Cytomegalovirus Infection and Immunity in Homosexual Men.

Peter Muir

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ABSTRACT

The prevalence and characteristics of cytomegalovirus (CMV) infection in homosexual men was investigated. CMV infection may affect various immune functions under different circumstances. The acquired immune deficiency syndrome (AIDS) has been reported with increased frequency in male homosexuals and the possibility that CMV infection may contribute to the observed immune impairment was considered. It is necessary to study CMV infection in the absence of AIDS in order to assess its individual contribution to immune deficiency. To this end a population of male homosexuals and heterosexual controls where no cases of AIDS had been reported was selected for study.

Virological, immunological and serological parameters of CMV infection were assessed. Virus isolation in cell culture was used to detect oral and urinary excretion of virus. Virus-specific and general cell-mediated immunity was investigated using lymphocyte proliferation assays and a range of serological assays were used. IgG antibodies to CMV were detected by indirect ELISA to determine the prevalence of past infection in the two study populations. Antibodies to CMV immediate early and early antigens were detected by indirect immunofluorescence, as an indication of the extent to which humoral immunity is stimulated during CMV infection. An IgM capture ELISA was developed to detect IgM antibodies to CMV. Together with data obtained from virus isolation studies, this provided an indication of the prevalence of current or

recent active CMV infection.

CMV infection was found to be highly prevalent in male homosexuals. This did not appear to be due to increased frequency of sexual contact within this group. Rather homosexual contact in itself would seem to result in efficient transmission of infection as evidence of past infection was highly prevalent among young, sexually inexperienced homosexuals. Active CMV infection was largely confined to this group.

There was little evidence of deficient cell-mediated immune function in the homosexuals studied as a whole or in those with active CMV infection. However CMV-specific humoral immune parameters indicated that CMV infection frequently resulted in a greater stimulation of immunity in homosexuals. This may reflect a reduced capacity to contain active viral replication in this group. Some defect in immune function which is not detected in lymphocyte proliferation assays may be responsible. Alternatively, the extent of viral replication may be determined to some extent by the route of infection.

The high level of pre-existing CMV infection in homosexuals, and its active state in many support the hypothesis that CMV infection may influence the development of immune deficiency both during and subsequent to infection with the causative agent of AIDS.

DECLARATION

The investigations and procedures that form the basis of this thesis were designed and carried out by the author except where stated otherwise.

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GENERAL INTRODUCTION

A number of viral infections of man and animals cause a suppression of immune functions. Included in this group are Epstein-Barr virus (EBV) infection (Mangi et al., 1974), the cytomegalovirus (CMV) infections of man (Ten Napel et al., 1980a; b) and of mice (Howard et al., 1974), acute and chronic hepatitis B virus (HBV) infections (Barnaba et al., 1983; Thomas, 1981), measles virus infection (Whittle et al., 1978), feline leukaemia virus (FeLV) infection (Essex et al., 1975; 1976), and in mice, lymphocytic choriomeningitis virus (LCMV) infection (Mims & Wainwright, 1968) and lactic dehydrogenase virus (LDV) infection (Howard et al., 1969). The range of immune functions impaired and the extent of their suppression differ in each case and the underlying mechanisms which may be responsible are diverse.

The acquired immune deficiency syndrome (AIDS) caused by the human T-lymphotropic retrovirus type III (HTLV III) is perhaps the supreme example of a viral-induced immunodeficiency in man. The cause of immune suppression in this case appears to be the elimination of a subgroup of T lymphocytes, however other factors may contribute prior to this late-stage event as only a proportion of infected individuals proceed to this phase of disease. AIDS often occurs in association with other viral infections including some of those mentioned above, in particular CMV and EBV infection. Of particular interest therefore is the possibility that such viral infections may predispose to greater pathogenic expression of HTLV III

infection, which may subsequently proceed to AIDS and related conditions with greater frequency.

This thesis examines the possibility that CMV infection may play such an accessory role by investigating the extent of CMV infection in a population which may be at increased risk for AIDS, namely male homosexuals. The group studied were free of AIDS and its related complex. This avoids the difficulty of determining whether CMV infection in symptomatic individuals contributes to immune deficiency, or is merely arising in an opportunistic fashion in the presence of immune deficiency due to other causes.

The following chapters lay the basis for understandimg the pathogenesis of CMV infection, of HTLV III infection, and their possible interactions in AIDS. Chapter 1 reviews aspects of the biology of CMV important in comprehending the nature of the virus-host interaction at the cellular level. This material also forms the basis on which much experimental work with CMV is designed. Chapter 2 considers epidemiological aspects of CMV infection, upon which much of our understanding of the pathogenesis of CMV infection lies. Chapter 3 extends the consideration of CMV pathogenesis in various clinical situations and provides a grounding for investigating its possible role in AIDS. Chapter 4 reviews microbiological, immunological and other pathological aspects of AIDS and discusses the small but rapidly growing literature on the pathogenesis of HTLV III infection.

INTRODUCTION

CHAPTER 1

THE BIOLOGY OF HUMAN CYTOMEGALOVIRUS

History and classification

In 1956 Smith reported the isolation and *in vitro* propagation of a virus from autopsy specimens of salivary gland and kidney tissue (Smith, 1956). These specimens were derived from two infants found to have salivary gland inclusion disease (cytomegalic inclusion disease) - a diagnosis made on the basis of the large intranuclear inclusions seen in histological sections (Pinkerton, 1950). The virus was implicated as a cause of the disease, as nuclear inclusions were seen in cells infected in culture which bore resemblance to the *in vivo* inclusions and were identical to those produced in mouse cell cultures infected with mouse salivary gland virus (Smith, 1954). Like the mouse virus, the human virus was species-specific and the latter could not be transmitted to suckling mice. This observation distinguished the human virus from that of the mouse, and also from herpes simplex virus (HSV) which may have been responsible for the inclusions in histological section (Pinkerton, 1950). The virus was also distinguishable from varicella-zoster virus (VZV) which induces similar inclusions in cell culture (Weller, 1953) by the presence of infectious virus in the culture medium.

Subsequent isolates were made from cultured adenoid tissue (the Ad 169 strain), liver biopsy tissue (Davis strain), and from urine (Kerr and Esp strains) and these viruses were shown to be related to the original isolates by their serological cross-reactivities and cell cultural similarities (Rowe et al., 1956; Weller et al., 1957). All, apart from the Ad 169 strain were isolated from infants with cytomegalic inclusion disease. Weller et al. (1960) coined the name "cytomegaloviruses" (CMV) for these viruses and those of other species previously known as salivary gland viruses or cytomegalic inclusion disease viruses. This change of nomenclature was in recognition of the fact that virus isolation could be made from numerous sites other than the salivary glands, and could not always be associated with any disease syndrome. Indeed, a large proportion of the population had serological evidence of past, undetected infection with human CMV.

Electron microscopy of infected cells suggested that CMV was enveloped, the internal capsid being 80-100 nm in diameter (Luse & Smith, 1958; 1959) however detailed characterisation was hampered initially by the low in vitro infectivity of CMV and its strong cell association (Benyesh-Melnick et al., 1966). This made the preparation of concentrated virus impractical. Improved preparation techniques which increased the sensitivity of electron microscopy (Smith & Melnick, 1962) allowed Smith & Rasmussen (1963) to determine that CMV was probably a DNA virus and was very similar to HSV in its size and capsid architecture.

These observations made a strong case for including CMV in the group known as the herpes type viruses. This classification was borne out in due course as the ultrastructure and biology of the virus was further elucidated.

CMV is one of four herpesviruses that regularly infects man (Timbury & Edmond, 1979). The others are HSV (the prototype of the group), VZV and Epstein-Barr virus (EBV). Herpesviruses have also been found in many other eukaryotic species (Plummer, 1967). This has undoubtedly been a major factor in stimulating research on CMV; much of the extensive literature that now exists was initially prompted by observations made in other herpesvirus systems, several of which lend themselves more readily to laboratory investigation.

The herpesviruses are structurally similar, but biologically diverse and exist in a wide variety of host-parasite relationships with perhaps an even greater variety of disease manifestations, ranging from the inapparent to the rapidly fatal. In common with at least some animal herpesviruses and the other three viruses of humans, CMV may exist in a carrier state following primary infection and this may give rise to a re-emergence of active infection at a later date (Jack, 1974; Plummer, 1973; Weller, 1971).

Strucutre and replication of CMV

The molecular biology of CMV has stimulated interest both as a model for investigating the control of eukaryotic

cell function and as a means of understanding the pathogenesis of CMV infection. In this context, an account of the structure, composition and replication of CMV serves also as an introduction to the antigenic complexity of the virus and as a rationale for certain aspects of experimental design. The molecular events have been studied at various levels; initially most investigation was of necessity indirect in approach, using light and electron microscopy, and immunofluorescent and immunoperoxidase staining techniques most commonly. These methods are now supplemented by sophisticated physical, biochemical and immunological techniques.

In an account of this nature certain points should be borne in mind:

a) Much of current knowledge in this area is derived from studies in in vitro experimental systems. Because of the species specificity of human CMV (as with most CMV's of other species) there is no animal model available. CMV infection in these systems differs fundamentally from that in an in vivo situation in a number of respects:

i) There are fewer physiological constraints on viral replication operative in an in vitro system.

ii) The cell types which permit productive infection in vitro (fibroblasts) are probably not the major cell types infected in vivo.

iii) The virus strains most commonly employed are those which have become adapted by serial passage to these culture conditions.

b) It is not uncommon for different investigators to obtain different results for similar experiments. Factors which may account for these discrepancies include the type of fibroblasts used (Wroblewska et al., 1981) and their physiological state (De Marchi & Kaplan,1977a), the strain of virus, multiplicity of infection (moi), the relative numbers of defective and non-defective particles in the viral inoculum, and the incubation conditions employed (Albrecht et al., 1980a; b; Stinski et al., 1979a). These variables will be referred to in greater depth in subsequent sections.

c) Our understanding of many of the molecular processes is based on evidence which is lacking in conclusive proof. It should therefore be remembered that some conclusions drawn from experimental data are probabilities rather than statements of fact.

Structure and composition

The herpesviruses are defined by their virion structure: the capsid has icosahedral symmetry and is constructed of 162 capsomeres. The genome is a single, linear, double stranded DNA molecule and the nucleocapsid is surrounded by a lipid envelope (Lwoff et al., 1962). The herpesviruses are large in comparison to most animal viruses; at 100-150 nm in diameter, only the poxviruses are larger (Fenner, 1968).

The genome of CMV is larger than that of most other herpesviruses, being approximately 150 x 10^6 daltons in molecular weight as determined by sedimentation rates in sucrose density gradients and contour length measurements by

electron microscopy (De Marchi et al., 1978; Geelen et al., 1978; Kilpatrick & Huang, 1977; Stinski et al., 1979a). The DNA of other herpesviruses is typically 90-100 x 10^6 daltons (Becker et al., 1968; Soehner et al., 1965). Smaller DNA molecules of about 100 x 10^6 daltons predominate in many CMV preparations but these are genetically incomplete and probably non-infectious (Kilpatrick & Huang, 1977; Stinski et al., 1979a). Restriction endonuclease (RE) analysis of the genome indicates the presence of four isomeric forms as some fragments are present in quarter-molar amounts (Kilpatrick & Huang, 1977). This is best explained by considering the genome as consisting of two unique sequences, one longer than the other, each of which is bounded by inverted repeat sequences. These repeats permit each segment to lie in either orientation relative to the other segment, RE fragments generated from the joint region being those found in submolar amounts. The isomeric variants probably arise during the replication process. A similar genome organisation is observed with HSV (Hayward et al., 1975b). With some other herpesviruses only one or two isomers exist (Honess & Watson, 1977).

The CMV genome consists of approximately 240 kilobase pairs and has considerable coding potential. It is known to specify at least 23 non-structural polypeptides involved in modulating host cell metabolism and viral replication (to be discussed subsequently) and at least 33 virus-specified structural polypeptides (i.e. associated with the free virion) have been identified to date (Kim et al., 1976). The

same number of structural proteins has been reported for HSV (Heine et al., 1974) and EBV (Dolyniuk et al., 1976). Although these proteins are predominantly structural in function, some enzymatic functions are associated with free virions (Mar et al., 1981) which may conceivably play some role in viral entry and initiation of replication. At least eight of the structural proteins are glycosylated and these are associated with the viral envelope (Stinski, 1976).

Given that the lipid component of the viral envelope is derived from cellular membranes (Kanich & Craighead, 1972b; Smith & De Harven, 1973) it is perhaps surprising that cytomegalovirions appear to contain no host cell polypeptides; antisera raised against purified virions do not react with uninfected cells (Oefinger & Arenius, 1983; Stinski, 1976). It is suggested that the presence of viral proteins in the membrane or on the surface of the capsid as it associates with the membrane sterically precludes the presence of other proteins in the membrane in the vicinity where envelopment occurs.

Variation in composition

The functional integrity of proteins are dependent on their amino acid sequence and conformation. Nevertheless, some degree of variation is frequently possible without substantial loss of function. Variation in CMV proteins can be demonstrated at two levels: the base composition of the DNA that encodes the proteins, and in the proteins themselves.

Variation in base composition of CMV DNA has been

demonstrated in two ways. The extent of DNA-DNA reassociation between heterologous strains of virus is usually less than that of the homologous strain and the percentage reassociation (relative to the homologous DNA) is an expression of the similarity of the two DNA types. Huang et al. (1976) demonstrated more than 80% reassociation between Ad 169 strain and all other human CMV's tested except strain Colburn, which, although a human isolate (Charamella et al., 1973), bears more resemblance to simian CMV strains (Huang et al., 1978; Kilpatrick et al., 1976). The technique is useful for distinguishing human CMV from non-human strains and other herpesviruses which show no significant DNA-DNA reassociation (Huang & Pagano, 1974).

Differences in DNA base composition are also reflected in the number and size of fragments generated by RE digestion of DNA, which is visualised by polyacrylamide gel electrophoretic separation (PAGE) of the restricted DNA. Unrelated human CMV isolates have similar but non-identical cleavage patterns (Kilpatrick et al., 1976) which are again useful for distinguishing CMV from other herpesviruses and have also been used epidemiologically to determine paths of transmission of infection (Huang et al., 1980; Spector et al., 1983).

Inter-strain variation in the composition of viral proteins can be detected as slight differences in the PAGE profiles of the proteins (Gupta et al., 1977) or, more commonly as differences in antigenicity. Antigenic variation is apparent as differences in neutralisation of infectivity

kinetics between homologous and heterologous strains (Andersen, 1970; Waner & Weller, 1978; Weller et al., 1960), in complement fixation (CF) testing using different strains as antigens (Huang et al., 1976; Medearis, 1964) and as differences in the lymphocyte proliferative response of immune individuals to different strains (Beutner et al., 1978; Gartner et al., 1982b). No strain differences have been detected by indirect immunofluorescene (IIF) with IgM antibody (Gartner et al., 1982a).

Several investigators have attempted to exploit the above differences in virus composition as a means of classifying or subgrouping human CMV isolates. Waner & Weller (1978) and Weller et al. (1960) have suggested that reciprocal cross-neutralization kinetics may provide a basis for such a grouping. Differences in RE cleavage patterns are probably too small and too continuous to be useful in this respect. Certainly, no subgrouping comparable with that of HSV is possible, where HSV type 1 (HSV 1) and HSV 2 show only 40% DNA homology by reassociation kinetics (Kieff et al., 1972), are readily distinguishable on the basis of PAGE profiles of RE digested DNA (Hayward et al., 1975a) and form two distinct serotypes (Pauls & Dowdle, 1967). In contrast, EBV isolates show little variation in their DNA composition (Kawai et al. (1973) or antigenic properties (Gerber et al., 1976). The significance of variability in CMV proteins will be discussed in later sections.

The CMV replication cycle

The CMV in vitro replication cycle proceeds efficiently to completion only in human fibroblasts (Rowe et al., 1956). Human epithelial cells (Figueroa et al., 1978; Knowles, 1976; Vonka et al., 1976), peripheral blood leukocytes and lymphoblastoid lines (Furukawa et al., 1979; St. Jeor & Weisser, 1977) yield only very low titres of infectious virus. A variety of other human and non-human cell types permit limited expression of the viral genome, but no detectable viral DNA (vDNA) replication or progeny virus release occurs (De Marchi, 1983b; Einhorn et al., 1982; Einhorn & Ost, 1984; La Femina & Hayward, 1983).

CMV replicates slowly in fibroblast culture in comparison with many other herpesviruses (Rapp & Benyesh-Melnick, 1963). At high moi the growth cycle of a typical laboratory strain lasts 7-8 days from initiation of infection to destruction of the cell monolayer, whereas that of HSV lasts 1-2 days (Smith & De Harven, 1973). Following entry of virus into a permissive cell type (i.e. one which allows the complete viral replication cycle to take place, giving rise to infectious progeny virus) the events can be grouped into three distinct, chronological phases; immediate early (IE), early and late phases (De Marchi et al., 1980) which are broadly analogous to the three phases of HSV replication (Honess & Roizman, 1974). Proteins synthesised in the IE phase regulate the synthesis of early proteins (Wathen et al., 1981; Stinski et al., 1982). Both these classes of proteins are synthesised before vDNA replication takes

place and in the presence of inhibitors of DNA synthesis. Late protein synthesis only occurs after vDNA replication is initiated (Stinski, 1978). Towards the end of the growth cycle virus particles at various stages of maturation are present in infected cells and infectious virus can be detected in the culture medium (Kanich & Craighead, 1972b).

Entry of virus into cells

The adsorption of CMV to cell membranes and its subsequent penetration has been studied electron microscopically by Smith & De Harven (1974). Adsorption of both enveloped and naked particles occurs, and a variety of human and non-human cell types are capable of adsorbing virions (Einhorn *et al.*, 1982). This is similar to the adsorption of HSV, which has been likened to the uptake of inert particles (Epstein *et al.*, 1964) but is in sharp contrast to the adsorption of EBV which is mediated by a receptor possibly unique to B lymphocytes (Jondal & Klein, 1973). There may however be adsorption mechanisms in addition to non-specific interactions as enveloped CMV particles are adsorbed preferentially over naked particles, and enveloped particles adsorb far more efficiently to some cell types than to others.

Adsorption of virus is rapidly followed by entry of virus into the cytoplasm. This may be direct, by fusion of the viral envelope with the plasma membrane, or indirect, by phagocytosis of the virions, singly or in groups, followed by fusion of the viral envelope with the membrane of the

phagosome (Smith & De Harven, 1974). Thus although non-enveloped particles can adsorb to the cell membrane and be phagocytosed they cannot enter the cytoplasm and are degraded on phagolysosome formation.

Within minutes of entering the cytoplasm viral particles are transported towards the nucleus and accumulate in the Golgi region and around nuclear pores. Up to this point, CMV and HSV particles behave similarly. However following entry, HSV nucleocapsids are rapidly disassembled and few can be seen two hours after penetration whereas CMV nucleocapsids appear to become coated following entry and retain their characteristic morphology for up to 36 hours (Cavallo et al., 1981; Smith & De Harven, 1974). This may play a part in extending the time scale of the CMV growth cycle over that of HSV.

Immediate early events

Within the first hour of infection a group of infected cell-specific polypeptides (ICSP) can be detected by indirect immunofluorescence in the nuclei of infected cells (Michelson-Fiske et al., 1977). These immediate early antigens (IEA) are defined as those ICSP's whose mRNA's are transcribed in the absence of de novo protein synthesis and are translated immediately on the removal of a cycloheximide protein synthesis block. They are encoded by the viral genome (De Marchi, 1981; Wathen et al., 1981). The presence of these proteins so shortly after the initiation of infection must be reconciled with the fact that CMV nucleocapsids remain intact

within the cytoplasm for much longer periods. It is possible that only a few nucleocapsids undergo disassembly, making the vDNA available as a transcriptional template and that those particles present at later times do not contribute to the replicative cycle, at least in the earlier stages.

Nine predominant IE proteins have been identified by PAGE to date (Wathen et al., 1981); the genes for these proteins have been mapped by hybridisation with labelled IE mRNA to Southern blots of restricted vDNA and are located primarily within restricted regions of the genome. Transcription of the remainder of the genome would appear to be blocked at this stage (Wathen et al., 1981; Wathen & Stinski, 1982).

The function of the IE proteins is only partially understood; none are incorporated into progeny virions (Stinski, 1978). Since proteins synthesised in the first two hours of infection control the transcription of early mRNA (De Marchi, 1981; Wathen et al., 1981) it is likely that IE proteins with or without additional host cell functions effect this regulation. The finding that interferon (IFN) pretreatment of infected cells, which selectively inhibits vmRNA translation also results in reduced early mRNA transcription (Stinski et al., 1982) enforces this view. The function of the corresponding proteins of HSV (the α proteins) include the regulation of early (β) mRNA synthesis (Honess & Roizman, 1974).

Early events

Approximately six hours after infection the synthesis of most IE proteins terminates although some are expressed constitutively (Jahn et al., 1984). A second group of non-structural proteins, the early proteins appear in infected cells at this time (Wathen et al., 1981). In common with the IE proteins, they are synthesised in the absence of vDNA replication (Stinski, 1978). At least 16 early proteins have been detected by radioimmunoprecipitation (Blanton & Tevethia, 1981). IE mRNA continues to be synthesised at early times but early mRNA is preferentially translated, possibly due to retention of IE mRNA in the nucleus (Wathen & Stinski, 1982). Transcription of early mRNA occurs from most regions of the genome, the block operative at IE times having been reversed, probably by an IE function (De Marchi, 1981; Wathen et al., 1981; Wathen & Stinski, 1982). Together with the IE transcripts, the early mRNA's account for about 12% of the coding capacity of the genome (Stinski, 1978). In contrast to most IE mRNA's transcription and translation of early mRNA is sustained and continues into the late phase (Wathen & Stinski, 1982). In common with most IE proteins, most early proteins accumulate in the nucleus (Reynolds, 1978). However at least one early protein associates with the plasma membrane (Tanaka et al., 1981).

During the early phase a number of changes take place in infected cells which are attributed to the activity of IE or early proteins. Around the onset of the early phase the fibroblastic morphology is lost and infected cells assume a

rounded shape (Cavallo et al., 1981; Furukawa et al., 1973; Smith & De Harven, 1973) possibly due to the inhibition of fibronectin synthesis (Ihara et al., 1982). This process is dependent on de novo protein synthesis but independent of vDNA synthesis and host cell viability, and only occurs at high moi. This early cytopathic effect (CPE) often appears as focal lesions in the cell sheet but can be more generalised at higher moi (Kanich & Craighead, 1972a). Where the virus is poorly adapted to growth in cell culture and the inoculum is small, as is usually the case with clinical sources of virus, development of this CPE is delayed for several days or weeks (Weller et al., 1957). It is probable that several rounds of replication are required to generate a sufficient titre.

At the same time as the appearance of early CPE, early nuclear inclusions are visible by electron microscopy as electron-dense spherical bodies with electron-lucent cores. These subsequently mature, the central areas becoming compartmentalised by the electron-dense material, and probably form centres of vDNA replication and, later on, nucleocapsid assembly (Cavallo et al., 1981).

