell proteins. It is possible that this may be due to direct transactivation of host cell genes by IE gene products of the virus (Everett, 1984). Thus these changes in TfR number and LDL binding may reflect some of the host cell changes directly induced by the virus to favour HCMV replication.
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