Clumping and margination of host cell chromatin takes place, but is transient (Cavallo et al., 1981; Ruebner et al., 1965) unlike HSV-infected cells which show chromatin reorganisation throughout the replication cycle (Smith & De Harven, 1973). This organisation of chromatin may be similar to the genetically inactive heterochromatin of highly differentiated cells (Berlowitz, 1965; Frenster et al., 1963); certainly HSV infection is associated with decreased

cellular macromolecular synthesis (Roizman et al., 1965). In contrast CMV infection results in a general stimulation of cellular metabolism, with induction of cellular DNA polymerase (Hirai & Watanabe, 1976) and thymidine kinase (Estes & Huang, 1977), increased cellular DNA synthesis (St. Jeor et al., 1974) and chromatin template activity (Kamata et al., 1978) and increased synthesis of cellular RNA (Tanaka et al., 1975) and protein (Stinski, 1977). Although these increases are not always detectable early in infection they are dependent on virus-coded early functions. In productively infected cells stimulation of host DNA synthesis is shut down before it reaches detectable levels (De Marchi & Kaplan, 1977a). This shutdown is accomplished by early gene function(s) (De Marchi, 1983a) but frequently does not occur in cells in the G_0 or G_1 phase of the cell cycle as a result of contact inhibition or serum starvation. Such cells synthesise cellular DNA at an increased rate but do not express detectable amounts of viral antigen, or release progeny virus (De Marchi & Kaplan, 1977a).

In one non-permissive cell type in contrast, host DNA synthesis is stimulated and most but not all early antigens are expressed (De Marchi, 1983b). Those not expressed presumably include those responsible for shutdown of cellular DNA synthesis. Another early gene function expressed in productively infected cells is a viral DNA polymerase (Huang, 1975a). Unlike HSV and VZV, CMV does not encode a viral thymidine kinase (Miller et al., 1977) and vDNA synthesis is probably dependent on increased expression of the cellular

thymidine kinase. Thus the way in which IE and early gene functions are expressed plays a major part in determining the outcome of infection. The incomplete viral replication cycle that takes place in non-permissive cells and non-productively infected permissive cells may be achieved by modulating the expression of one or more of these genes. This may result in cell death from subversion or inhibition of cellular biosynthetic apparatus by viral macromolecules (Riepenhoff-Talty & Flanagan, 1982), or in the elimination of virus. Human CMV infection of mouse (La Femina & Haywood, 1983) or guinea pig cells (Fioretti et al., 1973) usually results in limited IE and early protein synthesis but no vDNA replication, and vDNA and antigens are undetectable 24-48h after infection.

Late events

The late phase of the CMV replication cycle begins with the onset of vDNA synthesis. This is detectable 15-24h post infection depending largely on the sensitivity of the detection method (Goodheart et al., 1964; Stinski, 1978; St. Jeor & Hutt, 1977). In HSV-infected cells vDNA synthesis begins around 6h post infection (Levitt & Becker, 1967), the period when β proteins are synthesised most abundantly (Honess & Roizman, 1974). The extended early phase and delay in vDNA replication in CMV-infected cells accounts in part for the longer growth cycle. This may be because one or more events in the early phase proceeds slowly or inefficiently. Alternatively, there may be little vDNA present in the

nucleus to act as a template for vDNA synthesis due to the inefficiency of disassembly of input virus.

Prior to, or during vDNA replication some of the genomes lose their linear form and become "endless", either by circularisation, concatemerisation or, less likely, by random integration into cellular DNA (La Femina & Haywood, 1983). It is not known if only one or both genomic forms contribute to the replicative pool, however endless forms are not detectable in at least one non-permissive cell type, suggesting that their presence is associated with a complete replication cycle. Circular and concatameric genomes are a feature of the rolling circle DNA replication mechanism (Gilbert & Dressler, 1968) which could allow isomeric variants of the genome to be generated during cleavage of the concatamer, as has been demonstrated in HSV DNA replication (Jacob et al., 1979). vDNA synthesis continues throughout the late period, peaking at 24-36h post infection then increasing again from 48-72h post infection (Stinski, 1978; St. Jeor & Hutt, 1977). This biphasic pattern of DNA synthesis is consistently observed. The first peak may serve to increase the pool of template molecules for subsequent rounds of replication after a period of late gene transcription (Cavallo et al., 1981).

Early genes are still expressed during the late period and may be responsible for the continuous replication of vDNA as proposed by Wathen & Stinski (1982). The presence of these proteins is however increasingly overshadowed by that of the late proteins, which are synthesised sequentially and in

proportion to the extent of vDNA synthesis (Stinski, 1978).

While vDNA synthesis proceeds and late proteins, which include the structural proteins, accumulate in the nucleus, the CPE develops further. Infected cells assume a fibroblastic or epithelial morphology but are still considerably larger than uninfected cells, and polykaryocytosis is occasionally observed (Albrecht et al., 1980a; Cavallo et al., 1981; Smith & De Harven, 1973). Early in the late phase (24h post infection) a large cytoplasmic inclusion visible by light and electron microscopy is sometimes observed which displaces the nucleus, causing it to take on an elliptical or reniform shape (Albrecht et al., 1980a; Kanich & Craighead, 1972a; Smith & De Harven, 1973). This inclusion appears to consist of accumulated Golgi and other organelles possibly involved in viral protein synthesis, but is clearly not essential for virus production which can proceed in its absence (Kanich & Craighead, 1972a).

The nuclear inclusion, which was present during the early phase becomes larger and better defined in the late phase (Cavallo et al., 1981) and is visible by light microscopy in fixed, stained cells (Albrecht et al., 1980a). It continues to develop and by 48h post infection is compartmentalised into a number of electron-lucent areas separated by a meshwork of electron-dense fibrillar material (Cavallo et al., 1981). These may be areas of vDNA synthesis surrounded by accumulated viral structural proteins.

Capsids and nucleocapsids first appear in the nucleus around this time and are found particularly around and within

the nuclear inclusion (Cavallo et al., 1981; Smith & De Harven, 1973). These particles increase in number over the next 24h and also begin to appear in the cytoplasm (Kanich & Craighead, 1972b). Particles enter the cytoplasm by budding into the perinuclear cisterna and from there into vacuoles formed by evagination of the outer lamina, or into the cytoplasm via the rough endoplasmic reticulum. Naked nucleocapsids are also present in the cytoplasm, possibly arising from fusion of the membrane acquired at the inner lamina of the nuclear membrane with the outer lamina. Particles which do not retain the membrane acquired at the inner nuclear lamina become enveloped by budding into cytoplasmic tubules and vesicles.

Later on in the late phase electron-dense bodies of variable size arise in the nucleus and bud into the cytoplasm in the same way as viral particles (Kanich & Craighead, 1972b; Sarov & Abady, 1975). These dense bodies probably consist of excess, abberantly assembled structural proteins without a DNA core.

Late cytoplasmic inclusions arise late in infection (Kanich & Craighead, 1972a), some comprised of naked nucleocapsids and dense bodies, others of enveloped viral particles associated with cytoplasmic membranes and organelles.

Free virus appears in the extracellular environment from 120h onwards (Kanich & Craighead, 1972a; Smith & De Harven, 1973). Virus probably leaves the cytoplasm via vacuoles formed from cytoplasmic tubules and vesicles, which then fuse

with the plasmalemma, releasing virus into the exterior. Cell lysis does not appear to play an important part in release of infectious virus, except perhaps at very late times (7-8 days; Smith & De Harven, 1973). Cell lysis at earlier times frequently occurs with low passage, wild virus isolates but little infectious virus is released during this process (Kanich & Craighead, 1972a).

Persistent CMV infections

The ability of herpesviruses to persist in the host without any overt virus replication for long periods of time has led numerous investigators to study persistent infection in culture in an effort to explain this phenomenon in biochemical terms. In vitro persistent infections have been established, although mainly in cells of a different type or species from those infected in nature. The relevance of these laboratory phenomena to persistent infection in the natural host is debatable but what has become apparent is that the replication cycle of herpesviruses can be blocked at a number of stages, the result of which may be a persistent infection.

For the viral genome to persist it must replicate at least as often as cellular DNA. This may be accomplished by autonomous replication or by integration into a cellular chromosome. Expression of late antigens and virus production need not be an essential feature of persistence and once the DNA synthesis necessary to establish the persistent state has occurred, expression of IE and early genes may also cease.

EBV is well known for its ability to persist in vitro

(Zur Hausen *et al*., 1972). Persistently infected cells are of an immortalised phenotype. The viral genome persists in an episomal form, as it does in lymphoblastoid lines established from Burkitt's lymphoma tissue (Lindahl *et al*., 1976) and also occasionally in an integrated form (Anderson-Anvret & Lindahl, 1978). Expression of the genome in these cell lines is variable; some, termed producer lines express viral structural antigens and release varying amounts of infectious virus. Other non-producer lines exhibit a more restricted expression of the viral genome (Epstein & Achong, 1977).

The first persistent CMV infection to be established *in vitro* was in hamster fibroblasts which had been exposed to ultraviolet inactivated virus (Albrecht & Rapp, 1973). Cells thus infected were of a transformed phenotype and induced tumours in hamsters. Only a proportion of the transformants expressed late antigens and infectious virus could not be rescued. This suggests that an incomplete viral genome persisted in these cells. Around the same time, similar experiments were performed with HSV 1 and HSV 2 (Duff & Rapp, 1971; 1973).

More recently transformation of human fibroblasts has been achieved using an unusually slowly replicating CMV isolate (Geder *et al*., 1976). Inactivation of virus was not necessary in this case, however infectious virus could not be rescued. This too is suggestive of persistence of a defective viral genome.

Boldogh *et al*. (1977) have described a persisent human CMV infection of mouse cells in which no early antigens were

detectable, suggesting that the genome was replicated, possibly in an integrated form by host cell enzymes. As such this may represent a truly latent infection. The complete genome persisted; antigen expression could be regained by fusion of the infected cells with permissive human cells and virus production was observed on treatment of the heterokaryons with the base analogue 5-iodo-2' deoxyuridine.

Gadler & Wahren (1983) established a persistent infection in fibroblasts in the presence of phosphoformic acid, which selectively inhibits vDNA polymerase. Viral sequences were associated with high molecular weight species of DNA which had a density close to that of cellular DNA. The complete genome may have become integrated into cellular DNA. The resumption of virus production on removal of the inhibitor, even after long periods of culture indicates that it persisted by some means.

Of these persistent infections, those from which infectious virus can be recovered are potentially useful as models of <u>in vivo</u> persistence. Others may be relevant in considering the possible oncogenic role of CMV. It is evident that the viral replication cycle may be aborted at various stages. Factors which bear upon this are the nature of the infecting virus, the cell type and the metabolic state of the cells. Other factors which may exert additional influences in the host are the presence of immune functions, of hormones (Tanaka <u>et al</u>., 1984a; b) and of IFN (Stinski <u>et al</u>., 1982).

INTRODUCTION

CHAPTER 2

EPIDEMIOLOGY OF CMV INFECTION

The epidemiology of CMV infection can be studied by monitoring either the prevalence of current virus excretion or of CMV-specific antibody. Current infection in the individual, which may or may not be primary, is frequently demonstrable by detecting virus in body tissues, fluids and excretions, and by measuring the developing immune response to the virus.

Most individuals who have experienced infection in the past are readily recognised by virtue of the long term persistence of specific immune functions, most obviously the presence of serum IgG antibody. Such seropositive individuals comprise what is often referred to as the immune population. The term "immune" should not be taken to mean insusceptible to further active CMV infection; it merely indicates that virus-specific immune functions are demonstrable. Individuals with long-standing immunity to CMV can occasionally be shown to be excreting virus. This infection may be local, where virus is shed from one anatomical site only or may be generalised, usually in patients who are immunodeficient or immunosuppressed. It is frequently impossible to determine whether this virus excretion is due to persistence of active viral replication for months or years following primary infection, reactivation of endogenous latent infection, or reinfection from an exogenous source. Huang et al. (1980)

compared serial isolates from women and from their congenitally infected infants by restriction endonuclease analysis and found that isolates from the same woman or from mother-infant pairs were nearly always genetically related, even when isolations were up to seven years apart. Thus reinfection could be excluded as a cause of viral excretion and subsequent intrauterine transmission in seroimmune women in most cases.

In addition, there is evidence that reactivated infection, that is viral replication following an apparently non-productive phase of infection, can also occur. In pregnant women the frequency of isolation of virus increases with increasing gestational age (Montgomery <u>et al</u>., 1972; Reynolds <u>et al</u>., 1973) although this may actually be due to a reduced rate of excretion early in gestation compared with nonpregnant women (Stagno <u>et al</u>., 1975a). This pattern of excretion, together with the predominance of shedding from the cervix alone and the stability of antibody titres suggests that this type of infection is the result of localised, reactivated virus replication which is in some way influenced by maternal physiological factors. Knowles <u>et al</u>. (1982) in studying cervical excretion in non-pregnant women found that the majority of women who shed virus did so during the first half of the menstrual cycle. Excretion for such a brief period which again appears to be influenced by physiological factors will be further evidence that reactivation of infection can occur, if this limited survey is confirmed on studying larger numbers. Further evidence for reactivation of infection comes from studies of infection in allograft recipients and is discussed

below. The actual frequency with which reactivation occurs in healthy adults is unknown as the infection is asymptomatic. Figures of 4-14% for pregnant and post partum women (Knowles et al., 1982; Montgomery et al., 1972; Reynolds et al., 1973; Stagno et al., 1975a) must be regarded as minimums as isolation can only be attempted on a limited number of occasions and excretion resulting from reactivated infection may be transient or intermittent. In the above studies there was noted a general trend towards a lower frequency of excretion in older and more multiparous women. Although clinically inapparent, reactivated infection is of epidemiological significance in congenital and perinatal transmission of infection and probably in other transmission routes also.

Human CMV infection is ubiquitous in all populations (Krech, 1973) and can occur at any age. Infection can be transmitted by various routes, some of which are important in particular groups of individuals where infection may be associated with pathological syndromes. These infection routes and the clinical features sometimes associated with them are described below. A discussion of the pathogenesis of infection in different clinical settings is reserved for the following chapter.

CMV infection in infancy

Exposure to CMV in infants who have not been infected in utero may begin during delivery if virus is present in the birth canal. Infants infected from this source usually begin to excrete virus three to twelve weeks after birth (Reynolds et al., 1973). Excretion of virus occurs in about 10% of women at

some time during pregnancy and usually begins late in gestation, continuing into the post-partum period (Stagno et al., 1975a). The frequency of infection in the newborn developing during the post-natal period is higher when maternal cervical excretion occurs close to term; when excretion occurs in the third trimester and in the post partum 57% of infants become infected postnatally, compared to 12.5% when maternal excretion occurs in the first or second trimester, and less than 1% when mothers do not excrete or shed virus only in urine or saliva (Reynolds et al., 1973; Stagno et al., 1980).

The other main mode of transmission of infection to the neonate is breast feeding. Excretion of virus in colostrum and milk is more common than excretion from any other maternal site. Stagno et al. (1980) found a minimal excretion rate of 13% in one population and 30% of seropositive mothers may excrete from this site during the nursing period (Dworsky et al., 1982). The incidence of neonatal infection among infants fed infective breast milk is high: 58% and 82% respectively in the cohorts studied by Stagno et al. (1980) and Dworsky et al. (1982). In a community where most mothers were seropositive (98%) and most breast fed their offspring (88%) Stagno et al. (1982a) found that 40% of infants became infected during the first year of life. This population is typical of many in developing nations. The high prevalence of past infection in the adult population and of breast feeding among mothers undoubtedly accounts to a large extent for the relatively high infection rate within the first year of life in these communities; figures as high as 92% have been observed in other

underdeveloped countries compared wth 3-5% in communities with a high standard of living (Krech & Tobin, 1981).

The effect of CMV infection during the first year of life may be minimal, except in those born prematurely or with some other condition where the outcome may be fatal (Gurevich et al., 1981). Viral excretion continues for at least three years (Stagno et al.,1975b) but many infants remain asymptomatic throughout (Reynolds et al., 1973). Mild symptoms consisting mainly of respiratory infection or hepatosplenomegaly have been associated with the onset of infection in about a third of cases however (Kumar et al., 1975).

The prevalence of antibody to CMV in the population continues to increase with age beyond the neonatal period (Carlstrom, 1965; Krech & Tobin, 1981; Rowe et al., 1956; Stern & Elek, 1965; Weller et al., 1957). The rate at which this age-related increase proceeds varies considerably between countries and between communities within countries: in most third world countries the rate of exposure approaches 100% by the fourth year of life, whereas in many developed nations the prevalence in this age group is 4%.

The high frequency of exposure during infancy in underdeveloped nations is usually attributed to the socioeconomic factor; most of the population lives under crowded conditions and poor hygienic standards. Evidence from at least two sources suggests that the former of these is the more important determinant. Sarov et al. (1982) working in southern Israel found a higher than expected number of seroimmune children in kibbutz communities where hygienic standards are relatively high but contact between children is

constant. 76% of these children between one and two years of age were seropositive whereas only 54% of this age group had antibody in urban communities. A significant difference in antibody prevalence was observed among children from urban communities when grouped according to the extent of crowding in living quarters. In addition, the same investigators studied exposure to CMV among children in a nomadic community living under poor hygienic conditions and found that the major age-related increase in exposure coincided with the start of schooling, when the frequency of contact with other children increased.

The other evidence linking close contact to CMV infection rates in infants comes from the observations of Pass et al. (1982a) who found an extraordinarily high incidence of viral shedding (59%) in children attending day care centres, most of whom came from a high socioeconomic background. Saliva was frequently infective and this was deemed the most important vehicle of transmission, either directly between infants or via contaminated inanimate fomites. A relatively high prevalence of past infection is observed in infants in Sweden (Carlstrom, 1965; Carlstrom & Jalling, 1970) despite its reputation as having the highest living standard in the world. Weller (1971) has suggested that this may be due to the widespread use of nurseries in that country.

Community-acquired CMV infection in young children is similar to maternally acquired neonatal infection in some respects. Viral excretion is prolonged - for up to two years following infection. The long term effects appear to be

negligible but respiratory illness may be associated with viral excretion (Stern, 1968).

Community-acquired CMV infection after infancy

In developed nations where a high percentage of the population escape infection in early life, a significant increase in the infection rate often begins in the adolescent years and continues into adulthood (Collaborative study, 1970; Krech & Tobin, 1981). The prevalence of antibody in the population is 20-25% at the onset of puberty, rising to 50-60% in the fifth and sixth decades of life (Carlstrom, 1965; Stern & Elek, 1965). Considerable circumstantial evidence links this to the onset of intimate salivary and sexual contact in this age group. Virus can be found in saliva (Faix et al., 1893a; Weller, 1971), semen (Biggar et al., 1983; Lang & Kummer, 1972; 1975; Lang et al., 1974), and cervical secretions (Faix et al., 1983a; Knowles et al., 1982; Stagno et al., 1975a). CMV infection occurs more frequently in women undergoing examination for suspected sexually transmitted disease (STD) compared to women examined for other reasons, both in terms of cervical excretion frequencies (13% versus 0% respectively) and antibody prevalence (83% versus 50% respectively; Jordan et al., 1973). Infection is also more common in those, both male and female, with a past history of gonorrhoea. 90% of these patients are seropositive compared to about 60% of other STD clinic patients (Jordan et al., 1973; Mindel & Sutherland, 1984). In addition, CMV infection is highly prevalent in homosexual men, who are frequently more promiscuous than heterosexuals. 76-94% of homosexuals have antibody, compared to

around 50% of heterosexuals, and up to 8% of the former may be viruric at any one time; viruria is rarely detectable in heterosexuals (Drew et al., 1981; Goldmeier et al., 1983; Mindel & Sutherland, 1984; Mintz et al., 1983).

Primary CMV infection during adolescence and adulthood is probably most frequently asymptomatic, but may on occasion present as a mononucleosis-like syndrome clinically similar or indistinguishable from that caused by primary EBV infection (Klemola & Kaarianen, 1965). CMV is responsible for about half of heterophile-negative cases of mononucleosis in over 15 year olds (Klemola et al., 1969).

The prevalence of past infection continues to increase with age at an estimated mean rate of 2% per annum until the age of 50 (Kane et al., 1975). After this age there is a reduced chance of acquiring CMV infection and in developed nations 20-40% of the population may escape infection altogether (Carlstrom, 1965; Stern & Elek, 1965).

Individuals who have immunity from past CMV infection may be reinfected upon subsequent re-exposure. This may be more likely when antigenic differences between the reinfecting strain and the original strain to which immunity is directed are significant. The in vitro immune response of seropositive individuals is quantitatively and qualitatively different when measured against different strains of virus as discussed in Chapter I. This may be reflected in vivo by reduced susceptibility to infection with a heterologous strain as compared to the homologous strain. Huang et al. (1980) identified two women in whom CMV isolates were genetically

distinct from previous isolates from the same woman in one case and from the congenitally infected infant of the other woman - strong evidence of reinfection with different strains of virus. Viral excretion in sexually active adolescents and young adults may be due in part to reinfection resulting from frequent exposure, although persistence or reactivation of the primary infection may also be responsible.

Age, socioeconomic status, particularly with respect to the extent of crowding in living quarters, and degree of sexual activity then emerge as major factors that influence the probability of CMV infection occurring or having occurred in the past. There is no evidence that climate influences the transmission rate of CMV (Krech, 1973). The possibility that genetic factors may be of significance in this respect is largely unexplored. Differences of this nature between racial or ethnic groups may influence susceptibility to infection or the extent and duration of viral shedding which would in turn affect the size of the reservoir of infection. Luby & Shasby (1972) reported a highly significant sex difference in the rate of seropositivity; only 33% of males had antibody compared to 55% of females. As others have found no such association (Stern & Elek, 1965), this may be due to social factors peculiar to the population studied, for example, greater contact between women and infants who may be excreting virus. Infected infants are thought to be an important source of infection for seronegative parents, particularly mothers, in other communities (Stagno et al., 1984; Yeager et al., 1982).

The epidemiological behaviour of community-acquired CMV infection is similar to some extent to that of EBV and HSV 1

infection. The prevalence of both these herpesvirus infections increases with age and is influenced by socioeconomic factors. The influence of sexual activity is less prominent than with CMV infection however. Where the standard of living is high the proportion with antibody to HSV-1 increases from 20% in early infancy to 97% in those over 60 years old (Smith et al., 1967). Among poorer communities as many as 71% are seropositive by the second year of life and 90% of over 15 year olds have experienced infection in the past (Buddingh et al., 1953). The EBV infection rate is 20% by the second year of life in most western nations, and reaches 70-90% in the adult population (Henle & Henle, 1979). In many third world countries by contrast, over 80% of the population are seropositive by the age of two years, and by the sixth year the figure approaches 100% (Henle et al., 1969). The reason for the greater frequency of infection with HSV-1 and EBV compared to CMV infection is not known but may be due to differences in infectivity and a greater dependence on close person to person contact for transmission of CMV infection to occur. This may account for the closer relationship between CMV infection and sexual contact than is observed with other herpesvirus infections.

Congenital CMV infection

A prorportion of infants are found to have CMV infection at birth. Such congenital infection is acquired from the mother and can be diagnosed by isolating virus from the infant within one week of birth, or detecting specific IgM antibody in the cord serum.

The clinical spectrum observed in congenitally infected infants is broad. Many are apparently healthy and infection would be unrecognised in the absence of laboratory evidence, however a proportion of these infants show signs of some degree of central nervous system (CNS) damage during follow up (Peckham et al., 1983; Reynolds et al., 1974; Starr et al., 1970). Others have mild symptoms which usually include hepatosplenomegaly and slight disturbance of liver function during the neonatal period (MacDonald & Tobin, 1978; Montgomery et al., 1980). Cytomegalic inclusion disease at its most severe is manifest by a range of symptoms and abnormal laboratory tests including hepatosplenomegaly, liver dysfunction, jaundice, petechial haemorrhages, thrombocytopenia, leukopenia, pneumonitis, microcephaly, cerebral calcification, motor and sensorineural dysfunction and mental retardation. Those who survive this rare syndrome usually suffer severe and permanent CNS damage (McCracken et al., 1969; Medearis, 1964; Weller & Hanshaw, 1962). In all however, 7-10% of congenitally infected infants may be left with serious brain damage (Hanshaw, 1971; Peckham et al., 1983) and many more may be left with subtle mental deficiencies resulting in poor educational progress or behavioural problems (Williamson et al., 1982). Those with symptoms attributable to congenital CMV infection at or shortly after birth generally have a poorer long term prognosis in this respect. Virus is commonly excreted in the urine of congenitally infected infants for up to four years and occasionally for even longer (Stagno et al., 1975).

Infection of the foetus may be more likely to occur when maternal infection occurs late in pregnancy (Griffiths &

Baboonian, 1984b; Nankervis et al., 1984) and may also be more severe at this stage of gestation (Monif et al., 1972) but further investigation is required in this area as other groups have failed to demonstrate such an association (Griffiths et al., 1980; Stagno et al., 1982b). One problem is that very large numbers of pregnant women must be closely monitored in order to demonstrate a statistically significant relationship between gestational age at the onset of infection and involvement of the foetus. Long-term collaborative work will probably be necessary to this end.

By contrast, there is considerable evidence to support the hypothesis that congenital infection is more likely and is associated with greater morbidity and mortality when acquired as a result of primary maternal infection. Congenital CMV infection follows primary maternal infection in about 50% of cases (Stagno et al., 1982b; Stern & Tucker, 1973). The frequency with which congenital infection follows secondary or reactivated maternal infection is unknown as diagnosis of this type of infection is far less straightforward. Stern & Tucker (1973) documented non-primary infection in eight pregnant women, none of whom gave birth to infected infants. This may have been due to the small numbers involved as others have shown that transplacental transmission of infection can occur frequently in this situation (Stagno et al., 1973; 1977; Schopfer et al., 1978). Medearis (1982) has estimated a frequency of 6% for such an occurrence.

With respect to the relative effects of congenital infections resulting from primary and non-primary maternal

infection, Stagno et al. (1982b) found that of 33 infants infected following primary maternal infection five had associated symptoms at birth. In contrast none of 27 infants infected as a result of non-primary maternal infection had any symptomatology, although such an event is not unknown (Ahlfors et al., 1981; Embil et al., 1970). In addition, Stagno et al. (1982a) found that CMV infection which was probably congenital in origin was associated with 1% of neonatal deaths in a relatively serosusceptible population. No such link was evident in a highly immune population where the rate of congenital infection was high but was largely due to secondary or recurrent maternal infections.

The reported incidence of congenital CMV infection around the world varies from 0.6% to 2.5% of live births (Levinsohn et al., 1969; Montgomery et al., 1980; Peckham et al., 1983; Starr et al., 1970; Stern, 1968; Stern & Tucker, 1973). On further analysis it is apparent that congenital infection occurs more commonly in highly immune populations than in less immune populations (1.6% and 2.4% respectively; Stagno et al., 1977; 1982a; 1982b).

Thus CMV causes more congenital infections than rubella virus (Griffiths et al., 1980; Griffiths & Baboonian, 1984b). Transplacental transmission of maternal infection with the latter occurs with a higher frequency - in as many as 90% of serologically confirmed maternal infections in the first trimester (Rawls et al.,1968; Thompson & Tobin, 1970), falling to between 30% and 50% of cases thereafter (Cradock-Watson et al., 1980). However the actual incidence of rubella infection

during pregnancy is much lower than that of CMV infection; White et al. (1969) identified clinical rubella in 0.04-0.08% of pregnant women during a non-epidemic period and estimated an overall incidence of 0.12-0.14% to account for subclinical cases. (The frequency rises during rubella epidemics and Sever et al. (1969) reported clinically apparent infection in 2% of pregnant women in one such epidemic.)

Transfusion-associated CMV infection

Recipients of blood from seropositive donors are at risk of acquiring CMV infection. In premature infants born to seronegative women this infection occurs in 13.5% of those receiving blood from immune donors but does not occur in those transfused with blood from seronegative donors (Yeager et al, 1981). Half of these infections are complicated by serious or fatal pneumonia or hepatitis. In infants born to seropositive women transfusions do not appear to be an imporant source of infection.

In adults who receive blood transfusions CMV infection occurs in 7-9% (Kane et al., 1975; Prince et al., 1971). This infection is commonly asymptomatic but may be accompanied by a "postperfusion syndrome" which is usually similar to CMV mononucleosis in adults (Kaariainen et al., 1966; Lang et al., 1968) although fatal complications have been encountered (Lang & Hanshaw, 1969). Transmission of infection is particularly associated with fresh blood (Lang et al., 1968; Lang & Hanshaw, 1969), with leukocyte-rich fractions (Winston et al., 1980), with multiple transfusions, when infection occurs in up to 20%

of recipients (Prince et al., 1971; Stevens et al., 1970), with blood that has IgM antibody to CMV (Beneke et al., 1984), and possibly with blood from cytomegaloviruric donors (Kane et al., 1975).

Some investigators have found that pre-existing immunity appears to protect against transfusion-acquired infection (Henle et al., 1970; Paloheimo et al., 1968) while others have found no difference in infection rates among immune and non-immune recipients (Prince et al., 1971; Stevens et al., 1970). The fact that the infection rate is not greater in seropositive transfusion recipients suggests that reactivation of infection in response to transfusion does not occur often and that most infections are acquired from virus present in blood products.

CMV infection in the immunocompromised host

a) Renal and cardiac allograft recipients

CMV infection is more common in renal transplant recipients than in transfusion recipients, occurring in over 70% of patients who survive for at least one month after transplantation (Craighead et al., 1967). There are a number of possible sources of this infection. These patients are immunosuppressed in order to suppress allograft rejection and may therefore be more susceptible to reactivation of endogenous, latent infection, reactivation of latent virus in donor organ tissue or transfusion-acquired infection. Further analysis of the statistics of renal transplant associated CMV infections permits certain comments to be made regarding these

possibilities. Patients who have serological evidence of past infection excrete virus more commonly than other patients following transplantation; 80-90% of the former group become infected compared to 50-60% of the latter group (Betts et al., 1975; Craighead et al., 1967; Ho et al., 1975). Thus latent CMV infection appears to be a significant source of infection in seropositive individuals. This is in contrast to the situation in blood transfusion and may be related to immunosuppressive therapy or to complex immunological reactions in transplant patients.

Over 80% of seronegative transplant candidates who receive an allograft from a seropositive donor become infected compared to 30% of cases where both recipient and donor are seronegative (Ho et al., 1975). The transplanted organ therefore appears to be a major source of infection, at least in those without prior latent infection. Infection in those cases where both donor and recipient are serongative is probably acquired in most instances from transfused blood and blood products.

A similar picture of CMV infection emerges among cardiac transplant recipients. The actual figures are even higher, infection being almost universal in seropositive recipients and occurring in half of seronegative recipients who receive organs from seropositive donors (Preiksaitis et al., 1983). In this study infection in seronegative recipients could be reduced by using only serologically screened blood products for transfusion. Again, at least three possible sources of infection can be identified; endogenous latent infection, the donor organ and blood transfusions.

CMV infection in renal and cardiac transplant recipients is frequently more severe than in the general population, presumably due to their reduced immunocompetence. In renal transplant recipients symptoms arise predominantly but not exclusively in those with primary infection (Betts et al., 1975; Ho et al., 1975), the most common symptoms being hepatitis and pneumonia.

b) Bone marrow allograft recipients

CMV infection occurs in up to 60% of bone marrow transplant recipients (Meyers et al., 1975; 1980c; Neiman et al., 1973; 1977). Serological diagnosis of infection in these patients is often unsatisfactory due to their profound immunosuppressed state and viral excretion is frequently not detectable even in the presence of disseminated infection (Neiman et al., 1973). The source of this infection is not known with certainty. Neiman et al. (1977) found that patients with antibody prior to transplantation were three times more likely to shed virus post transplant. Meyers et al. (1975) demonstrated an association between the incidence of interstitial pneumonia, which is frequently associated with CMV infection, and a positive donor serology. However a subsequent survey by this latter group (Meyers et al., 1980c) suggested that most infections were not attributable to the recipient or the donor but were acquired from other exogenous sources such as transfused blood products.

In these patients all infections are primary-like (Meyers et al., 1980) due to the ablation of immunological memory

resulting from total body irradiation. Infection is often associated with interstitial pneumonia, which is commonly fatal.

c) Patients with acquired immune deficiency syndrome (AIDS)

Homosexuals with AIDS frequently have elevated IgG and IgM antibody titres to CMV, however the significance of serological data in this situation is doubtful as B cell function is profoundly abnormal (Lane et al., 1983). CMV is nevertheless excreted in a high proportion of cases; 30% of those with Kaposi's sarcoma are viruric (Friedman-Kien et al., 1982) and in those with severe opportunistic infection virus shedding is almost universal (Gottlieb et al., 1981). Reinfection resulting from multiple exposure occurs and may be superimposed upon an ongoing infection. This has been shown by the isolation of genetically distinct strains of virus from different anatomical sites in the same patient (Drew et al., 1984; Spector et al., 1984). Reactivation of infection is also likely. Dissemination of infection occurs in the absence of immune restraint and pulmonary involvement contributes to the high mortality rate (Follansbee et al., 1982; Mildvan et al., 1982).

Healthy homosexuals do not usually excrete virus after the age of 30 (Drew et al., 1981), however older men who have haematologic findings consistent with immune dysfunction which may be a prodromal stage of AIDS, are occasionally virusemenic (Biggar et al., 1983). Thus the emergence of active CMV infection may take place early in the evolution of the syndrome and may therefore be pathogenetically involved in some cases.

d) Other immunosuppressed patients

Patients who are immunosuppressed but are not significantly exposed to exogenous infection (those not receiving transfusions or organ allografts) often have active CMV infection. Cytomegalic inclusions have been found at autopsy, most frequently in the lungs, of adults with malignancy (Rosen & Hajdu, 1971) and in those receiving corticosteroid therapy (Evans & Williams, 1968). Cytomegalic inclusions are rarely found in adults. This infection, which probably represents persistence or reactivation of endogenous viral replication, was asymptomatic and was not implicated as a cause of death.

Conclusion

The epidemiological pattern of CMV infection that emerges is similar to that of several other herpesviruses. Transmission facilitated by contact with infective tissues and secretions is a feature of HSV and EBV infection. Congenital transmission is more unusual but is also known to occur in CMV infection of the guinea pig (Choi & Hsiung, 1978; Kumar & Nankervis., 1978) and the mouse (Johnson, 1969) and in alcelaphine herpes virus type 1 infection of the wildebeest (Plowright et al., 1960).

Latency is a feature of herpesvirus infections and in the immune population reactivation is an important source of recurrent infection with HSV (Nahmias & Roizman, 1973) and VZV (Hope-Simpson, 1965). In immunosuppressed patients these reactivations become more frequent and severe (Gallagher &

Merigan, 1979; Montgomerie _et al_., 1969; Sokal & Firat, 1965) and reactivation of EBV infection also occurs (Cheeseman _et al_., 1980; Spencer & Andersen, 1972). Reinfection with HSV has been confirmed by RE analysis and it is likely that reinfection with VZV is possible, at least in immunosuppressed patients (Sokal & Firat, 1965).

Data from epidemiological studies in different patient groups gives information regarding the site(s) and mechanism(s) of viral latency and the pathogenesis of infection, and will be alluded to frequently in the following chapter.

CHAPTER 3

THE PATHOGENESIS OF CMV INFECTION

The relationship between CMV and the host is essentially a dynamic one. The ability to contain infection is dependent on the capacity to develop and maintain effective antiviral mechanisms, and while CMV infection may be quiescent and apparently inconsequential for long periods, physiological changes in the host are liable to disturb this balance. Our understanding of the factors which determine the expression of this host/virus relationship is limited by certain aspects of the virus and its host interaction. Firstly, because of its species specificity no animal model is available for studying infection with human CMV. Although similar herpesviruses have been found in many other species, their classification as CMV's is often based on their cytopathic effects and species specificity; no other similarity to the human virus is necessarily implied. Nevertheless some animal CMV infections serve as useful models provided that their dissimilarities with human CMV infection are borne in mind. Most extensively studied are the CMV infections of the mouse and guinea pig.

A second limitation arises from the fact that active CMV infection in most humans is unrecognised. As most investigations are limited to individuals with clinically apparent infection, or to those at increased risk of active

infection, much current knowledge in this area pertains to unusual manifestations of CMV infection. Active infection has been studied mainly in those with a mononucleosis or postperfusion syndrome, in congenitally and neonatally infected infants, in pregnant women and in immunocompromised patients. It is not known how this information relates to infection in other milieux. The presence of clinical signs and symptoms in association with infection may result from a host response which is distinctly different from that in those who remain asymptomatic. Alternatively the differences in response may be quantitative rather than qualitative.

In this chapter current concepts regarding the host/virus relationship are discussed. Particular emphasis is given to the interaction of CMV with the immune system - the effect of CMV infection on immune function, the immune response mounted in the face of viral challenge, and the influence of this response on the virological and clinical expression of CMV infection. As the nature of this interaction and its outcome is dependent to some extent upon the setting in which it occurs, the pathogenesis of infection is discussed in the contexts of those settings which are widely recognised and well studied. In order to understand the interaction between CMV and the immune system, it is necessary first of all to consider certain aspects of immunological function of importance in controlling viral infections. This discussion of CMV infection in various settings will provide a basis for subsequent discussion on CMV infection in homosexual men and its relationship with and contribution to immune impairment.

The immune response in viral infections

Viral replication results in the synthesis of proteins and other macromolecules which are foreign to the host and many of these molecular species will elicit a specific immune response. The generation of immune responses has been studied extensively in the mouse using in vitro but also in vivo experimental systems. Characterisation of the human immune response has to a large extent followed in the wake of the murine studies. The main features are comparable in both species.

The induction of an immune response requires that antigen be presented to the immune system in an appropriate form. Antigen must be presented in association with major histocompatibility complex (MHC) antigens. In the human and in the mouse, these antigens are of two main classes: Class I antigens (HLA-A, -B and -C in the human, H2-K and -D in the mouse) and Class II antigens (HLA-DR in the human, Ia in the mouse). Antigen present on the surface of infected cells is usually associated with Class I MHC antigens as, with the exception of B cells, activated T cells, specialised antigen presenting cells, bone marrow precursor cells and spermatozoa, most cell types do not normally express Class II antigens (Ko et al., 1979; Wernet, 1976; Winchester et al., 1977). Antigen presentation in association with Class II antigens is accomplished by specialised cells expressing Class II antigens. Traditionally regarded as the most important antigen presenting cells are the mononuclear phagocytes (Bergholtz & Thorsby, 1977). These are present in peripheral blood as monocytes which subsequently localise in various tissues where they undergo

morphological changes and become fixed macrophages comprising the reticuloendothelial system. Some other cells are also capable of antigen presentation, including endothelial cells (Hirschberg et al., 1980), Langerhans cells (Braathen & Thorsby, 1980) and dendritic cells (Crow & Kunkel, 1982). There is evidence that these cells are actually more effective than monocyte-macrophages at antigen presentation (Braathen & Thorsby, 1983; Van Voorhis et al., 1983) but as most studies have concentrated on antigen presentation by adherent mononuclear leukocytes, these cells will be referred to in this account.

Different T lymphocyte subpopulations recognise and respond to antigen in association with different MHC class products. Antigen in association with Class I antigens is recognised by T lymphocytes which perform suppressor and cytotoxic functions mainly (Engleman et al., 1981; McMichael et al., 1977). In the human these T cells can be identified by reaction with the monoclonal antibody OKT8 (Reinherz et al., 1979b) and will be referred to as T8+ lymphocytes. T cells with helper and inducer functions recognise antigen in association with Class II MHC products (Engleman et al., 1981) and are identifiable by reaction with the monoclonal antibody OKT4 (T4+ lymphocytes; Reinherz et al., 1979a). The correlation between surface marker expression and effector function is not absolute as both T4+ suppressor cells (Thomas et al., 1981) and T8+ helper cells (Friedman et al., 1981) have been described.

Interaction of antigen on the surface of a presenting cell with memory T4+ cells specific for the presented antigen

results in stimulation of the presenting cell to produce interleukin 1 (IL 1) and in sensitisation of the T4+ cell resulting in a shift from the resting (G0) phase to the G1 phase of the cell cycle (Stadler et al., 1981) and the onset of synthesis of type II (immune or gamma) IFN and other lymphokines (Hecht et al., 1983). At the same time, some of these lymphocytes become responsive to IL 1, and others to IL 2 (Palacios, 1981). In the presence of IL 1, IL 1-responsive lymphocytes produce IL 2 (Mizel & Ben Zvi, 1980) which acts as a second signal for antigen-primed T8+ as well as T4+ cells, driving them into the S phase of the cell cycle (Stadler et al., 1981), leading to clonal expansion of antigen-specific lymphocytes. This results in greatly increased release of lymphokines necessary for the maturation or enhancement of a variety of immune functions as described below.

The interaction of MHC antigens with T cell membranes is also the initial activation signal in the mixed lymphocyte reaction (MLR). In the autologous MLR T4+ lymphocytes proliferate in response to HLA-DR antigens on non-T cells (Engleman et al., 1980). In the allogeneic MLR T4+ lymphocytes proliferate in response to allogeneic HLA-DR antigens and T8+ lymphocytes respond to allogeneic non-DR antigens, probably to MHC Class I antigens, although this latter response is dependent to a large extent on T4+ lymphocyte responses (Engleman et al., 1981), possibly for IL 2 and other helper factor production (Mannel et al., 1983; see below). In the MLR monocytes are not required for antigen production, only for IL 1 production (Berlinger et al., 1976).

These MHC antigen-T cell membrane interactions which lead to T cell activation and induction of responsiveness to IL 1 or IL 2 can be mimicked _in vitro_ by T cell mitogens such as phytohaemagglutinin (PHA), concanavalin A (Con A) and the pan T cell-reactive monoclonal antibody OKT3 (Palacios, 1982). PHA stimulates T4+ cells preferentially while Con A induces a comparable response in both subsets (Reinherz _et al_., 1979a). In mitogen stimulated cultures lymphocytes of both subsets may release IL 2 (Fishbein _et al_., 1983; Luger _et al_., 1982) although T4+ cells are probably the main IL 2 producers under most antigenic stimuli (Palacios, 1982).

Antigen- or mitogen-primed T8+ lymphocytes, as well as releasing lymphokines involved in immune regulation (Haynes & Fauci, 1977; Krammer _et al_., 1982), are capable of maturing into cytotoxic lymphocytes (CTL's). This is dependent on the presence of IL 2 and two other lymphokines produced by activated T4+ cells (Biddison _et al_., 1981; Larsson, 1981; Mannel _et al_., 1983). Mature CTL's recognise and kill cells expressing the appropriate antigen in expression with Class I MHC antigens (McMichael _et al_., 1977) or cells expressing foreign Class I antigens. CTL's arising during viral infections in the mouse have been shown to be extremely sensitive and may be directed against antigens which are not usually considered to be expressed in appreciable amounts on the cell surface, such as influenza virus nucleoprotein (Townsend & Skehel, 1984) and viral polymerase (Bennink _et al_., 1982), ectromelia virus early proteins (Ada _et al_., 1976) and the immediate early proteins of HSV (Pfizenmaier _et al_., 1977) and murine CMV

(Reddehase & Koszinowski, 1984; Reddehase et al., 1984b). The action of these effectors may therefore be important in eliminating infected cells prior to the assembly and release of progeny virus.

The recognition of antigen by B cells is fundamentally different from T cell recognition and is not MHC-restricted. The initial event in B cell activation is the interaction of the relevant antigen with specific immunoglobulin present on the B cell membrane. Activated B cells are rendered responsive to IL 1 (Howard et al., 1983) and the T lymphokine B cell growth factor (BCGF) which induces B cell proliferation (Muraguchi & Fauci, 1982). The capacity of these cycling B cells to secrete antibody is dependent on the activity of a further two T lymphokines (Nakanishi et al., 1983). This accounts for the requirement of T cell helper functions in the development of humoral immune responses.

In addition to these acquired, specific immune responses, several innate immune functions may be of importance during viral infections. Natural killer (NK) cells are medium sized non-T lymphocytes and may provide important cytotoxic functions against virus-infected cells. Their function is not genetically restricted or antigen-specific. The way in which NK cells recognise infected cells is not understood, but may be mediated by foreign viral antigens (Bishop et al., 1983) or by cellular targets such as the transferrin receptor (Vodinelich et al., 1983), the expression of which may be increased in virus-infected cells (Borisiewicz et al., 1985). This would also explain the potent anti-tumour cell activity of NK cells

(Herberman et al., 1975) and provides a basis for understanding their proposed role in regulating cell proliferation and differentiation. Although active NK cells are present in peripheral blood in the absence of infection or immune stimulation, their function is greatly enhanced by immune IFN (Weigent et al., 1983b). NK-like functions are also mediated by mononuclear phagocytes.

Killer (K) cells are similar to NK cells but employ a different recognition mechanism. Antibody-coated target cells are susceptible to K cell lysis and interaction of the Fc component of the coating antibody with the K cell Fc receptor is necessary for this to occur (Melewicz et al., 1977). While IgM cannot participate in this antibody-dependent cellular cytotoxicity (ADCC) in itself, it can greatly enhance IgG-mediated ADCC, probably by promoting effector-target contact (Perlmann et al., 1981). This may be important in the early stages of an immune response when little IgG antibody is present. ADCC may also be mediated by monocyte-macrophages and polymorphonuclear leukocytes (PMNL's; Kohl et al., 1977; MacDonald et al., 1975).

Type I IFN may be produced in varying amounts depending on the infecting virus. Unlike type II IFN, type I IFN is not a product of the specific immune response but is produced mainly by leukocytes (alpha IFN) and fibroblasts (beta IFN) in direct response to virus. Both types of IFN may inhibit viral replication by direct effects on infected cells, although the two types vary in relative activity against different viruses (Rubin & Gupta, 1980). Both IFN types potentiate NK cell

activity (Herberman et al., 1979), although type I IFN alone gives only a weak enhancement (Weigent et al., 1983a). Type I IFN may also enhance CTL function (Zarling et al., 1978), ADCC mediated by K cells (Warren et al., 1980) and PMNL's (Hokland & Berg, 1981), and the phagocytic and natural killing activities of monocyte-macrophages (Huang, 1977; Schultz et al., 1977; Stanwick et al., 1980). The effect of type II IFN on these functions is not known, with the exception of monocyte-macrophage mediated tumour cell killing which is enhanced (Schultz & Kleinschmidt, 1983). Type II IFN also induces increased Class II MHC antigen expression in macrophages (Steeg et al., 1982) which may enhance antigen-presenting function.

Immune responses are controlled by a number of negative feedback mechanisms. This is necessary to control the inflammatory response and limit tissue damage. Some feedback mechanisms act by recruiting or activating suppressor T cells. Suppressor cells may be antigen-specific (Uytdehaag et al., 1979) or non-specific (Kasakura et al., 1983b; Haynes & Fauci, 1977) in their effects and may act on other T cells, B cells or antigen-presenting cells (Kasakura et al., 1983b; Warrington et al., 1983). The way in which suppression is mediated is not fully understood; one mechanism is the inhibition of IL 2 production (Chouaib & Fradelizi, 1982). There is evidence that both direct cell to cell contact (Kasakura et al., 1983a) and release of soluble mediators (Uytdehaag et al., 1979; Warrington et al., 1983) can result in suppression in different circumstances. The induction of suppressor cells may be mediated by the T lymphokine(s) suppressor cell induction

factor (Eardley et al., 1978; Kasakura et al., 1983a) or by prostaglandins produced by monocytes when present in high numbers (Chouaib & Fradelizi, 1982). Prostaglandins also inhibit NK and K cell activity (Droller et al., 1978).

Macrophages can suppress in vitro immune responses in much lower numbers than monocytes (Rinehart et al., 1979) and may have an important in vivo regulatory effect later in the inflammatory response following localisation and maturation of monocytes at the site of infection. This suppression is mediated by soluble mediators which act directly on effector cells or by inducing suppressor cells which probably act by direct cell contact.

NK cells can suppress helper T cell function in an analogous way to suppressor T cells (Arai et al., 1983). IFN's, as well as potentiating immune functions, can suppress B cell function (Brodeur & Merigan, 1975; Sonnenfeld et al., 1978) and monocyte to macrophage maturation (Lee & Epstein, 1980), and can activate T cells to release soluble mediators of immune suppression (Aune & Pierce, 1982).

An appreciation of these events has led to a clearer understanding of immune dysfunction in various pathological situations and is important in understanding immunological events in both CMV infection and in AIDS and related conditions.

CMV-mononucleosis

The mononucleosis syndrome is diverse in its clinical manifestations but has been defined as "an acute infectious

disease characterised by fever, enlargement of the lymphatic glands and changes in the blood, especially lymphocytosis." (Tidy, 1934). Most cases are caused by EBV (infectious mononucleosis [IM]; Niederman et al., 1968) and can usually be diagnosed in the laboratory by the presence of heterophile antibody. Heterophile-negative cases of mononucleosis are nevertheless common and diagnosis is justified in the absence of heterophile antibody provided other features are consistent, namely the persistence for at least ten days of a blood condition where at least 50% of leukocytes are mononuclear cells and where at least 10% of these are atypical (Klemola et al., 1970). One half to two thirds of such cases are caused by CMV; the remainder are due mainly to EBV but occasionally to other viruses or Toxoplasma gondii (Horwitz et al., 1977; Klemola et al., 1970).

Primary infection may present as a mononucleosis syndrome usually only in individuals after the onset of puberty and in adults (Klemola et al., 1970). The interval between infection and onset of symptoms is impossible to ascertain in community-acquired infections but is on average 3-4 weeks in transfusion-associated infections, assuming that infection occurs at the time of transfusion (Ten Napel & The 1980a). The major clinical features are fever of 2-5 weeks duration, lymphocytosis and abnormal liver function tests, often accompanied with hepatosplenomegaly, and occasionally with rash, haemolysis and jaundice. Unlike mononucleosis caused by EBV or other agents pharyngitis or significant lymph node enlargement are not present (Horwitz et al., 1980; Kaariainen

et al., 1966; Klemola & Kaariainen, 1965; Klemola *et al.*, 1969; 1970; Lang & Hanshaw, 1969; Lang *et al.*, 1968).

During the acute phase of CMV-mononucleosis, virus is readily isolated from buffy coat cultures (Lang *et al.*, 1968). This virus is associated predominantly with adherent cells from the PMNL-rich and mononuclear leukocyte-rich fractions. Virus cannot be isolated from red blood cells, plasma or leukocyte lysates (Carney & Hirsch, 1981; Rinaldo & DeBiaso, 1983; Rinaldo *et al.*, 1977). This evidence points to the PMNL and/or the monocyte-macrophage cell type as the main reservoir of virus in the blood during viraemia. *In vitro* attempts to infect blood cells have found the monocyte to be the most permissive host cell (Einhorn & Ost, 1984). The association of virus with these cells may be due to phagocytosis of virus released at the site(s) of viral replication. That this association is not merely passive however is suggested by the fact that virus cannot be recovered from leukocyte lysates. In addition, monocytes from viraemic patients have suppressor cell properties similar or identical to those of monocytes actively infected with CMV *in vitro*, but distinct from those of monocytes passively exposed to virus (Carney & Hirsch, 1981; see below). PMNL function is not altered during mononucleosis (Rinaldo *et al.*, 1979).

Virus is often, although not always detectable in saliva and urine during the acute phase (Rinaldo & DeBiaso, 1983; Rinaldo *et al.*, 1977). Leukocyte-associated viraemia may be responsible for dissemination of virus to anatomical sites distant from the site of initial infection. Viral antigens have

been demonstrated in liver tissue during CMV-mononucleosis (Sacks & Freeman, 1984) and involvement of the liver, kidneys and salivary glands is well recognised in other CMV - related syndromes to be described subsequently.

During convalescence virus can be isolated rarely from leukocytes, occasionally from saliva and frequently from urine (Rinaldo & DeBiaso, 1983). Viruria can persist for long periods; frequently for two years, and longer on occasion (Klemola et al., 1969). Virusemenia likewise has been shown to persist for more than a year following primary infection (Lang et al., 1974).

The acute phase of infection is characterised by an array of haematologic and immunological peculiarities. A range of unusual antibody reactivities are present, including erythrocyte autoantibodies, rheumatoid and antinuclear factors, cold agglutinins and cryoglobulins (Kantor et al., 1970). This resembles the polyclonal stimulation of IgG and IgM synthesis that occurs in IM (Wollheim & Williams, 1966). In both cases this may result from the direct activation of immunoglobulin secretion by B cells following exposure to EBV or CMV. This has been clearly demonstrated in vitro (Hutt-Fletcher et al., 1983; Rosen et al., 1977), although there is no evidence that CMV infects B cells in vivo or in vitro.

The cause of lymphocytosis in CMV-mononucleosis may also be similar to that in IM. In the acute phase of IM lymphocytosis is evident both in the increased numbers of circulating lymphocytes and in the lymphocytic infiltration of lymphoid and many other organs and tissues (Carter 1972),

accounting for the enlargement of lymph nodes, liver and spleen. Lymphocytosis appears to be due to an initial proliferation of B cells resulting from infection with and activation by EBV, which is subsequently overshadowed by a massive proliferation of T cells in response to infection (Mangi et al., 1974). In addition to eliminating cells actively infected with virus this response may be important in curtailing B cell proliferation and hyperactivity (Carter, 1975). Of relevance in this respect is the observation that increased numbers of circulating lymphocytes in IM is at least partly due to elevated numbers of T8+ lymphocytes (De Waele et al., 1981; Reinherz et al., 1980).

Although CMV does not infect lymphocytes, at least during acute mononucleosis, it may stimulate their proliferation as suggested by the in vitro experiments cited above, and the proliferation of regulatory T cells may also be in response to B cell hyperactivity in this instance. Indeed increased numbers of T8+ lymphocytes are also observed in CMV-mononucleosis (Carney et al., 1981; Rubin et al., 1981). A vigourous T cell response to viral antigens in other cells (e.g. PMNL's or monocytes) may also contribute to lymphocytosis.

The presence of numerous "atypical" lymphocytes is also reminiscent of IM. In the latter these have been shown to be mainly T cells (Sheldon et al., 1973). The appearance of these cells is due to their activated state. During acute IM high numbers of virus-specific cytotoxic T cells circulate (Rickinson et al., 1977a; Svedmyr & Jondal, 1975), and these account for at least some of the atypical lymphocytes (Crawford

et al., 1981).

Accompanying these abnormalities in lymphoid cell populations in both IM and CMV-mononucleosis is a generalised state of reduced immuno-competence. This hyporesponsive state is demonstrable in vitro as a reduced ability of peripheral blood lymphocytes to proliferate and produce lymphokines in response to certain mitogens and recall antigens (Carney et al., 1983; Levin et al., 1979; Mangi et al., 1974; Rinaldo et al., 1980; Ten Napel & The, 1980b; Wainwright et al., 1979) and as an inability to develop cytotoxic effector lymphocytes following in vitro sensitisation (Carney et al., 1983). This defect may result in part from the imbalance of T cell subsets caused by increased and decreased numbers of T8+ lymphocytes and T4+ lymphocytes respectively (Carney et al., 1981). Homeostatic regulation of immune responses is dependent on the balance between these subpopulations (Reinherz & Schlossman, 1980) and a preponderance of T8+ cells resulting from a vig o rous immune response may be accompanied by a large release of soluble mediators of DTH suppression ("antigenic competion"; Mangi et al., 1974). Wainwright et al. (1979) have partially characterised a serum factor present during acute IM which inhibits DTH responses in vitro and may mediate this effect in vivo.

Such a factor has not been demonstrated in CMV-mononucleosis (Rinaldo et al., 1980) and in this case there is evidence that alternative or additional immunosuppressive mechanisms may operate, arising from the interaction of CMV with monocytes. Monocytes play a role as helper cells for

lymphocyte proliferation responses to concanavalin A (Con A) and recall antigens in CMV-mononucleosis as shown by the further reduction in proliferation that occurs following their depletion (Rinaldo & DeBiaso, 1983). These monocytes, or a subgroup of them, however may also have a suppressive effect during acute infection. Preculturing lymphocytes from acutely infected patients prior to stimulation leads to a partial recovery of the Con A response which can be abrogated by the addition of fresh autologous adherent mononuclear cells (Rinaldo & DeBiaso, 1983; Rinaldo et al., 1979). Preculture has been shown to selectively eliminate the preponderant con A-hyporesponsive suppressor T cells, thus enriching for T4+ lymphocytes which proliferate normally in response to Con A (Carney et al., 1983). It therefore appears that monocytes can facilitate the proliferative response of T8+ cells while inhibiting the response of T4+ cells. This inhibition is associated with monocytes infected with CMV in vivo and a similar effect on the Con A response of precultured lymphocytes from healthy donors can be achieved with autologous monocytes infected in vitro (Carney & Hirsch, 1981). This may be due to interference with the antigen-presenting capacity of monocytes to T4+ lymphocytes, stimulation of the release of prostaglandins or other substances resulting in the recruitment of suppressor cells, or to other effects of CMV infection on monocytes. Non-productive infection of monocytes, without expression of late viral antigens also results in a similar alteration of monocyte function (Rice et al., 1984).

The hyporesponsive state of T8+ lymphocytes and their

inability to give rise to cytotoxic effector cells may be due to prior immunological commitment resulting from their activated state; mononuclear cells, particularly T8+ cells have elevated spontaneous proliferation rates (Carney et al., 1983; Rinaldo & DeBiaso, 1983; Rinaldo et al., 1980). In addition, a high proportion of lymphocytes express HLA DR antigens, characteristic of immunologically activated cells during the acute phase (Carney et al., 1983).

The differential activity of infected monocytes on T cell subpopulations may be responsible in vivo for both the activation of and proliferation of suppressor T cells and the suppression of immune responses to recall antigens which are dependent on helper T cell function. Monocytes with enhanced suppressor function have been observed in some other infectious diseases of man (Ellner et al., 1978; Katz et al., 1979; Piessens et al., 1980).

These immunological abnormalities are maximal during the acute phase. Lymphocytosis and hypergammaglobulinaemia disappear early in convalescence (Kantor et al., 1970). Other features normalise much more slowly; although T8+ lymphocytes are no longer activated, their numbers are still elevated (Carney et al., 1981) and lymphocyte responses remain below average for over two years in some cases (Ten Napel & The, 1980b).

During the second week following the onset of symptoms a CMV-specific humoral immune response becomes detectable. Specific IgM is always produced in previously immunocompetent individuals with the mononucleosis syndrome and can be detected

in serum around this time (Kangro et al., 1982; Rasmussen et al., 1982). Viral glycoproteins are major targets of this response (Pereira et al., 1982b) and IgM is the major class of antibody that reacts with early and late membrane antigens by immunofluorescence (Landini et al., 1984). This IgM frequently has complement fixing (CF) activity (Booth et al., 1980; Cremer et al., 1975) and a complement-dependent cytolytic activity in vitro (Betts & Schmidt, 1981). IgM antibodies to late, and to a lesser extent early intracellular antigens are also demonstrable (Pereira et al., 1982b; Riggs & Cremer, 1980).

The IgG response becomes apparent at the same time as or slightly delayed with respect to the IgM response (Kangro et al., 1982). This response consists mainly of the IgG1 and IgG3 subclasses, with the latter usually appearing first (Linde et al., 1983), and is directed against various structural and non-structural antigens including membrane glycoproteins (Pereira et al., 1982b), nucleocapsid antigens (Cremer et al., 1975; Schmitz et al., 1980a), intracellular late antigens (The et al., 1974) early antigens (Landini et al., 1984) and pre-early nuclear antigens (Gartner & Orstavik, 1984). Neutralising antibody titres are often delayed with respect to other measurements of antibody (Andersen, 1970; Carlstrom et al., 1968). This is not simply due to the relative insensitivity of this test as neutralising titres rise as CF titres fall. Neutralising antibodies may be reactive with different antigens, or alternatively may be of a different IgG subclass from CF antibody.

Specific serum IgA titres usually rise concomitantly with

IgM and IgG tires, although they are generally lower in magnitude (Levy & Sarov, 1980). Both IgA subclasses are produced (Linde _et al_., 1983) and this response has also been shown to be directed against early and late antigens (Riggs & Cremer, 1980).

This humoral response reaches its maximum during the acute phase of infection. Maximum IgM titres are present 3-6 weeks after onset of symptoms (Kangro _et al_., 1982) and coincide with the viraemic phase (Rasmussen _et al_., 1982). Titres decline during convalescence and are usually undetectable after three or four months (Kangro _et al_., 1982), although low titres may persist for longer (Lang _et al_., 1974; Rasmussen _et al_., 1982). Other antibody responses often remain elevated throughout convalescence; IgA may decline in parallel with IgM but can also persist for much longer on occasion (Levy & Sarov, 1980). IgG titres persist indefinitely, although not at the elevated titres observed during acute infection (Andersen, 1970; Klemola _et al_., 1969) and IgG antibodies directed against some antigens may decline more rapidly than others; antibody to early antigens declines during the convalescent phase in some cases (The _et al_., 1974).

In contrast, the development of the specific DTH response is markedly delayed relative to the humoral response (Ten Napel & The, 1980a); its appearance coincides with the start of the recovery of other proliferative responses to mitogens and recall antigens (Levin _et al_., 1979). Suppression of the virus-specific DTH response during the acute phase may then be mediated by the same factors that cause general

immunosuppression. The specific proliferative response is usually negative during the first month after onset and does not reach levels comparable to those of healthy, immune persons until after the third month (Carney et al., 1983; Levin et al., 1979), when the response may actually be higher than average for a time (Pollard et al., 1978). Other proliferative responses begin to recover during this period also but as already discussed, often do not attain levels comparable to controls for many months. The developing immune response to CMV during convalescence may thus prevent the complete restoration of other responses by a mechanism analagous to antigenic competition as proposed to take place in IM by Mangi et al. (1974).

MHC-restricted and nonrestricted cytotoxic responses in CMV-mononucleosis have not been studied in depth to date. In IM cytotoxic effector cells are present during the acute phase; there is a rapid proliferation of NK cells (Lipinski et al., 1979) and K cells (Jondal, 1976). EBV-specific cytotoxic T cells are also present early in infection - this response is thought to contribute to the suppression of other T cell functions, as specific DTH responses are absent during acute IM also. These CTL's are unusual however as they are not HLA-restricted, being able to kill all EBV genome-containing lymphoid lines (Lipinski et al., 1979; Rickinson et al., 1977; Svedmyr & Jondal, 1975) and many appear to lose characteristic T cell markers following in vitro activation (Svedmyr et al., 1974). Typical HLA-restricted CTL's only appear during convalescence (Misko et al., 1980; Moss et al., 1978; Rickinson

et al., 1979; 1980). Comparison of CMV-mononucleosis with IM may be misleading in this instance.

Cytotoxic responses in murine CMV infection have been studied and may be comparable to the situation in primary infection in the human. Primary CMV infection in the mouse also results in a general suppression of DTH responses (Howard *et al.*, 1974; Osborn *et al.*, 1968). Cytotoxic responses, in contrast, are elicited rapidly. On the third day after infection numbers of splenic NK and K cells increase rapidly (Quinnan & Manischewitz, 1979). The rapid nature of this response may be facilitated by the IFN response which is present on the second day (Grundy *et al.*, 1982; Quinnan & Manischewitz, 1979). These responses are transient, being undetectable after six days, and are replaced by an H-2 restricted CTL response demonstrable in splenic and peripheral blood lymphocytes on the fourth or fifth day and in lymph node cells on the sixth day (Ho, 1980; Quinnan *et al.*, 1978; 1980). This response is maximal 7-10 days after infection, correlating with the fall of infectious virus titres in the spleen, and declines to undetectable levels 2-3 weeks after infection. It is noteworthy that these responses precede the formation of specific antibody, which becomes detectable 8-10 days after infection (Manischewitz & Quinnan, 1980).

Cellular cytotoxic responses to CMV infection in humans who have received bone marrow allografts have been studied and will be discussed subsequently, however it is worth stating at this point that specific cytotoxic responses develop relatively quickly in those with non-fatal CMV infection despite their

general immunosuppressed state and often precede the rise in antibody titres (Quinnan et al., 1981; Rook et al., 1984).

In view of these examples then, it is not unlikely that thymic and non-thymic cytotoxic responses develop rapidly during the course of CMV-mononucleosis. As already stated, the generation of cytotoxic effector T cells following in vitro sensitisation with allogeneic cells is suppressed in acute mononucleosis (Carney et al., 1983). It is of interest however that murine CMV infection also inhibits primary and secondary cytotoxic responses early in infection when the CMV-specific CTL response is present (Ho, 1980). Again an antigenic competition effect where the CTL response results in excessive negative feedback inhibition which suppresses cytotoxic as well as proliferative responses may be operative in both the murine infection and in CMV-mononucleosis.

Mononucleosis is an unusual clinical response to primary CMV infection. Whether or not the virological and immunological features are also unusual, qualitatively or quantitavely, is not known and any attempt to construct a comparable picture of events in healthy adults who have asymptomatic primary infection remains largely speculative at present as such cases are rarely identified and followed prospectively. Whether infection is asymptomatic or not may be determined by a variety of factors such as the size of the infecting dose, the route of infection, the timing of the immune response and various host factors including age.

Congenital CMV infection

Transmission of infection to the foetus occurs in a proportion of women who experience CMV infection during pregnancy. This has been associated with foetal loss in 15% of primary maternal infections during early gestation (Griffiths & Baboonian, 1984b) although it could not be ascertained if this was actually due to intrauterine infection. Liveborn congenitally infected infants may manifest signs and symptoms of cytomegalic inclusion disease or may be asymptomatic initially, developing late onset disease during the neonatal period. Most infections however remain subclinical throughout (Chapter 2). Aspects of maternal infection which may influence the risk of transmission to the foetus and its outcome are discussed subsequently.

Whether symptomatic or not, congenitally infected infants excrete virus in high titre in urine, although the former shed significantly higher titres during the first months of life (Griffiths et al., 1982a; Pass et al., 1983c; Stagno et al., 1975b). The amount of virus excreted falls with increasing age, but most are still viruric at five years of age. Salivary viral excretion is also prominent but is less chronic and generally ceases by two years of age (Pass et al., 1983c). Little information on the occurrence of viraemia is available however Lang & Noren (1968) reported that three symptomatic cases studied were all viraemic, in one case for at least five months. Virus can readily be recovered at autopsy from numerous organs, in particular the kidneys, liver, lungs, pancreas, thymus and thyroid, but also from brain and skin (Benyesh-

Melnick _et al_., 1964; Medearis, 1964). These infants therefore carry an extremely large viral load and, at least in the most severely affected, disseminated infection occurs. Extensive lytic viral replication may by itself account for much of the teratogenic potential of CMV.

It is not known what factors influence the extent of viral replication in the foetus. Insofar as the extent of excretion is reflected in pathological consequence, it would seem that gestational age at the time of infection is important. Later gestational ages are characterised by the developing foetal immune system and lower cell division rates, both of which may limit viral replication. The extent of placental infection may also determine the size of the initial infecting dose of virus and consequently the extent of foetal viraemia.

Most congenitally infected infants (89%) have detectable levels of CMV-specific IgM in cord blood, provided the detection system is sufficiently sensitive (Griffiths _et al_., 1982a) and most of those with symptoms either at birth or later in infancy have significantly elevated titres. Specific IgM titres may then reflect antigenic load, however it was noted by this group that IgM titres did not always correlate with urinary virus titres, suggesting that a vigourous immune response might also contribute to symptomatology and sequelae. Additional evidence implicating the antiviral immune response in the pathology of congenital infection comes from studies of immune complexes in infected infants (Stagno _et al_., 1977b). Circulating complexes are frequently found in infected infants but only occasionally in others, and these complexes are

generally larger in those with symptomatic infection. Larger complexes, the formation of which may be favoured by conditions of antigen excess, again may simply reflect a greater viral load, nevertheless such complexes may also accumulate more readily in body tissues. In a limited number of fatal cases studied IgG and CMV antigen deposits were demonstrated by immunofluorescent staining of sections of renal glomeruli and tubules. Immune deposits at this site and elsewhere (e.g. the choroid plexus) could result in inflammatory reactions resulting in tissue damage. IgG and IgM antibodies may also contribute to tissue damage by their participation with complement, K cells and monocyte-macrophage cells in cytotoxic reactions directed against infected cells.

Other aspects of humoral immunity are also elevated in congenitally infected infants by comparison with normal immune adults (Stagno et al., 1975b). CF antibody titres are high at birth and often remain so, particularly in those with symptoms. Fluorescent antibody titres against early and late antigens are both high in titre initially; the former remain elevated during the first two years then decline while the latter show only a slight drop over the next five years. Despite this vigourous production of antibody, there is evidence that the humoral response to infection may be qualitatively deficient as Pereira et al. (1983) found that sera from symptomatically infected infants failed to precipitate many polypeptides precipitated by other sera in an RIP assay during the first year of life. An alternative explanation of these results is that antibody to these antigens was present, but was involved in complexes

formed in vivo and was therefore not free to react with radioactive precursor-labelled proteins in the RIP assay. After the first year sera from these infants precipitated CMV polypeptides for longer than sera from asymptomatic infants and also precipitated additional polypeptides. This elevated humoral response to infection argues strongly against the hypothesis that the inability of these infants to limit infection results from induction of immunological tolerance following exposure during foetal life.

In comparison, the lymphocyte proliferation response to CMV antigens is markedly deficient in such infants. The response is usually entirely absent during most of the first year; an increasing proportion become responsive with increasing age thereafter although the response remains low compared with that of immune adults and many remain unresponsive for five years or more (Gerhz et al., 1977; 1982; Okabe et al., 1983; Pass et al., 1983c; Reynolds & Dean, 1978; Reynolds et al., 1979; Starr et al., 1979). Lymphokine production in response to CMV antigens is also impaired (Fiorilli et al., 1978; Starr et al., 1979). This however is not due to a generalised suppression of cell-mediated immunity as occurs in CMV-mononucleosis in adults. Lymphocyte responses to mitogens and recall antigens are comparable to those of age-matched controls (Gehrz et al., 1982; Pass et al., 1983c; Reynolds et al., 1979; Starr et al., 1979) and the uptake of live vaccines is normal in these infants (Reynolds et al., 1978). Although some immunological and haematological features are reminiscent of mononucleosis, such as elevated total IgG

and IgM levels (Griffiths et al., 1982a; Pass et al., 1980a; Reynolds et al., 1974), lymphocytosis (Pass et al., 1980a) with an increased proportion of T cells (Schauf et al., 1976) and elevated numbers of suppressor T cells resulting in a low helper/suppressor phenotype ratio (Pass et al., 1983b), these findings are not consistently observed and are far less striking than findings in CMV-mononucleosis.

Immunosuppression associated with congenital CMV infection, then is substantially different from that observed in CMV-mononucleosis as it is CMV-specific and is frequently of considerably longer duration in the former. The mechanism(s) whereby this immune suppression is effected are not apparent. The immunological immaturity of the foetus and neonate cannot account entirely for the defect as lymphocyte responses to HSV in infants with perinatal HSV infection, although low, are usually detectable in the newborn period. (Pass et al., 1981a).

Unresponsiveness is not due to an inability to synthesise IL-2 as the addition of exogenous IL-2 has no effect on the in vitro proliferation response, suggesting that few antigen responsive cells are present or that antigen presentation to antigen-primed lymphocytes is defective (Pass et al., 1984). The frequency of lymphocytes in peripheral blood capable of proliferating in response to CMV is low in congenitally infected infants compared to adult controls as shown by Hayward et al. (1984) using a limiting dilution lymphocyte proliferation assay. Low responder frequencies were also observed in infants perinatally infected with HSV compared to immune adults, however, suggesting that this may be an age

related deficiency. Those infants with congenital CMV infection may have even fewer responder cells resulting in the complete absence of the proliferation response.

A low frequency of responder cells may be due to defective generation or functional capacity of these cells, or to defective accessory cell function. With regard to this last possibility Hayward et al. (1984) found that monocytes from infected infants presented antigen normally to maternal lymphocytes, however others have reported that monocyte-derived macrophages from such infants inhibited the proliferation of lymphocytes from control donors in a CMV-specific fashion (Coleman et al., 1982). Other factors which have been implicated in suppressing lymphocyte proliferation are unidentified soluble serum components (Reynolds & Dean, 1978; Coleman et al., 1982). It is difficult to envisage how soluble factors, with the exception of specific antibody, could mediate antigen specific suppression of lymphocyte reactivity however.

Another possible mechanism of suppression of CMV-specific lymphocyte proliferation is the induction of specific suppressor T cells. Lymphocytes with such properties have been identified in other pathological conditions; lymphocytes from patients with chronic hepatitis B virus infection give low proliferative responses in general, but the response to hepatitis B surface antigen (HBs Ag) is further suppressed or completely absent (Barnaba et al., 1985). This response can be improved by eliminating T8+ cells, suggesting that activated suppressor cells are present. When lymphocytes from low responders are stimulated with HBs Ag and irradiated they

significantly inhibit the response of fresh autologous lymphocytes to HBs Ag but not to other antigens.

The presence of activated, specific suppressor cells in CMV-infected infants may account for the lack of proliferation following stimulation in some infants, and in most under one year of age. Others with low responses may have high numbers of circulating suppressor precursors which are rapidly activated following antigen stimulation. This could be decided by experiments similar to those described above. Viral infection of specific subgroups of lymphocytes may also result in CMV-specific immune suppression. Until these possibilities are addressed using sensitive biochemical, molecular and immunological techniques the precise mechanism of this immune suppression remains enigmatic.

The corresponding age-related increases in the proportion of congenitally infected infants who develop a positive CMV-specific proliferative response and the proportion who cease to excrete virus, taken together with the observation that the lymphocyte response defect is most pronounced in those with symptomatic infection (Pass et al., 1983c; Reynolds et al., 1979) suggests that the absence of the proliferative response is related to the persistence of infection and its pathological consequence. This does not however establish that immune unresponsiveness is causally related to the duration or extent of infection. Certainly this cannot be the sole factor in determining outcome as there is no absolute correlation between the two. Unresponsiveness may persist long after the cessation of excretion and may equally be resolved despite

continuing excretion.

Immune and innate cytotoxic responses may be of greater importance in containing infection but are poorly studied in this patient group. The number of circulating NK and K cells are very low (0.5% of mononuclear cells) during the newborn period and increase in number slowly thereafter for at least 30 years (Abo et al., 1982). The ability of peripheral blood lymphocytes to mediate natural and antibody-dependent cytotoxicity against HSV-infected targets is markedly reduced for at least the first year of life in comparison with adults (Kohl, 1983). Although absolute numbers of T4+ and T8+ lymphocytes are elevated at birth (Thomas & Linch, 1983), there is evidence that some T cell-mediated cytotoxic functions are deficient (Campbell et al., 1974).

Curtailment of CMV infection in infants may then be achieved through the combined effects of various immune functions including the DTH response and cytotoxic responses mediated by T, NK and K cells as well as phagocytic cells. The slow rate of acquisition of some of these functions during infancy may explain the prolonged periods of shedding.

CMV infection in infancy

As shown in seroepidemiological surveys CMV infection is common during infancy especially in poorer nations. Infection can only be confidently diagnosed as perinatal or postnatal when virus excretion is shown to be absent during the first week of life but present at a later date. Most information on this type of infection relates to infants who become infected

shortly after birth. The more limited information that exists regarding infection in older children indicates that this situation is more similar to infection in younger infants than in adolescents or older children. Infection in the neonatal period is usually subclinical (Pass *et al*., 1981a; Stagno *et al*., 1975b). Infection acquired later in infancy, although also subclinical in most, is more often clinically apparent. The most common manifestations are hepatosplenomegaly, liver dysfunction, pneumonitis and haemolytic anaemia (Gehrz *et al*., 1982; Hanshaw *et al*., 1965). This may argue for a protective role for maternal antibody in modifying the outcome of infection, as some have observed in neonatal HSV infection (Yeager *et al*., 1980). No long-term sequelae have been reported in otherwise healthy infants infected after birth (Pass *et al*., 1981a).

Infected infants excrete virus in saliva and urine (Pass *et al*., 1982a; Tamura *et al*., 1980b). The amount of virus shed in urine is similar to the amount shed by congenitally infected infants older than three months old and is generally persistent for at least three years (Stagno *et al*., 1975b).

The humoral immune response to infection appears to be intact in these infants. Specific IgM is present and this response is of similar duration to the IgM response in CMV-mononucleosis (Chiba *et al*., 1980). Secretory IgA with *in vitro* virus-neutralizing capacity is present in saliva (Tamura *et al*., 1980b). Serum IgG has complement fixing activity and is directed against various groups of viral antigens including PENA, EA and LA including viral glycoproteins, although the

response to the first of these groups of antigens may be relatively delayed in onset (Chiba et al., 1980; Pereira et al., 1983; Stagno et al., 1975b). These IgG responses generally persist at moderate to high titre throughout the first year following infection and at lower titres thereafter, despite the persistence of viruria (Stagno et al., 1975b).

Infection acquired early in life is not accompanied by the haematological abnormalities or general cell-mediated immunosuppression observed in CMV-mononucleosis. Circulating T cell numbers are normal (Gehrz et al., 1982) as are lymphocyte proliferative responses to mitogens and recall antigens (Gehrz et al., 1982; Okabe et al., 1983; Pass et al., 1981a; Reynolds et al., 1979). CMV-specific cell-mediated immunity however is markedly suppressed. Lymphocyte proliferation and lymphokine production in response to CMV is absent in most and low in the remainder (Fiorilli et al., 1982; Gehrz et al., 1982; Pass et al., 1981a). Only one group (Okabe et al., 1983) has reported positive responses in postnatally infected infants under one year of age. The proportion of infected infants who respond increases with age; a significant proportion are still unresponsive at four years of age or older however (Pass et al., 1981a).

This response to infection is similar to that in congenital infection and may result from similar or identical mechanisms. Although both the cessation of excretion and the development of a proliferative response to CMV are associated with increasing age, one is not always predictive of the other (Pass et al., 1981a). This is also similar to congenital

infection and suggests that the activity of several antiviral functions is responsible for the eventual elimination of infection in postnatally infected infants also.

The virus carrier state

During convalescence from primary CMV infection in healthy adults the haematological picture normalises, immunological responsiveness gradually returns and virus excretion falls and is eventually terminated although this may not be achieved for many months. These events are accompanied by changes in the virus-specific immune response; some immune functions are lost during convalescence while others remain and still others only become fully developed during this period.

That CMV is not completely eradicated from the host following infection in most if not all cases is undoubtable in view of the evidence discussed in Chapter 2 that replication of the virus which caused the original infection may resume and cause a re-emergence of infection, and that infection can be acquired from immune individuals and transfusions and transplanted organs derived from immune donors. It is not known however, in what state virus persists, nor how it escapes immune destruction during the interim period. Low grade viral replication may continue in tissues protected from the immune system; for example, persistence of virusemenia may be explained on this basis. However in most immune individuals infectious virus cannot be detected for most of the time. Moreover, _in vitro_ studies of CMV replication considered in Chapter 1 have shown that the replicative cycle can be

reversibly blocked at various stages. One or more of such mechanisms may operate in vivo and allow the persistence of virus in a non-replicative state.

Latency is usually defined as an ongoing virus-host relationship in which infectious virus is undetectable (Jack, 1974). As a working definition latency is often regarded as a state of infection where infectious virus is not released from infected cells and cannot be detected in cell-free homogenates, but can be rescued in vitro by explant culture or cocultivation (Openshaw, 1983). CMV infection in immune individuals fits the first of these definitions but this may be due to the inadequacy of current detection methods. Whether CMV can remain truly latent or can only persist by replicating at undetectable levels cannot be readily ascertained at present as it is not known with any degree of precision where the virus is harboured.

The site of latency in some other herpesvirus infections is known and consequently more is understood regarding the level of viral expression in these instances. The demonstration of HSV 1 in the spinal ganglia of mice which could only be rescued by organ explant culture (Stevens & Cook, 1971) was rapidly followed up with similar observations in man (Baringer & Swoveland, 1973; Bastian et al., 1972). Subsequent experiments in mice have shown that the neuron is the latently infected cell-type (Cook et al., 1974; Kennedy et al., 1983; McLennan & Darby, 1980). Even with this knowledge, and the availability of animal models the molecular mechanism of latency has not been elucidated. Stevens & Cook (1971)

failed to detect viral particles in latently infected ganglia. The virus may not be completely dormant however as virus-specified thymidine kinase is expressed at detectable levels up to 60 days after initiation of infection and possibly for longer at undetectable levels (Yamamoto et al., 1977) whereas infectious virus cannot be recovered from homogenised ganglia beyond two weeks after infection (Stevens & Cook, 1971; Walz et al., 1974). In addition, Baringer & Swoveland (1973) found occasional viral particles during detailed examination of ganglial cells. Such attempts to determine the extent of viral expression in latently infected cells are of course limited by the methods available. DNA replication or antigen production which proceeds slowly or only occurs in a few cells may well escape detection.

There is also evidence that HSV may persist in other tissues and cell types including trigeminal nerve roots in man (Warren et al., 1982), the sciatic nerve and the footpad in guinea pigs (Scriba, 1977) and in mice the footpad (Al-Saadi et al., 1983) and the eye (Openshaw, 1983).

The ability of EBV to persist in the host is well recognised and the B cell is known to harbour virus. Tonsillar lymphocytes from immune donors contain low amounts of viral DNA (Gerber et al., 1972) and a proportion express EBNA but not early antigens or viral capsid antigen (Veltri et al., 1976), a level of viral expression consistent with latency (Epstein & Achong, 1977). The EBNA-positive cells have since been shown to be B cells (Veltri et al., 1977). In addition peripheral blood lymphocytes from immune donors are capable of giving rise to

EBV-transformed lymphoblastoid lines in culture (Chang et al., 1971; Gerber & Monroe, 1968; Nillson et al., 1971). These in vitro transformants do not arise directly from the outgrowth of latently infected lymphocytes; activation of lytic infection in the latter gives rise to free virus which subsequently transforms uninfected B cells in vitro (Rickinson et al., 1975; 1977b). The physical state in which the viral genome persists in latently infected lymphocytes has not been demonstrated but is possibly the same as that in in vitro transformed and in vivo derived lines, namely multicopy episomes possibly with some copies integrated into host cell chromosomes.

Latency in murine CMV infection has also been studied in some depth. Latent virus can be rescued from spleen cells by explant culture or cocultivation with permissive mouse cells (Olding et al., 1975; 1976; Wise et al., 1979) and can be detected by DNA hybridisation in spleen cells at a level of 3-4 genome copies per 100 cells (Olding et al., 1976). By cocultivation of fractionated spleen cell populations B cells have been found to be one site of latency (Olding et al., 1975) however latent CMV has also been detected in peritoneal macrophages by cocultivation and by DNA hybridisation at a level of 4-7 genome copies per 100 cells (Brautigam et al., 1979). Latent virus has also been detected by explant culture in the salivary gland and prostate gland (Cheung & Lang, 1977) and by DNA hybridisation in salivary gland cells (Olding et al., 1976) and spermatogenic cells (Dutko & Oldstone, 1979).

The situation with regard to latency in human CMV infection is far less clear. The epidemiology of infection in

recipients of blood or blood product transfusions and in renal and cardiac allograft recipients indicates that some cells in the transferred tissues are harbouring potentially infectious virus. The particular implication of leukocytes in transfusion-acquired infection is consistent with the known involvement of these cells in active infection. Most attempts to demonstrate latent virus directly have proved unsuccessful however. With rare exception (Diosi et al., 1969) CMV cannot be cultured from the buffy coat cells of blood from immune donors, even when the donor was implicated in a transfusion-acquired infection (Bayer & Tegtmeier, 1976; Kane et al., 1975). Viral DNA has occasionally been detected in low amounts, however in leukocytes of non-viraemic donors (Pagano,1975) although this finding has not been repeated by others.

Attempts to reactivate latent CMV infection in renal tissue by explant culture with or without cocultivation have proved uniformly negative (Naraqi et al., 1978). This may not be surprising however as these techniques also failed to reveal virus in kidneys actively infected as shown by the presence of infectious virus in cell-free homogenated tissue. It may be argued that the risk of infection acquired from renal and cardiac allografts is due to latently infected donor leukocytes perfusing the organ at the time of transplantation. The increased frequency of infection in these patients over other transfusion recipients may result from their immunosuppressed state which in turn may result in a reduced ability to eliminate virus-infected cells.

The difficulty in demonstrating the presence of latent CMV

in humans may indicate that the mechanisms responsible for restricting viral expression are different from those in the herpesvirus infections described above. Alternatively, latent CMV may reside in a smaller number of cells, or in a cell type which has a short lifespan when cultured in vitro. Another possible reason arises from the fact that only fibroblasts are fully permissive for CMV replication in culture whereas latency may well involve another cell type. This could result in inefficient in vitro reactivation. The ability to induce a permissive state in other cell types using hormones (Tanaka et al., 1984b) and base nucleotide analogues (St. Jeor & Rapp, 1973) may thus aid in the search for sites of CMV latency.

In the absence of overt viral infection virus-specific immune functions are the only readily demonstrable indications of past infection with herpesviruses. Both humoral and cell-mediated immune functions may be present and will now be described. Certain aspects of innate immunty are also discussed. Although not CMV-specific, these functions may be of importance in maintaining the latent state.

During convalescence from primary infection the CF antibody titre to CMV falls (Andersen, 1970; Medearis, 1964) although this decline may not become apparent for at least several months after resolution of infection (Carlstrom et al., 1968). CF antibody persists indefinitely in most individuals (Betts et al., 1976; Griffiths et al., 1978) but some workers have observed fluctuation of titres with time (Waner et al., 1973). Similarly, neutralizing antibody titres also persist at detectable levels in all or most of the immune population and

correlate well with CF titres (Plummer & Benyesh-Melnick, 1964; Stalder & Ehrensberger, 1980).

CF and neutralizing antibodies are directed primarily against late, structural viral antigens (Pereira et al., 1982b). A large proportion of the antibody measured in the indirect immunofluorescence, ELISA and RIA IgG assays is also reactive with these antigens (Forghani & Schmidt, 1980; Sarov et al., 1980; Torfason et al., 1981).

Antibodies to immediate early, pre-early nuclear and early antigens are often present at high titre during active CMV infection and may decline during convalescence. Because of this many have regarded these antibody reactivities as markers of acute or recent infection (Musiani et al., 1984; The et al., 1974) as was proposed for antibody to early antigen in EBV infections (Henle et al., 1971). It is now known however that the decline of these antibody titres during convalescence is not consistently observed and many individuals without evidence of current or recent active infection have detectable, and often high IgG titres to these antigens (Friedman et al., 1982a; b; Gartner & Orstavik, 1984; Griffiths et al., 1980a; Landini et al., 1984). The reason why particular antibody reactivities persist after infection in some cases but not others is not known.

The significance of serum IgA antibody to CMV has not been fully evaluated in this respect. Initial studies have shown that most healthy immune adults do not have detectable IgA titres (Levy & Sarov, 1980; Pinku et al., 1982; Strand & Hoddevik, 1984) but it was noted in one study (Levy & Sarov,

1980) that IgA may persist considerably longer than IgM following primary infection. Further studies are required to clarify the relationship of CMV-IgA to active and latent infection.

Investigations of specific DTH responses in healthy individuals of longstanding immunity have produced variable findings. Many have found that lymphocytes from all or most of such individuals proliferate in response to CMV antigens (Pollard et al., 1978; Schirm et al., 1980; Starr et al., 1979; 1980; Waner & Budnick, 1977). In contrast, others have found that lymphocytes from approximately half of seropositives are unresponsive in this assay (Ten Napel & The, 1980a; Ten Napel et al., 1977). Still others have found that while most immune adults give a positive response, the response may be absent in a proportion of adolescents (Faix et al., 1983a) and children (Beutner et al., 1978). In these last two studies negative responses in seropositive persons could be explained by fluctuations in responsiveness with time in the former and, in the latter, greater strain specificity of the response in the children studied than has been observed in adults (Starr et al., 1980; 1981). Ten Napel and colleagues (Ten Napel & The, 1980a; Ten Napel et al., 1977) found that immune individuals who also had antibodies to early antigens were more consistently responsive and gave responses which were generally greater in magnitude than did seropositives without antibody to early antigens. Most others, however have found that the presence or magnitude of the response correlates neither with the magnitude of antibody titres nor with the presence of any

specific antibody reactivities (Beutner et al., 1978; Faix et al., 1983a; Schirm et al., 1980; Starr et al., 1979; Waner & Budnick, 1977). In older immune individuals (60 years and over) there may be a diminishing of the proliferative response while antibody titres remain high (Ten Napel & The 1980a).

The lymphocyte proliferation assay systems used by the investigators cited above vary in detail with respect to culture conditions, cell density, the source of serum or plasma, the strain of virus and the antigen preparation used. While no single factor appears to account for the discrepant results obtained by different groups, it is clear that standardisation of the assay and identification of those variables which bear upon the response are necessary in order to assess the value of this test as an indicator of cell-mediated immunity.

Other assessments of DTH responses based on measuring lymphokine production in response to CMV antigen have also been used. Production of migration inhibition factor as measured by a leukocyte migration inhibition test has been reported to occur with lymphocytes from immune donors but only a few individuals were tested (Fiorilli et al., 1978; 1982). Gamma IFN production has also been used to assess CMV-specific DTH responses (Starr et al., 1980). While lymphocytes from most immunocompetent seropositive individuals are responsive, the amount of IFN produced in response to antigen does not correlate with the magnitude of the proliferative response and maximal IFN titres are obtained with different antigen concentrations from those which induce optimal proliferation.

These two assays may therefore be assaying different populations or maturational stages of immune lymphocytes.

Activated HLA-restricted CTL's are not present in the peripheral blood of latently infected individuals (Kirmani et al., 1981). This is analogous to the situation in latently infected mice (Ho, 1980; Quinnan et al., 1978; 1980). Lymphocytes from individuals latently infected with EBV on the other hand are capable of mediating regression of EBV-induced transformation in vitro, and HLA-restricted T cells are responsible for this effect (Moss et al., 1978). These however are secondary CTL's generated from memory T cells following prolonged contact with target cells as regression does not take place until 1-2 weeks after initiation of transformation. By presensitising splenic lymphocytes from CMV-immune mice using UV-irradiated target cells as stimulator cells Ho & Ashman (1979) were able to demonstrate secondary CTL's which had been generated in vitro. The generation of secondary CTL's from peripheral blood lymphocytes from immune human donors has also been demonstrated using CMV-infected autologous fibroblasts as stimulator cells (Borysiewicz et al., 1983).

A proportion of circulating lymphocytes are the null cells which include NK and K cells. Under appropriate conditions, both these cell types can lyse CMV-infected target cells in vitro and their combined activity accounts for most if not all of the cytotoxicity of lymphocytes against infected cells (Kirmani et al., 1981). It was concluded in this study that both NK and K cells from immune persons were cytotoxic for cells expressing CMV antigens and that the responsible

effectors from non-immune lymphoctes were NK cells. However the results clearly showed that specific antibody enhanced the cytotoxicity of both immune and non-immune lymphocytes. This is to be expected as K cells themselves have no immune specificty; the specificity of antibody-dependent cellular cytotoxicity (ADCC) is determined entirely by the specificity of the antibody coating the target.

Cytotoxic lymphocytes such as these which do not require to be presensitised with antigen for their activation may provide an early means of eliminating lytically infected cells before virus is released. Their presence may therefore contribute to the maintenance of the latent state.

Reactivation of CMV infection and reinfection

Much of our current information regarding CMV reactivation and reinfection is based on findings in pregnant women and in immunosuppressed allograft recipients and is discussed in those contexts. The remaining information is derived in the main from studies on patients attending STD clinics and much of this pertains to male homosexuals. As sexual activity may be an important determinant of both reinfection and reactivation extrapolation from this data to the general population may not always be valid.

Reactivation and reinfection are considered together here as they cannot normally be distinguished in practice. The events which precede and ensue from these two types of infection may however be different. This is perhaps more likely to be the case if reinfection is caused by a strain of virus

which is substantially different in antigenic composition from that to which pre-existing immunity is directed. Under these circumstances exposure to exogenous virus without any local or systemic changes in the immune or physiological status of the host may be sufficient for reinfection to occur. In reality however, these events may occur simultaneously, particularly if exogenous virus is associated with immunomodulating agents such as blood products (Goeken et al., 1982) or seminal plasma (James & Hargreave, 1984).

While evidence in pregnant women and immunosuppressed patients discussed below indicates that reactivation of infection is clearly influenced by the host immune status and possibly by hormonal factors, it is not known whether subtle changes in these parameters are necessary for subclinical reactivation in healthy individuals. Reactivation of a productive viral replication cycle at the molecular level may conceivably occur in response to fluctuations in cellular physiology which are independent of gross physiological parameters. Whether such an event could lead to sufficient viral replication to result in a detectable reactivation of infection without concurrent changes in host physiology is nigh impossible to ascertain.

Following infection an antigenically foreign virus may be expected to elicit a greater immune response than a reactivation of the original strain or a reinfection with a similar strain. The timing and nature of this immune response may determine the subsequent course of infection. The site of initial infection may or may not be different in reinfections

and reactivations and this too may bear upon the course of infection.

Frequencies of excretion reported vary widely depending on patient demography and the number of anatomical sites from which viral culture was attempted. Where necessary, excretion rates given below have been adjusted from the original publications to give percentages which relate to the number of immune persons rather than to the total number studied.

In the only study of non-pregnant women where cervical, urinary and salivary excretion of virus were all considered, 33% of seroimmune women shed virus from at least one site, and 24%, 10% and 4% from the cervix, urine and saliva respectively (Faix et al., 1983a). This study was conducted among adolescent women who are known to have the highest excretion rates; in studying an older age group Stagno et al. (1975a) and Knox et al. (1979) reported cervical and urinary excretion rates of 10% and 4% respectively. A higher cervical excretion rate (16%) has also been observed in seroimmune women attending STD clinics (Jordan et al., 1973).

Most of this excretion cannot be due to primary infection, even assuming a high seroconversion rate and prolonged persistence of excretion in many of these women. The relative extents to which reactivation and reinfection contribute to this excretion cannot however be determined.

Among male homosexuals reported frequencies of viruria in the seropositive majority range from 8-9% (Drew et al., 1981; Mintz et al., 1983) to 20% (Greenberg et al., 1984; Lange et al., 1984). Virusemenia is reported to occur in 20% (Biggar et

al., 1983; Lange et al., 1984) to 40% (Mintz et al., 1983). Again these figures cannot be explained by high primary attack rates as many of the men studied had been practising homosexuals for 10 years or more and would presumably have seroconverted in the distant past. The frequency of virusemenia in a cohort of males not selected for homosexuality or promiscuity by contrast was 2% (Lang & Kummer, 1975), a figure consistent with the probable primary infection rate. (This figure relates to the total number studied as no serology was performed.)

All this evidence suggests that the genital tract is a common site of reactivation and/or reinfection in both sexes and that some of these infections may be restricted to this site. In the only one of these studies where viraemia was sought none was detected (Faix et al., 1983a). These infections are almost always asymptomatic, however two cases of non-primary infection associated with hepatitis or a mononucleosis-like illness in male homosexuals have been reported (Coutinho et al., 1984).

The immune response to reactivation and reinfection has been studied little. Coutinho et al. (1984) found that 4-5% of seropositive male homosexuals per annum showed four-fold or greater increases in CMV-IgG titres and Lange et al. (1984) found that 66% had elevated IgG titres and that 30% had specific IgM. This latter parameter correlated strongly with the presence of viruria. On the other hand, Mintz et al. (1983) studying a different homosexual population found that IgM titres fluctuated with time but were positive in 95% of those

with IgG on at least one occasion and did not correlate with with virus excretion. Although antibody responses to non-primary infection in the general population have not been studied, these findings are unexpected as studies in healthy pregnant women with asymptomatic non-primary infections show that rises in antibody titre are uncommon (Stagno et al., 1975a) and IgM responses are absent (Griffiths et al., 1982b).

Low or inverted T4+/T8+ lymphocyte subset ratios similar to those observed in CMV-mononucleosis have been reported in CMV-excreting homosexuals (Greenberg et al., 1984) but these abnormalities are common in promiscuous homosexuals, at least in the USA (Wallace et al., 1982) and others have found no correlation with CMV excretion (Lange et al., 1984). All this raises the question as to whether these individuals are fully immunocompetent, and this matter will be discussed subsequently.

Only one group (Faix et al., 1983a) has studied CMV-specific cell-mediated immunity in healthy, non-pregnant women with non-primary infections. In this study (which was conducted among adolescents with a high excretion rate) CMV-specific lymphocyte proliferation was absent in over 30% of seropositive women, however this bore no relationship to viral excretion. Lymphocyte responses were found to fluctuate with time, whereas mitogen responses were normal throughout. In these individuals the specific lymphocyte response may partially reflect some other immune function(s) which may be a more important determinant of immunity to infection and which may be defective or reduced in excreting women. This is

suggested by the correlation between increasing age and reduced virus excretion and by the increased number of adults who give positive lymphocyte proliferation to CMV, despite no correlation between excretion and lymphocyte reactivity. An age-related decrease in the prevalence of CMV excretion is also observed in homosexual men (Biggar et al., 1983; Drew et al., 1981). Other immune responses have not been studied in these groups.

CMV infection during pregnancy

CMV infection in the pregnant woman has received much research with the aim of identifying factors important in facilitating transplacental transmission of infection. It is not known however to what extent infection in this setting resembles or differs from infection in other healthy adults. Physiological changes occurring during pregnancy may alter the frequency of reactivation or modify the course of infection. Pregnancy hormones may influence viral replication directly by their effects on infected cells (Chong & Mims, 1984; Koment, 1985; Tanaka et al., 1984a; b) or indirectly by modifying aspects of immune responsiveness (Kasakura, 1971; Stimson et al., 1980). While some modification of maternal immunity may be necessary to prevent rejection of the histo-incompatible foetus, this alteration may be very subtle or specific. The virtual absence of MHC class I and II antigen expression on the syncytiotrophoblast which forms the materno-foetal interface (Faulk & Temple, 1976; Goodfellow et al., 1976) may preclude the necessity for generalised suppression of MHC-restricted

immune responses. At least some blocking factors present in pregnancy plasma specifically inhibit the one-way MLR of maternal lymphocytes against paternal lymphocytes only (McIntyre & Faulk, 1979).

In view of this it is perhaps not surprising that many have found no generalised impairment of cellular immunity as detectable in total T cell numbers and proliferation responses to mitogens and recall antigens (Faix et al., 1983b; Gehrz et al., 1977; Reynolds et al., 1979; Starr et al., 1979). There is no general agreement on this matter however (Birkeland & Kristoffersen, 1980; Blecher & Thompson, 1976; Finn et al., 1972) and other aspects of immunity such as the MLR are reported to be increasingly suppressed during the course of gestation (Kasakura, 1971). Other evidence of selective immune suppression is the observation of spontaneous remission of some autoimmune phenomena such as rheumatoid arthritis during pregnancy (Ostensen & Husby, 1983) and the altered pathogenesis of certain viral infections during pregnancy. This may be manifested as an increased rate of infection or reactivation of infection, increased severity or mortality of infection, or deficient in vitro immune responses to viral antigens. This is true of infection during pregnancy with hepatitis viruses (Christie et al., 1976; D'Cruz et al., 1968), influenza virus (Greenberg et al., 1958), poliovirus (Siegal & Greenberg, 1955), rubella virus (Thong et al., 1973), polyoma viruses (Coleman et al., 1983), VZV (Mendelow & Lewis, 1969), and occasionally with HSV (Peacock & Sarubbi, 1983).

Reported CMV excretion rates in pregnant women range from

around 10% (Knox et al., 1979; Montgomery et al., 1972; Stagno et al., 1975a) to 20% (Faix et al., 1983b; Nankervis et al., 1984). The higher prevalence in these last two reports is due to the restriction of the study populations to adolescent women; increasing age is associated with lower excretion, as is increasing parity. Viral shedding from the cervix is more common than urinary shedding [rates of 8-10% versus 3-5% respectively were reported by Knox et al. (1979), Montgomery et al. (1972) and Stagno et al. (1975a)]. Higher excretion prevalences are observed during the third trimester and postpartum period than during early pregnancy. Stagno et al. (1975a) and Knox et al. (1979) found that excretion rates during the third trimester and post partum were not substantially different from those in nonpregnant women. Knowles et al. (1982) however found a higher incidence of cervical excretion in post partum women when compared to nonpregant women. Further study is required to clarify the relative frequencies of excretion in pregnant and nonpregnant women, taking into account age race, parity and gestational age.

CMV infection during pregnancy, whether primary or not is usually asymptomatic; primary infection is rarely associated with a mononucleosis syndrome (Faix et al., 1983b; Griffiths et al., 1978; Kumar et al., 1984; Stagno et al., 1982b). Virus is usually excreted in low amounts only but excretion may be persistent, continuing for three years or more following delivery (Nankervis et al., 1984). Viraemia is not usually detectable (Kumar et al., 1984; Pass et al., 1982b).

Infection of the placenta is probably necessary for intrauterine transmission to occur. While it is known for congenitally infected infants to be delivered from mothers whose placentae were free of infection (Nankervis et al., 1984) this may be due to resolution of placental infection some time between transmission and delivery. If the placenta is infected at term, it is likely but not inevitable that foetal infection will have resulted. Transmission is more likely to occur and possibly to be of greater clinical consequence to the foetus if maternal infection is primary as discussed in Chapter 2. Transmission is also more likely in those women who excrete higher titres of virus in urine and in those nonprimary infections associated with significant rises in antibody titre during gestation (Nankervis et al., 1984). Excretion of virus from the cervix or oropharynx only may represent localised reactivation of infection resulting in little immune stimulation and with low risk to the foetus, at least during intrauterine life. Those women who transmit CMV infection in utero are also those who excrete virus in higher titre and for longer periods in the post partum (Pass et al., 1982b). In the study of Pass et al. (1982b), two of these risk factors, namely primary infection and urinary viral excretion, were related; women with primary infection more frequently excreted virus in higher titre and for longer periods than those with nonprimary infection. In the study of Nankervis et al. (1984) however, these factors were unrelated as most infections were nonprimary. This suggests that there may be other factors in addition to primary infection which are responsible for

increased viral excretion and increased immune stimulation in some women. Some women may be susceptible to reactivation with a greater degree of viral replication, reflected in increased urinary excretion of virus and increased risk of transmission. This cannot be the sole determinant of risk to the foetus however as transmission does not necessarily result even when viraemia is present during gestation or when the placenta becomes infected (Faix et al., 1983b; Kumar et al., 1984). That some women may have a propensity to transmit infection to the foetus is suggested by two observations. Firstly, immune women from poorer communities transmit CMV infection more often than women from more affluent communities (1.5% versus 0.5% respectively; Stagno et al., 1982b). Secondly, in a recent study in London (Griffiths & Baboonian, 1984a) the only case of congenital infection among infants born to immune women occurred in an infant whose older sibling was also congenitally infected despite pre-existing maternal immunity. In view of these observations the authors speculate with Stern (1977) that some women are at increased risk of transmitting infection in utero and that infection occurring in early life, which is the norm in developing nations may be one cause of this maternal defect. Other maternal factors which appear to affect the frequency and duration of excretion are age and parity, as already mentioned (Montgomery et al., 1972; Pass et al., 1982b; Stagno et al., 1975a). The finding that transmission is more likely to occur when maternal infection takes place during the final trimester (Griffiths & Baboonian, 1984b; Nankervis et al., 1984) may be due to greater suppression of immunty to CMV

at this stage (see below) or may be due to changes in the structure of the placenta, as proposed by Nankervis et al. (1984).

Humoral immune responses to CMV infection during pregnancy are well delineated. Primary infection elicits seroconversion and an IgM response of up to four months duration (Griffiths et al., 1982b). Reactivated infection may be associated with a rise in antibody titre but most are not (Nankervis et al., 1984; Stagno et al., 1975a) and no IgM response is present (Griffiths et al., 1982b).

Current knowledge regarding cellular immunity to CMV infection during pregnancy is incomplete as most studies have been limited to the post partum period, and primary and nonprimary infections are generally not distinguished. Gehrz et al. (1981a; b) followed seroimmune women throughout pregnancy and found that CMV-specific proliferative responses became increasingly depressed with increasing gestational age. Responses to PHA were normal throughout; responses to recall antigens however also became depressed, but not to the same extent as CMV responses. The responses to CMV and other recall antigens reappeared around four months and three months respectively after delivery although the former remained low for longer periods. It was suggested that this may allow reactivation and transmission of infection to the foetus. Although this is consistent with the findings of increased frequency of CMV excretion towards the end of pregnancy, none of the women in this study actually excreted virus, and Faix et al. (1983b) found that while proliferative responses to CMV

were frequently low or absent during pregnancy, this did not correlate with the presence or absence of viral excretion. Suppression of the CMV- specific proliferation response during pregnancy, then, is not necessarily related to active infection.

This complicates the interpretation of lymphocyte proliferation results in post partum women. As expected from the above studies, women who were excreting late in pregnancy or who gave birth to infected infants showed a marked depression of this response post partum (Gehrz et al., 1977; Reynolds et al., 1979; Starr et al., 1979), however these women were not compared with post partum women without recent infection. Presence or absence of the response did not correlate with a history of transplacental transmission or with current virological status, but in women with congenitally infected infants the response was on average lower in those with symptomatic infants than in those with asymptomatic infants. It cannot be determined whether this reduced response was causal or consequential to the type of congenital infection, or a reflection of some other immune defect. It is possible that reduced proliferative responses and symptomatic congenital infection are both more likely when maternal infection is primary.

Mechanisms whereby lymphocyte proliferative responses are suppressed during pregnancy are not apparent. It is not clear whether suppression of the CMV-specific response is a reflection of a more generalised immune defect associated with pregnancy or is the result of a specific defect as observed in

congenitally and postnatally infected infants. Limited evidence indicates that serum factors and monocyte-derived macrophages may suppress CMV-specific lymphocyte proliferation in an antigen specific manner as described in congenitally infected infants (Coleman et al., 1982).

It is clear from all this that lymphocyte proliferation studies alone give little or no indication as to why CMV infection reactivates in some pregnant women and results in intrauterine infection in a proportion of these women. Investigation of other cellular immune parameters may reveal specific defects in immunity. Such studies are virtually absent in the literature. Rola-Pleszczynski et al. (1977) found that lymphocyte-mediated cytotoxicity was on average lower in mothers of congenitally infected infants. Comparison of these findings with results in other pregnant and post partum women would be necessary to establish any relationship to infection or transmission. The nature of the cytotoxic cell type participating in this assay was not determined. The specificity of cytotoxicity is most consistent with an NK-like function.

In summary, the CMV-immune pregnant woman may be susceptible to reactivated infection. This susceptibility is greatest in the period before and after delivery but how this compares with excretion rates in other women is not entirely clear. Pregnancy is associated with a decreased lymphocyte proliferation response to CMV which may be partly or entirely CMV-specific. This defect is of unknown cause and bears little or no apparent relationship to reactivation of infection or transmission to the foetus. Immune, hormonal and other factors

which influence these events have not been elucidated. Primary infection is associated with a higher incidence of transmission, however other virological and immunological aspects may be similar to those in other adults with primary infection.

CMV infection in the immunocompromised host

Several patient groups fall within the category of immunocompromised patients. Those discussed here are mainly those patients iatrogenically immunosuppressed in connection with renal, cardiac or bone marrow transplantation. Many features of CMV infection in these patients are also true of infection in other compromised hosts, such as those receiving radiation or chemotherapy for malignant disease (Agatsuma et al., 1979). Patients with acquired immune deficiency syndrome (AIDS) are discussed in the following chapter.

a) Renal and cardiac allograft recipients.

Renal and cardiac transplant patients are susceptible to infection with a variety of viruses including all four human herpesviruses (Cheeseman et al., 1980; Gallagher & Merigan, 1979; Kanich & Craighead, 1966; Lopez et al., 1974a). Of these, CMV infection is the most important, both numerically and clinically (Fiala et al., 1975). As discussed in Chapter 2, CMV infection occurs in up to 100% of initially seropositive and in 40-60% of initially seronegative renal and cardiac recipients.

CMV infection is detectable by isolating virus from urine, throat washings, leukocytes or bronchial washings, or by

demonstrating specific IgM or rising antibody titres. Viral excretion or viraemia begins on average around five weeks post transplant when infection is primary and around eight weeks post transplant in other types of infection (Pass et al., 1983c) however a significant proportion of patients with serological evidence of secondary infection do not become viruric (Sarov et al., 1984). Most infections are due to reactivations of latent virus either from the donor organ or from the recipient, although some may be transfusion associated. The shorter interval between grafting and excretion in primary infections indicates that reactivation from the donor organ may occur more easily or may lead to a detectable viral infection more readily in the non-immune host. Viral excretion is prolonged; half of long-term survivors are still shedding two to five years post transplant and although the frequency of shedding falls thereafter, excretion has been documented as long as 14 years after transplantation (Cheeseman et al., 1979b).

The most common presenting symptom of CMV infection in these patients is fever. This is frequently accompanied by other signs and symptoms however, the most important being pneumonia, leukopenia, hepatitis and, in renal transplant recipients graft failure (Fiala et al., 1975; Marker et al., 1981; Rubin et al., 1977; Simmons et al., 1974). Symptoms are more likely to occur in those with primary infection; 80% of primary infections are accompanied by clinical manifestations compared to only 30% of nonprimary infections (Betts et al., 1975; Ho et al., 1975; Whelchel et al., 1979). Clinically

apparent infection is also strongly associated with viraemia which is demonstrable in 40-60% of infected patients (Cheeseman et al., 1979a; Fiala et al., 1975). The incidence of CMV reactivation, viraemia and symptomatology are all greater in those patients who receive antithymocyte globulin (ATG) as part of their immunosuppressive regime (Cheeseman et al., 1979a; Pass et al., 1980b; 1981b; Preiksaitis et al., 1983; Schooley et al., 1983). ATG results in a greater reduction in T cell numbers and suppression of T cell function than other immunosuppressive agents (Thomas et al., 1977). Viraemic infection would thus appear to be favoured when infection is primary and when immunosuppression is greater and this in turn results in greater dissemination of infection.

The frequency and severity of CMV-pneumonia in transplant patients (Hill et al., 1964) may be due to the extreme permissiveness of alveolar macrophages for CMV replication (Drew et al., 1979). An inflammatory response to infection in the lungs may then result not in viral clearance but in amplified viral replication.

The pathogenesis of renal graft dysfunction is less clear. Rejection episodes occur more frequently in patients with primary infection and with secondary infections where an IgM response is present. IgM titres are also temporally associated to rejection episodes in a proportion of cases (Sutherland & Briggs, 1983). Some have postulated that CMV may cause or enhance rejection (David et al., 1972). It is also possible that allogeneic reactions occurring during rejection may enhance CMV reactivation or replication as has been

demonstrated in a murine model (Wu et al., 1975). Of relevance in this respect is the finding that CMV infection is more common and occurs after a shorter interval in recipients of cadaveric organs which are less likely to be as well matched to the recipient as allografts from living related donors (Pass et al., 1978; 1980b). Defining the association between CMV infection and rejection is further complicated by the fact that not all renal graft dysfunction is due to classical rejection. Glomerulopathy is more common in symptomatic and viraemic CMV infections (Richardson et al., 1981; Schooley et al., 1983) and may be due to viral replication in the kidney or to circulating immune complexes. While this condition may be clinically indistinguishable from rejection in the absence of biopsy evidence, resolution of glomerulopathy, unlike rejection follows a decrease in immunosuppressive therapy. This highlights the necessity of close virological monitoring of these patients to enable allograft dysfunction due to viral infection to be distinguished from rejection when considering therapeutic measures.

The cause of leukopenia in association with CMV infection is also unclear. While leukopenia due to immunosuppression may predispose to CMV infection, onset of CMV infection is often closely associated with a further reduction in leukocyte count which remains low even when immunosuppresive therapy is discontinued (Rubin et al., 1977). In this situation the risk of superinfection is high and prognosis is poor. Lytic infection of leukocytes is unlikely to be directly responsible for leukopenia as only a small proportion of cells are

infected. Virus is isolated mainly from the PMNL-rich fraction although also to a lesser extent from mononuclear cells (Fiala et al., 1975); a single report documents the presence of CMV in a very low percentage (0.00003%) of T cells (Garnett, 1982). IgM lymphocytotoxic antibody is produced in varying amounts in response to CMV infection (Baldwin et al., 1983) and this may contribute to leukopenia. In many of these patients CMV and other herpesvirus infections are associated with reduced numbers of circulating T4+ lymphocytes, increased numbers of T8+ lymphocytes and inverted T cell subset ratios (Schooley et al., 1983) which may result from events similar to those occurring in the mononucleosis syndrome. In recipients of allografts from living related donors where lower dose immunosuppression is often used these alterations have only been observed in CMV infections (Schooley et al., 1983). This can occur without resulting in absolute lymphopenia but this may not be true of the more severely immunosuppressed patients.

The immune response to CMV infection has been well studied in renal and cardiac transplant recipients. A vigourous humoral response to infection is observed in most patients which may be due to elimination of regulatory T cells or, more likely, to the reduced ability to restrict viral replication resulting in greater antigenic stimulation of B cells as suggested by Lopez et al. (1974b). In some patients the antibody response to infection is poor. Fatal dissemination of infection is common in such patients (Pass et al., 1983c; Rasmussen et al., 1982; Simmons et al., 1974). This may indicate a protective role for antibody in modifying infection in transplant patients or may

reflect a more profound cellular immune dysfunction with little helper T cell function in those who do not mount a humoral response. That antibody itself may contribute to protection or recovery from infection is suggested by the reports that some CMV infections appear to resolve following administration of hyperimmune globulin (Nicholls et al., 1983). This finding parallels that in a murine model (Shanley et al., 1981).

Primary CMV infection in renal and cardiac transplant recipients is usually accompanied by a specific IgM response (Pass et al., 1983a; Rasmussen et al., 1982; Sutherland & Briggs, 1983). Peak IgM titres coincide with the onset of viral excretion and with symptoms when present, but unlike patients with mononucleosis or pregnant women with asymptomatic infection, IgM titres frequently persist for a year or longer. The IgA response has not been studied in transplant recipients with primary CMV infection. Since humoral immunity is generally intact, this is likely to be present early in infection also.

The IgG response takes place concomitantly with the IgM response in primary infections (Pass et al., 1983a). This response is directed against similar targets as the response in other primary infections. CF titres and antibody titres to early and late antigens rise sharply following the onset of viral shedding (Pass et al., 1983a). The response to pre-early nuclear antigens however may lag behind other responses in these patients (Gartner & Orstavik, 1984). IgG titres generally peak at around 20 weeks after transplant and remain stable throughout follow-up (Pass et al., 1983c).

In patients with nonprimary CMV infection, the timing and

nature of the immune response is less predictable. Serological evidence of infection precedes viruria in about two thirds of patients and the appearance of specific serum IgA is often the first serological response to infection detectable (Sarov et al., 1981; 1982; 1984). In other patients the appearance of serum IgA coincides with a rise in IgG titres and overall over 90% of patients with recurrent infection produce IgA.

IgM is also produced in around 40% of nonprimary infection (Pass et al., 1983a; Sarov et al., 1984; Sutherland & Briggs, 1983). It has been speculated that an IgM response may be more common in reinfections acquired from the donor organ than in reactivations of virus latent in the recipient. The appearance of IgM is temporally related to a rise in IgG titres in most cases and to the appearance of IgA in some cases. The IgM response postdates the appearance of IgA in many however (Sarov et al., 1984). IgG titres do not generally approach those seen in primary infection (Pass et al., 1983b) but remain relatively high for long periods (Roenhorst et al., 1985). IgM and IgA titres frequently persist for a year or more.

Cell-mediated immunity has also been thoroughly investigated in renal and cardiac transplant patients. The aim of immunosuppressive therapy in these patients is to suppress cell-mediated immune functions specifically, which include those responsible for graft rejection. This treatment may result in a reduction of the proliferative response of PBL to mitogens (Agatsuma et al., 1979; Lopez et al., 1974b) but this is usually only demonstrable in those receiving ATG at the time of testing (Pass et al., 1981; Rand et al., 1976). The defect

can be overcome in vitro by culturing PBL in pooled plasma instead of autologous plasma (Lopez et al., 1974b) indicating that some immunosuppressive agents may act by suppressing lymphocyte activation or proliferation. As will be seen however these agents also act by blocking the maturation of functional effector cells from thymic precursors. Lymphocyte reactivity is maximally depressed during the first three months after transplantation and gradually returns to normal or near normal levels as immunosuppression is reduced to maintenance levels.

The development of antigen-specific reactivity following transplantation follows a similar time course to that described above for mitogen responsiveness. In one study (Rand et al., 1976) HSV-specific responses were maximally depressed during the first three months - the period when HSV reactivations are most common - and normalised thereafter. In contrast the return of VZV-specific responsiveness was delayed beyond this early period. Reactivated VZV infections were uncommon in this patient cohort, suggesting that return of immune responsiveness may be dependent on active infection and immune stimulation. This is consistent with the results of Haahr et al. (1979) who found that HSV-specific responses were actually elevated during the first year in comparison with healthy controls and fell thereafter in parallel with the declining frequency of HSV infections.

This pattern of returning immune reactivity does not extend to CMV-specific immunity however. Although most CMV infections occur during the early months after transplantation, specific lymphocyte proliferative responses do not return to

normal for at least two years, irrespective of whether infection is primary or not (Agatsuma _et al_., 1979; Haahr _et al_., 1979; Pollard _et al_., 1978; Roenhorst _et al_., 1985). Pass _et al_. (1981b) found that persistence of unresponsiveness was more pronounced in those receiving ATG. Immunosuppressive therapy may then aggravate lymphocyte unreactivity to CMV but would not seem to be the sole cause as the return of the CMV-specific response is delayed beyond the recovery of other responses, even in those receiving ATG. The development of a lymphocyte response to CMV bears some temporal relationship to the cessation of viral excretion in these patients suggesting that this may be one effector function responsible for curtailing residual viral replication. In particular the proliferative response to CMV-infected cells has been found to be lower in those who had experienced secondary infections during the first six months post transplant compared with those who had not become infected during this period and this parameter only returned to normal in long term survivors (Roenhorst _et al_., 1985). The response to CMV virion antigens was low in both groups of long term survivors in this study. This may suggest that reduced responsiveness to infected cell antigens is more likely to result in reactivated infection than reduced responsiveness to virion antigens only, but induction of a long-term suppression of the reponse to the former following reactivation may also be possible.

Prolonged suppression of the proliferation response to CMV appears from these results to occur following active CMV infection and may be partly CMV-specific although admittedly

this is difficult to ascertain in these patients. A general impairment of immunity is likely to occur in those infections associated with leukopenia and this may not be apparent in proliferation assays using standardised lymphocyte numbers. The frequency of superinfections in those with CMV infection testifies to such an impairment.

In contrast to the delay in the development of the DTH response to CMV infection, cytotoxic responses are elicited early in infection in most cases. These responses have been studied in renal transplant patients by Rook et al. (1984). Serious or fatal CMV infections were confined to those who did not mount a cytotoxic response. This response was mediated by CTL's and/or NK and K cells. NK and K cell activity was depressed throughout the first three weeks after transplant compared to pretransplant levels in most patients. (Activated CTL's were also absent prior to transplantation as in most latently infected individuals.) Following infection activated cytotoxic cells developed rapidly - prior to excretion and to the rise in antibody titres in the majority, even when infection occurred early after transplantation. Patients with CTL responses also showed generally higher levels of NK and K cell activity although in some patients these latter responses developed in the absence of a CTL response.

The absence of a cytotoxic response was associated with large dose methylprednisoline administration between five and 14 days previously to treat rejection episodes. The five day delay before this treatment suppressed cytotoxic function indicates that this drug acts primarily on cytotoxic

precursors. (ATG was not used in these patients.) Patients with cytotoxic responses did not suffer prolonged viraemia, pneumonitis or superinfections and had a significantly lower incidence of leukopenia and graft dysfunction. These responses therefore appear to be effective in restricting viral replication and curtailing viraemia, although not prolonged viral excretion. Far from resulting in graft rejection as has been speculated, these responses protect against it.

Cytotoxic responses were not the only protective mechanisms operative in these patients as most of those without them eventually recovered, despite complications. The only patient who died in this study was one of the two who failed to mount both cytotoxic and humoral responses to infection. Cheeseman et al. (1979a) reported that administration of human-leukocyte IFN prolonged the interval between transplantation and onset of virus excretion and reduced the incidence of viraemia. This beneficial effect may have been due to potentiation of cytotoxicity mediated by NK and K cells (Herberman et al., 1979) and CTL's (Zarling et al., 1978), however the diverse functions of IFN may also have resulted in other protective mechanisms.

b) Bone marrow allograft recipients.

The plight of bone marrow transplant patients is in some ways similar to that of other transplant patients, only greater. In addition to chemotherapy in order to suppress graft versus host (gvh) disease, these patients receive total body

irradiation prior to transplantation aimed at eliminating bone marrow haematopoeic precursor cells. This results in a state of profound immunodeficiency in the initial period after transplant (Gale et al., 1978), followed by a gradual return of immunocompetence as the donor marrow establishes and assumes functional capacity. During marrow regeneration T8+ cells reconstitute more rapidly than T4+ cells resulting in a low T4+/T8+ ratio (Schroff et al., 1982; Singer et al., 1983). During the first few months after transplant, IL 2 production by lymphocytes is very low, probably due to a lack of functional IL 2-producing lymphocytes. IL 2 production remains lower than normal after this period for at least two years, due in part to the excess numbers of suppressor cells which inhibit IL 2 production (Azogui et al., 1983).

During this period infectious complications are common and CMV emerges as a major pathogen. Interstitial pneumonitis is frequently associated with CMV infection, however pulmonary cytotoxicity due to gvh disease, chemotherapy, irradiation and other infections, particularly Pneumocystis carinii are also implicated in this condition (Meyers et al., 1975) and it is often difficult to identify a primary etiology even when pulmonary CMV infection is present. Interestingly in one study of 100 syngeneic transplant recipients (Appelbaum et al., 1982) no instances of CMV associated pneumonia were recorded, despite similar excretion rates to recipients of allogeneic marrow transplants. This may have been due to differences in the incidence of gvh disease, in the degree of immunosuppressive therapy used for its treatment or prevention, or in the rate of

immunological reconstitution between these two groups.

There is no difference in the incidence or severity of infection between those who were immune prior to transplant and those who were not (Meyers et al., 1980c). All infections in these patients are primary-like and this is also evident in the serological response to infection. Skinhoj et al. (1984) showed that most infections during the first five months were associated with both seroconversion or rising IgG titres and specific IgM production. It was not clear from this study whether the serological response coincided with the onset of CMV infection or the return of immunocompetence however, particularly those infections during the very early post transplant period. Failure to mount this response was recorded in five of 16 patients and was associated with high dose chemotherapy at the time of infection. All these patients succumbed to CMV-associated pneumonia.

CMV infection is associated with particularly low mitogen responsiveness in comparison with uninfected patients (Meyers et al., 1980c; Quinnan et al., 1981; 1982a). This may be related to the similar situation in CMV-mononucleosis, but it is also possible that emergence of CMV infection and mitogen unresponsiveness have a common cause, such as gvh disease or its treatment. CMV-specific lymphocyte proliferation responses are absent or significantly depressed after transplant and usually do not develop until CMV infection occurs irrespective of the pretransplant immune status of the recipient or the donor Meyers et al., 1980c). Even when infection does occur the response is usually delayed for three weeks or more in relation

to the serological response (Quinnan et al., 1981). This is still more rapid than the appearance of the response reported in renal transplant recipients however it is not known if the assays used are comparable. The delay in the response may be due to suppression of proliferative responses in general induced by CMV infection or may be due to the relatively low numbers of helper T cells. While this latter possibility is likely to contribute to the unresponsive state, CMV infection may also exert an additional immunosuppressive influence as a limited study of patients with active VZV infection indicates that VZV-specific proliferation responses are elicited rapidly following infection (Meyers et al., 1980a). In marrow transplant recipients surviving over 200 days proliferative responses to VZV, HSV and CMV are similar to those of normal individuals (Meyers et al., 1980a; b; c).

As with other transplant recipients, the generation of cytotoxic responses occurs rapidly in marrow transplant recipients following active CMV infection and has been studied by Quinnan et al. (1981; 1982a). The HLA-restricted CTL response is often detectable prior to seroconversion and is transient, declining after recovery. This response is rapidly followed by NK and K cell responses in most patients. In some patients these are the only cytotoxic responses demonstrable. These responses persist for longer than the CTL response and may be important in maintaining the infection-free state. Unlike renal transplant recipients, failure to elicit a cytotoxic response is uniformly associated with a fatal outcome in these patients. Low NK and K cell activity is also evident

prior to the onset of fatal infection.

These results indicate that cytotoxic responses are important in promoting recovery from CMV infection in marrow transplant recipients also. Protective roles for other immune responses cannot however be discounted. Winston et al. (1982) has reported that administration of immune plasma throughout the period of greatest susceptibility to CMV infection does not reduce the frequency of infection but does reduce the incidence of symptoms, particularly interstitial pneumonitis.

INTRODUCTION

CHAPTER 4

THE ACQUIRED IMMUNE DEFICIENCY SYNDROME

The outbreak of opportunistic infections and Kaposi's sarcoma (KS) in 1978 among male homosexuals in certain American cities has presented one of the greatest challenges of our times to all branches of medical science. Numerous speculations on the etiology of this acquired immune deficiency syndrome (AIDS) have been put forward in an attempt to explain its apparent sudden onset in epidemic proportions and its frequency amongst particular population groups. Many of these theories have envisaged a multifactorial etiology and most include one or more infectious agents as important factors.

The prominence of CMV infection in the syndrome was recognised in the earliest observations. A description of the epidemiology, microbiology and immunology of AIDS is necessary to establish the background against which this CMV infection is set. Although the primary etiology of AIDS is now known to be the retrovirus human T-lymphotropic virus type III (HTLV III; also known as lymphadenopathy-associated virus [LAV]), a consideration of previous hypotheses of etiology is also beneficial as it underlines the complex interactions that occur in AIDS. Furthermore the identification of the primary causative factor does not rule out the possibility that other factors may also be important or even essential to the pathogenesis of AIDS following the initial retrovirus

infection. In the present context, such a discussion also emphasizes the prominence of CMV infection, both prior to and during the evolution of the syndrome.

AIDS was first identified in promiscuous male homosexuals (Follansbee et al., 1982; Gottlieb et al., 1981; Mildvan et al., 1982; Masur et al., 1981; Siegal et al., 1981) and most cases in the west have arisen among this group. Other groups at risk are intravenous drug abusers (Gold et al., 1982; Masur et al., 1981; 1982), recipients of blood transfusions (Curran et al., 1984) and blood products, in particular factor VIII (Evatt et al., 1984), Haitian immigrants to the USA (Pitchenik et al., 1983; Vieira et al., 1983), sexual contacts of individuals in these groups (Masur et al., 1982; Pitchenik et al., 1984) and infants born to women at risk (Oleske et al., 1983; Scott et al., 1984). An increasing number of cases are being identified outside the USA and the disease is now known to be endemic in several central African countries (Piot et al., 1984; Van de Perre et al., 1984) with an estimated frequency of up to eight cases per 10 000 of the population per annum in one city and a female to male ratio approaching unity. Transmission appears to be predominantly among promiscuous heterosexuals in Africa. This is suggestive of an epidemic at a more advanced stage even than that observed in the USA and is consistent with an African origin for AIDS. The common denominators in most of these groups are intimate or parental contact with blood and its derivatives, semen or other body fluids. The apparent exception of the Haitian group may be due to difficulties in evaluating this group for risk factors.

Cases may present with a range of opportunistic infections. AIDS was initially recognised by the unusually high frequency of Pneumocystis carinii in previously healthy homosexual men (Gottlieb et al., 1981; Masur et al., 1981). Other protozoan, fungal and bacterial infections seen include Toxoplasma gondii, Entamoeba histolytica, Cryptococus neoformans, Isospora belli, Candida albicans, Nocardia species, Mycobacterium tuberculosis and M. avium intracellulare, Pseudomonas aeruginosa and Staphylococcus species (Gottlieb et al., 1981; Greene et al., 1982; Follansbee et al., 1982; Masur et al., 1981; Mildvan et al., 1982; Ng et al., 1984). Viral infection and reactivation is extremely common; CMV infection is almost universal (Gottlieb et al., 1981; Quinnan et al., 1984) as is EBV infection (Quinnan et al., 1984). HSV and VZV infections are also common, and are a cause of significant morbidity (Quinnan et al., 1984; Siegal et al., 1981). Polyoma virus (England et al., 1984; Miller et al., 1982) and adenovirus infections (Brodie et al., 1984) have also been described.

KS is the most common malignancy encountered in AIDS (Drew et al., 1982; Friedman-Kien et al., 1982; Urmacher et al., 1982). This is unlike the classical KS seen in elderly or iatrogenically immunosuppressed individuals which usually runs a slow, indolent course, and is more similar to the aggressive, often lymphadenopathic form seen most commonly in young male and less commonly female individuals in parts of central Africa (Slavin et al., 1970; Taylor et al., 1971) which is also associated with immunosuppression (Master et al., 1970). The development of KS has been confined mainly to male homosexuals

with AIDS where it is observed in a third of cases (Guinan et al., 1984). 10-20% of African cases have KS (Piot et al., 1984; Van de Perre et al., 1984) but few cases have been reported in drug addicts and Haitians (Guinan et al., 1984) and none in haemophiliacs (Evatt et al., 1984). The likelihood of KS occurring may then be influenced by other factors more prevalent among homosexuals, as well as by genetic factors (Friedman-Kien et al., 1982). Non-Hodgkins lymphoma has also been documented less frequently in AIDS patients (Ziegler et al., 1984).

Death in the AIDS patient most commonly results from pneumonia caused by opportunistic infections, notably P. carinii (Centers for Disease Control, 1983) and CMV (Macher et al., 1983). These two infections often occur together (Follansbee et al., 1982; Gottlieb et al., 1981).

The occurrence of these opportunistic infections and neoplasms is highly suggestive of an impaired immune system and immunological investigation of AIDS patients bears this out. Patients show varying degrees of anergy in the DTH test reactions (Friedman-Kien et al., 1982) and in vitro proliferative responses to T cell mitogens and recall antigens are significantly depressed (Gottlieb et al., 1981; Masur et al., 1981). Leukocytes function poorly both as responders and stimulators in the allogeneic and autologous MLR (Gupta & Safai, 1983; Masur et al., 1981;1982). These in vivo and in vitro tests of immune function are dependent largely on T4+ lymphocyte function. Analysis of leukocyte populations reveals a reduction in total lymphocyte numbers and total T cell

numbers (Masur et al., 1981) and an inversion of the normal T4+/T8+ lymphocyte numbers ratio which is due mainly to a selective, sometimes almost complete elimination of T4+ lymphocytes (Friedman-Kien et al., 1982; Gottlieb et al., 1981; Mildvan et al., 1982). The percentage of T8+ lymphocytes is increased as a result but absolute numbers are normal or somewhat depressed. This is not due to sequestration of T4+ lymphocytes in lymphoid tissue as analysis of lymph node lymphocyte populations reveals the same selective depletion of T4+ lymphocytes (Modlin et al., 1983). Low or inverted T4+/T8+ lymphocyte ratios of a less striking nature have also been observed in those with ARC[1] (Cavaille-Coll et al., 1985; Hersch et al., 1984; Mathur-Wagh et al., 1984), in healthy homosexuals (Detels et al., 1983; Fahey et al., 1983; Kornfeld et al., 1982; Wallace et al., 1982), promiscuous women (Wallace et al., 1983) and in treated haemophiliacs (Menitove et al., 1983) but except in those with ARC, this is due mainly to elevation of T8+ lymphocyte numbers in most cases. These changes may be related to the incubation phase of AIDS or may be due to other causes.

The extent of the reduction in T4+ lymphocyte numbers is generally reflected in the degree to which immune function is impaired in these groups. *In vitro* and *in vivo* DTH responses and mitogen-induced proliferative responses are maximally suppressed in those with fulminant AIDS, intermediate in those with ARC and least affected but still significantly lower than normal in healthy homosexuals (Cavaille-Coll et al., 1984;

1 ARC = AIDS-related complex.

Gluckman et al., 1985; Hersch et al., 1984; Kalish et al., 1984; Mathur-Wagh et al., 1984; Stahl et al., 1982). This results from low IL 2 production following antigenic or mitogenic stimulation of lymphocytes (Alcocer-Varela et al., 1985; Gluckman et al., 1985; Tsang et al., 1984).

In healthy homosexuals this may be due to the presence of high numbers of T8+ suppressor cells, however in patients with AIDS an intrinsic defect in the function of the remaining T4+ cells appears to be responsible as the response of purified T4+ lymphocytes in the autologous MLR is very low (Gupta & Safai, 1983). In addition there is no absolute correlation between T4+/T8+ lymphocyte ratios and IL 2 production capacity (Gluckman et al., 1985).

There is also an abnormal response to IL 2 of lymphocytes from patients with AIDS or ARC, as addition of exogenous IL 2 results in only a partial restoration of proliferative responses (Gupta et al., 1984; Lifson et al., 1984; Murray et al., 1985). This is due to deficient expression of IL 2 receptors on lymphocytes following activation (Gluckman et al., 1985; Prince et al., 1984; Tsang et al., 1984). This may be due to the presence of suppressive factors in the sera of AIDS patients which appear to act directly on stimulated lymphocytes, possibly by masking the IL 2 receptor (Hennig & Tomar, 1984). Reduced antigen-presenting capacity may also contribute to poor DTH responses, and reduced class II MHC antigen expression on antigen-presenting cells has been reported (Belsito et al., 1984; Heagy et al., 1984).

The reduction in the number and functional capacity of T4+ lymphocytes would seem to be the major cause of immune

dysfunction in AIDS and ARC however. In view of the central role of this cell in immune responses, it is not surprising that a variety of other immune and non-immune functions are impaired. Lymphokine production by stimulated lymphocytes is low (Murray et al., 1984) and this is likely to be related to the reduced NK cell activity (Reddy et al., 1984; Rook et al., 1983), the absence of CTL responses to viral infections (Rook et al., 1983), reduced monocyte adherence (Hersch et al., 1984) reduced class II MHC antigen expression on accessory cells and possibly to reduced PMNL function (Valone et al., 1984).

The observation that B cell numbers were approximately normal in AIDS (Mildvan et al., 1982) and that serum immunoglobulin levels were elevated (Mathur-Wagh et al., 1984; Stahl et al., 1982) initially led to the conclusion that B cell function was relatively unimpaired in AIDS. Closer investigation indicates otherwise however. PWM-driven immunoglobulin synthesis by B cells is defective and this is not solely due to lack of helper functions as T-independent mitogens such as EBV also fail to induce a normal response and coculture of B cells with normal T cells only occasionally corrects the defect (Lane et al., 1893; Pahwa et al., 1984). Primary B cell responses cannot be generated in vivo or in vitro following immunisation (Lane et al., 1983) and frequently no IgM response to CMV is demonstrable despite disseminated infection (Dylewski et al., 1983).

This B cell defect may be due to suppression by T8+ cells, and infiltration of the latter into the follicular centre (B cell zone) of lymph node follicles has been observed (Modlin

et al., 1983). It is perhaps more likely however that the defect is intrinsic and may result from non-specific in vivo activation of B cells. This would result in prior commitment and inability to respond to further in vitro stimulation. That B cells are activated in AIDS is shown by the high rate of spontaneous immunoglobulin secretion in vitro (Lane et al., 1983), and this is the probable cause of the hypergammaglobulinaemia. This of course places serious limits on the value of serology in diagnosing infection in AIDS patients. B cell activation is perhaps an important cause of the lymph node hyperplasia which characterises the prodromal, lymphadenopathic phase (Mathur-Wagh et al., 1984) and frank lymphoma is often preceded by this condition (Levine et al., 1984).

There are also signs of profound dysregulation of other immune control functions and of haematopoiesis in AIDS. Thymic dysplasia is often evident at autopsy (Elie et al., 1983) and anaemia and thrombocytopenia are common (Kalish et al., 1984). Levels of the thymic hormone alpha 1-thymosin are elevated perhaps due to lack of feedback inhibition from functional T4+ lymphocytes (Hersch et al., 1983; 1984). Serum levels of beta-2 microglobulin are also elevated (Zolla-Pazner et al., 1984) and an unusual, acid-labile alpha IFN is often present at high concentration in AIDS and ARC (De Stefano et al., 1982; Eyster et al., 1983). This may be due to antigenic stimulation (Balkwill et al., 1983) but acid-labile IFN has also been found in patients with systemic lupus erythematosis (Preble et al., 1982) and may be related to the occurrence of autoimmune phenomena in AIDS also (Morris et al., 1982).

Most of the features of AIDS and related conditions described above were elucidated without identifying their root cause(s), although numerous speculations were offered in this area. Attempts to answer the question of etiology had to account for certain features of the syndrome. Firstly, the evidence pointed strongly towards the involvement of an infectious agent, as clustering of cases was noticed and there were clear examples of cases which could be linked by sexual or parenteral contact to other cases (Auerbach et al., 1984). The initial geographic restriction to North American cities was also suggestive of a transmissible cause and the high frequency of occurrence in homosexuals and drug addicts was very reminiscent of HBV epidemiology.

The wide spectrum of sexually transmitted diseases prevalent among homosexuals allowed much speculation over the etiology of AIDS, however the second factor which had to be accounted for was the apparent sudden onset of the epidemic. The question arose of whether a new pathogen or a virulent strain of a known pathogen had appeared, or whether changes in the lifestyle of those at risk were responsible, perhaps in concert with other common infectious agents. While the search for new pathogens continued others addressed the second of these possibilities.

AIDS was initially recognised in the male homosexual population and it was probable that its occurrence in other groups resulted from contact with this group. The appearance of AIDS is set against a background of rapidly changing lifesyle among homosexual groups, particularly in the USA, with

increased promiscuity and mobility between communities in North American cities. This was accompanied by an increase in sexually transmitted diseases (Darrow et al., 1981) and it was thought possible that multiple infections may act in combination or synergism to produce the immunological changes seen in AIDS. Some infections common in homosexuals are known to have immunosuppressive effects, most notably CMV and EBV (Chapter 3) and CMV was considered by many to be a prime candidate (Durack, 1981; Gottlieb et al., 1981). Evidence of past CMV infection is very common in male homosexuals (Chapter 2), particularly in the USA where over 90% are seropositive (Drew et al., 1981). Amongst those who are seronegative on initial testing over 80% become infected per annum (Mintz et al., 1983). Such a high prevalence made it difficult to establish a causative role, however the high frequency of viruria (7-8%) and even higher frequency of virusemenia (35%; Drew et al., 1981; Mintz et al., 1983) suggested that CMV immunity was diminished in this group. This would result in frequent reactivation of infection which may induce further immunosuppression and susceptibility to opportunistic infection. The development of KS in which a role for CMV has been proposed on virological and epidemiological grounds (Boldogh et al., 1981; Drew et al., 1982; Giraldo et al., 1975; 1978) and the near universality of active CMV infection in patients with advanced AIDS supported this view.

A role for EBV in the induction of AIDS was not considered to the same extent initially. This may have been because information on active EBV infection in those with AIDS or in those at risk was lacking, or because the occurrence of B cell lymphoma which has been linked with EBV infection in other

immunocompromised individuals (Hanto _et al_., 1981) and other pathology attributable to EBV infection was seen less often than KS or disseminated CMV infection. However, it has since been shown that active EBV infection, like CMV infection is virtually universal in AIDS and ARC (Quinnan _et al_., 1984). Perhaps surprisingly, excretion was also almost universal in healthy homosexuals in this study. In addition the finding of polyclonal B cell activation (Lane _et al_., 1983), the recognition of the prodromal syndrome characterised by lymph node hyperplasia (Mathur-Wagh _et al_., 1984) and the demonstration of EBV DNA at high copy number (Lipscomb _et al_., 1983) and EBV antigens (Quinnan _et al_., 1984) in lymph node cells is also strongly suggestive of active, morbid EBV infection.

While CMV and EBV infections may result in an immune suppression of considerable duration, they are however normally self-limiting in otherwise healthy persons. Although T4+/T8+ lymphocyte ratios in AIDS and ARC are superficially similar to those in the mononucleosis syndrome, this is due to reduced T4+ cell numbers in the first two conditions and mainly to elevated T8+ cell numbers in the last. Furthermore the intrinsic defect in T4+ lymphocyte function is not observed in CMV or EBV infections. This would suggest that the immunosuppression seen in AIDS is not primarily due to infection with these viruses. CMV or EBV infection may predispose to some other immunological assault or may reactivate or gain fuller pathogenic expression subsequent to such an event, but additional factors seemed necessary to explain the progressive and permanent immune

dysfunction in AIDS.

Multiple CMV infections are likely to occur in promiscuous homosexuals and have been demonstrated in patients with AIDS (Drew et al., 1984; Spector et al., 1984). Repeated CMV infections or concurrent infection with EBV or other agents may cause a greater immune suppression than is normally seen in the mononucleosis syndrome. It would be predicted that this too however would result from stimulation of T8+ suppressor cells rather than an intrinsic T4+ cell defect.

An abnormal immune response may result when infectious agents are encountered via an unusual route. The rectal mucosa is fragile and easily breached during rectal intercourse when agents present in faeces or semen may be directly introduced into the circulation (Detels et al, 1983). Exposure to semen by itself may be a significant cause of immunosuppression. Spermatozoa are capable of suppressing mitogen - induced lymphocyte proliferation in vitro, possibly due to the presence of mitogen receptors on spermatozoa resulting in absorption of mitogen and reduction of the proliferative signal to lymphocytes (Marcus et al., 1978). As the interaction of mitogen with lymphocytes can mimic the interaction of MHC products with lymphocytes during an immune response this effect may be operative in vivo. Seminal plasma also contains a variety of immunomodulating agents which are probably important in suppressing immune responses against spermatozoa that would result in infertility. When encountered outside the reproductive system these components may suppress the function of lymphocytes and macrophages (James & Hargreave, 1984).

Immunosuppression may also follow immunisation against antigens on spermatozoa which cross-react with lymphocyte membrane antigens (Sonnabend <u>et al.</u>, 1984) and it has been suggested that the introduction of allogeneic leukocytes during rectal intercourse may induce local graft versus host (gvh) reactions if, on account of semen- or virus infection-induced immunosuppression they were not immediately rejected (Shearer <u>et al.</u>, 1983). Comparable events have been demonstrated in mice injected with parental lymphocytes and immunosuppression is the end result in this model (Shearer & Polisson, 1980). In support of these theories it has been reported that healthy homosexuals with a history of frequent passive (receptive) rectal intercourse frequently have anti-sperm antibody, have high MLR activity against lymphocytes from regular active partners compared to lymphocytes from other individuals, but low activity in a xenogeneic MLR which measures the ability of lymphocytes to induce a local gvh reaction in an immunosuppressed animal, and are more likely to have low T4+/T8+ lymphocyte ratios (Detels <u>et al.</u>, 1983; Mavligit <u>et al.</u>, 1984). Repeated occurrences of this nature may induce cycles of immunosuppression resulting in reactivation of CMV and EBV infection, which would themselves contribute to immunosuppression and result in a self-sustaining, irreversible phase (Sonnabend <u>et al.</u>, 1984).

Other factors considered of relevance to the etiology of AIDS in male homosexuals were the use of amyl and butyl nitrite drugs (Goedert <u>et al.</u>, 1982), oestrogens (Couthino, 1982) and steroids (Neumann, 1982). The susceptibility of other groups to

AIDS was thought to be due to other predisposing factors acting in synergy with viral or other infections, giving an end result similar to that in homosexuals. Drug addicts are known to be immunocompromised frequently (Brown et al., 1974) and the immunological immaturity of the neonate may increase susceptibility. Investigation of immune parameters in haemophiliacs reveals abnormal T cell subset ratios in a proportion (Lederman et al., 1983; Menitove et al., 1983) which have been attributed to the presence of potentially immunosuppressive components in factor VIII preparations (Ablin & Gonder, 1984; Lee et al., 1984).

A major shortcoming of these hypotheses which favoured a multifactorial etiology was their inability to explain the sudden onset of the AIDS epidemic in different groups at around the same time, and its occassional occurrence in individuals outwith recognised high risk groups where an isolated contact with an affected or at risk person appears to have resulted in transmission. It became increasingly clear that a common denominator, probably an infectious agent, was likely to be present in every case.

Several observations suggested a retroviral etiology. Although animal retroviruses have been studied mainly with a view to understanding their role in oncogenesis it was frequently noticed that retroviral infections resulted in immunosuppression and degenerative disease as well as malignant disease. The incubation phase of experimental leukemia in inbred mice is characterised by suppression of both humoral (Peterson et al., 1963) and cellular immune mechanisms (Dent et al., 1965). Infection of cats with feline leukemia virus

(FeLV), which is a naturally occurring, horizontally transmissible virus (Hardy et al., 1976) is frequently followed by viral persistence associated with lymphopenia (Essex et al., 1975; 1976), anaemia (Onions et al., 1982), suppression of humoral immune responses (Trainin et al., 1983) and a marked susceptibility to other infections (Essex et al., 1975; 1976).

Virion components of FeLV and other retroviruses are known to suppress mitogen-induced proliferation directly (Hebebrand et al., 1977; Weislow et al., 1981). This may cause an initial, local immunosuppression preventing viral clearance, followed by a systemic immunosuppression with the onset of viraemia (Mathes et al., 1979). Alternatively, immunosuppression may result from infection of lymphoid tissue or of bone marrow precursor cells. Experimental infection of kittens with FeLV results in bone marrow and lymph node lesions (Hoover et al., 1973), severe thymic atrophy (Anderson et al., 1971; Perryman et al., 1972) and suppression of cellular immune responses (Perryman et al., 1972).

The human T cell leukaemia viruses (HTLV I and HTLV II) are recently discovered human retroviruses (Kalyanaraman et al., 1982; Poiesz et al., 1980) which infect and transform T4+ lymphocytes specifically (Essex et al., 1982; Hattori et al., 1981). Evidence of sexual transmission exists (Schupbach et al., 1983) and serological evidence of infection is common in parts of Africa (Hunsmann et al., 1983; Saxinger et al., 1984) where aggressive KS is also found, and in the Caribbean (Schupbach et al., 1983). The cell tropism and geographical distribution of HTLV I suggested a possible

involvement in AIDS and a search for HTLV I infection in AIDS patients and in high risk groups was initiated. Proviral DNA sequences were detected in fresh T cells from two of 33 AIDS patients (Gelmann et al., 1983) and antibodies to HTLV I membrane antigens were found in 25% of AIDS cases, 26% of ARC cases, up to 19% of haemophiliacs at risk and only 1% of controls (Essex et al., 1983a; b). The difference in antibody prevalence among those with, or at risk of AIDS compared to controls was greater than that observed for any other viral infection. The failure to demonstrate antibody in all AIDS cases may have been due to waining of the humoral immune response as infected T4+ cells became depleted resulting in reduced antigenic challenge and impaired antibody production.

An HTLV I etiology for AIDS was not consistent with all the information however. The low frequency of HTLV I detection or isolation, the absence of antibody to virus core proteins and the virtual absence of AIDS in southern Japan despite the high prevalence of HTLV I exposure in that region (Hinuma et al., 1981) suggested that HTLV I was not the cause of AIDS, but that another antigenically related virus may be responsible. Subsequently, similar but serologically distinct retroviruses were isolated from a patient with ARC (Barre-Sinoussi et al., 1983) and from two sibling haemophiliacs, one of whom had AIDS (Vilmer et al., 1984). Characterisation of these isolates was initially hampered by the lack of a permissive cell line as isolation attempts in primary human lymphocyte culture resulted in accelerated terminal differentiation of infected cells, cell death and loss of virus (Gallo et al., 1984). A permissive T cell line has now

been found, allowing viral detection and isolation from the concentrated supernate of primary T cell cultures on a more reproducible basis (Popovic et al., 1984) and initial isolation attempts were positive in 86% of ARC cases, 36% of AIDS cases, 5% of healthy homosexuals considered to be at low risk, three of four mothers of infants with AIDS and none of over 100 heterosexuals (Gallo et al., 1984). Such a correlation between the virus carrier state and the presence of or capacity to transmit AIDS or ARC is strong evidence that theses retroviruses, collectively known as HTLV III or LAV were causative of these two conditions, and were not merely opportunists. The less than 100% isolation rate was considered due to the low numbers of T4+ cells in many specimens, particularly those from patients with advanced AIDS.

HTLV III membrane antigens were found to be cross-reactive with those of HTLV I and HTLV II whereas the core proteins were antigenically distinct (Barre-Sinoussi et al., 1983; Schupbach et al., 1984) which may explain the earlier findings of antibodies to HTLV I membrane antigens in patients with AIDS or ARC or in those at risk. Analysis of the HTLV III antibody prevalence in patients and high risk groups strengthened the case for an HTLV III etiology for AIDS. Antibody was present in 75% of AIDS cases, over 90% of ARC cases and none of controls (Brun-Vezinet et al., 1984a; Sarngadharan et al., 1984). The less than 100% detection rate may have been due to test insensitivity or to drops in antibody titres following the depletion of infected cells or to B cell malfunction. Antibody has also been demonstrated in AIDS patients from the UK (Cheingsong-Popov et al., 1984) and Africa (Brun-Vezinet

et al., 1984b), in blood donors implicated in transmission by transfusion (Jaffe et al., 1984), in other persons suspected as a resevoir of infection (Laurence et al., 1984), and in the UK, in 59% of homosexuals with symptoms other than those which constitute AIDS or ARC, 42% of case contacts, 17% of homosexuals at risk, 34% of factor VIII recipients, 15% of drug addicts but in no heterosexuals or random blood donors (Cheingsong-Popov et al., 1984).

A surprisingly high seropositivity rate of 67% has been found in sera collected from Ugandan children in 1973 (Saxinger et al., 1985) indicating that HTLV III or a similar virus existed some time before the recognition of AIDS. It is highly possible that AIDS was present in Africa before 1978 and probable instances have been diagnosed retrospectively from 1976 and 1977 (Bygbjerg, 1983; Brun-Vezinet et al., 1984b; Vandepitte et al., 1983). Antibody was present in 5% of healthy mothers in Zaire in 1980, in 7% of hospitalised patients in 1983 (Brun-Vezinet et al., 1984b) and in 2% of healthy Zambians in 1984 (Bayley et al., 1985) but in 20% of healthy Ugandans in 1984 (Bayley et al., 1985). This would indicate that HTLV III has been established for some time in Uganda and has recently spread into other African countries.

The status of aggressive KS in Africa with respect to HTLV III infection has recently been clarified. Antibody has been demonstrated in 92% of cases from Uganda and Zambia compared to only 17% of classical KS cases (Bayley et al., 1985).
Consistent with the view that HTLV III infection has only recently been introduced to Zambia is the observation that

aggressive KS was first recognised in that country in 1983 (Bayley, 1984).

These results present a strong case for considering HTLV III as etiologic in the development AIDS (including aggressive KS) and ARC. An understanding of the pathogenesis of HTLV III infection in these conditions and in those who remain asymptomatic can only be partial at the present time. Primary infection is associated with an acute glandular fever-like illness in some cases (Cooper et al., 1985; Lancet, 1984; Tucker et al., 1985). The initial infection is frequently or always followed by viral persistence (Laurence et al., 1984). The virus shows a strong T4+ lymphocyte tropism (Gallo et al., 1984; Klatzmann et al., 1984a) and the T4 antigen is a component of the virus receptor (Dalgleish et al., 1984; Klatzmann et al., 1984b). The low numbers and functional capacity of T4+ lymphocytes in AIDS and ARC may then be directly due to viral infection and elimination. Low numbers of T4+ cell numbers may also occur in the asymptomatic carrier state (Goedert et al., 1984) although this is not always the case (Laurence et al., 1984). Initial findings indicate that low T4+ numbers and the presence of additional constitutional symptoms in patients with lymphadenopathy is more likely in those with detectable HTLV III viraemia (Kaplan et al., 1985). Virus has also been demonstrated in semen (Ho et al., 1984; Zagury et al., 1984) as might be expected from epidemiological considerations, and also in saliva, both cell-free and cell-associated (Groopman et al., 1984). The importance of saliva as a vehicle of transmission is not known but clearly cannot be discounted in view of this finding. Retroviral

particles have also been visualised by EM in macrophages (Gyorkey et al., 1985) and follicular dendritic cells (Armstrong et al., 1985). The presence of viral particles in macrophages probably resulted from phagocytosis as complete particles were present in phagocytic vacuoles but budding of virus from dendritic cells was clearly discernable. Productive infection of macrophages may also occur following phagocytosis and these cell populations may be a reservoir for viral persistence and dissemination to lymphocytes (Armstrong & Horne, 1984).

It is estimated that approximately 7% of healthy seropositive individuals will develop AIDS each year, and that around 13% will develop ARC (Goedert et al., 1984). The factors which influence the likelihood of developing AIDS or associated conditions following HTLV III infection are unknown at present. It is possible that many of those factors previously considered as possible causes of AIDS will be found to be important as cofactors which influence the expression of HTLV III infection. The identification of the causative agent and the development of diagnostic assays allows such issues to be addressed.

INTRODUCTION

OBJECTIVES AND SCOPE OF PROJECT

This project was initiated at a time when the cause of AIDS and associated conditions was unknown. Some considered the characteristic immune abnormalities to be the net result of several forces acting together while others suspected a single agent to be pre-eminently responsible, although the presence of other cofactors was generally agreed to be important. In order to test these hypotheses it was considered necessary to establish the prevalence of possible causative or contributory factors in populations at risk for AIDS, but prior to its onset. It was therefore decided to investigate CMV infection and immunity in healthy male homosexuals in what may be considered an "AIDS-free" region. While several surveys of a similar nature have been undertaken elsewhere since the appearance of AIDS (Biggar et al., 1983; Couthino et al., 1984; Drew et al., 1981; Lange et al., 1984; Mintz et al., 1983; Quinnan et al., 1984) these studies have been conducted among populations where AIDS or its prodromal stages was known or strongly suspected to be present. Consequently it is unknown how many of those regarded as healthy homosexuals were actually in early, asymptomatic stages of the syndrome, in the absence of any definitive diagnostic test for AIDS or its putative agent(s).

The present study was conducted during the period before any case of AIDS was reported in Scotland. It was therefore thought probable that at least most of the homosexual

population would not be in the incubation phase of AIDS. This population then, provided an opportunity to assess the prevalence and effects of factors suspected of contributing to AIDS in individuals, many of whom in different geographical circumstances might be considered to be at high risk for AIDS.

In determining which factors should be assessed in this project, consideration was given to those parameters which were suitable for studying in large numbers of individuals. It was decided to study CMV infection and immunity for the following reasons:

a) CMV was considered by many to be important in the etiology and/or pathogenesis of AIDS.

b) In view of the influence of immune mechanisms on the expression of CMV infection, studying the latter may give an indirect indication of the functional capacity of the former.

c) Methods for determining the prevalence of previous and current CMV infection, and for assessing CMV-specific immune responses are suitable for studying the numbers anticipated.

d) Immune responses to CMV have been characterised in other groups, allowing comparisons with the group under study to be drawn.

CMV excretion from the throat and in the urine was assessed as isolation specimens from these sites are readily

available. Serological investigations included screening for CMV-IgG antibodies as an indication of past infection, screening for CMV-IgM as an indication of current or recent infection, and screening for antibodies to CMV IEA and CMV EA. The significance of these last two antibody reactivities has not been fully clarified (Chapter 3) but they have been found at increased frequency or in greater titres among patient groups where CMV infection is more prevalent, namely renal transplant patients (The et al., 1977) and patients with rheumatoid arthritis (Male et al., 1982). Thus, although not of diagnostic significance, they may provide information of epidemiological value.

Cellular immune parameters were also studied in this project. Lymphocyte proliferation responses to CMV antigens were determined as this measurement of immunity has been shown to be low in some groups of CMV-infected or CMV-susceptible patients. Mitogen-induced proliferation responses were also considered to be of interest. If active CMV infection contributes to the pathogenesis of AIDS, either by causing an initial depression of cellular immunity or by exacerbating a pre-existing immune dysfunction in individuals at risk for other reasons, then this may be detectable as reduced proliferative responses to mitogens in those with active CMV infection, or in those who are active homosexuals, (if homosexuality is, in itself associated with reduced immune capacity).

In view of the fact that the pathogenesis of ARC and AIDS is likely to be complex, the identification of the primary etiologic agent does not nullify the value of such

investigations. It does however provide a means of testing the original assumption that the population under study was relatively AIDS-free, and may enable comparisons to be made between those with and without HTLV III infection.

It is anticipated that this work will be of relevance when considered in relation to other investigations into the pathogenesis of AIDS. Such studies may eventually permit the identification of prognostic indicators which would have an impact on the management of and advice given to those in whom HTLV III infection has been diagnosed. An understanding of the pathogenesis of AIDS may also give insight into the processes by which opportunistic infections arise, and procedures by which they may be managed in other immunocompromised patients.

MATERIALS, METHODS AND STUDY POPULATIONS

Study population and conduct of survey

The population under study consisted of homosexual and heterosexual males who attended the STD clinic at the Department of Genitourinary Medicine, Edinburgh Royal Infirmary as outpatients between May 1983 and May 1984. Whenever possible both homosexuals and heterosexuals were enrolled into the survey on each clinic session. Individuals who were known to be hepatitis B virus surface antigenaemic at the time were excluded. In other respects individuals were approached and enrolled for study on a random basis. Consenting volunteers were studied under code, without knowledge of sexual preference or other particulars until the completion of the majority of laboratory investigations, except the serological analysis of HTLV III infection which was only performed on specimens from homosexuals.

A throat swab, clean-catch urine specimen and peripheral venous blood sample was sought from each volunteer. Throat swabs were immediately placed in viral transport medium and blood samples were immediately heparinised (10-15 IU heparin per ml of blood). All specimens were placed in sterile containers and were processed in the laboratory on the day of collection.

Miscellaneous specimens

Other heparinised blood specimens were obtained from healthy laboratory personnel. This group was used in the

initial characterisation of some of the assays. Some were also studied longitudinally on two or more occasions in order to assess time-dependent fluctuations in some of the parameters under study.

Serum specimens from patients in whom acute viral and other infections had been diagnosed were from the Virus Diagnostic Laboratory, Bacteriology Department, Edinburgh University, provided by Dr. E. Edmond and from the Regional Virus Laboratory, City Hospital, Edinburgh, provided by H. Cubie. Retrospective serum specimens from some of the study population were from the Hepatitis Reference Laboratory, Bacteriology Department, Edinburgh University, provided by Dr. J.F. Peutherer.

Cell culture

Human embryo fibroblasts (HEF) were propagated at 37C in glass or plastic Roux flasks. Growth medium (GM) consisted of Eagles minimal essential medium (EMEM; Flow Laboratories, Irvine, Scotland) supplemented with 6% heat-inactivated newborn calf serum (NBCS; Flow Laboratories), 2 mM L-glutamine (Gibco, Paisley, Scotland), 300 IU/ml benzyl penicillin (Glaxo), 0.3 mg/ml streptomycin sulphate (Evans) and 1-2 ml/100ml 8% sodium bicarbonate solution to give the correct pH. Maintenance medium (MM) was the same as GM in all respects but contained only 2% NBCS. HEF monolayers were allowed to grow to confluence in 100 ml GM and were then passaged by briefly exposing the washed monolayer to a solution of 1% trypsin and 0.02% versene in Dulbecco medium

(DM) at 37C. The detached monolayer was resuspended in GM and reseeded into two or three Roux flasks.

For cryopreservation of HEF's trypsinised cells were pelletted and resuspended in 50% NBCS, 40% GM and 10% dimethyl sulphoxide (DMSO), 1 ml per contents of each Roux flask. The suspension was cooled slowly to -70C in 1 ml aliquots then transferred into liquid nitrogen the following day. To retrieve cryopreserved HEF's, 1 ml aliquots were rapidly warmed to 37C and seeded into Roux flasks with 100 ml GM. GM was changed the following day.

Tube cultures were prepared by seeding tissue culture tubes with 2×10^5 HEF's in 1 ml GM and incubated in stationary racks until confluent. GM was then replaced with MM and tubes were transferred to a roller drum. Medium was changed every 2-3 days.

HEF's were checked periodically for mycoplasmal contamination by incubation in solid, semi-solid and diphasic pleuro-pneumonia-like organism (PPLO) agar at 37C under anaerobic conditions. Cultures were subcultured and examined twice weekly for two weeks.

Virus and antigens

The Ad169 strain of human CMV (Rowe et al., 1956) was used in all experiments. Virus was propagated in HEF's. Monolayers were infected while subconfluent as this favours productive infection in most cells with optimum antigen production and progeny virus release (Chapter 1). Monolayers were infected with virus in a small volume at a multiplicity

of infection (moi) of approximately one. After a one hour adsorption period at 37C monolayers were washed with DM before adding 100 ml MM. Control cultures were prepared in an analogous manner, omitting the exposure to virus. MM was changed after no more than seven days.

When infected cultures showed 100% cytopathic effect (CPE) and disintegration of the monolayer was evident (usually 8-10 days post infection) the culture supernatant was removed, clarified by low speed centrifugation (2000 rpm, 15 min in a bench centrifuge), aliquoted and stored at -70C. Stock virus was also checked for mycoplasmal contamination. Virus was titrated in tube culture. Tubes showing >50% CPE after seven days culture were scored positive and the 50% tissue culture infective dose ($TCID_{50}$) was estimated by the method of Reed & Muench.

Glycine extract (GE) antigen was prepared by infecting cells as described above. When infected cultures showed 100% CPE with disintegration of the monolayer, cells from infected and control cultures were dislodged with glass beads, without aspirating the culture medium. Cells were pelleted from the resulting suspension by centrifugation at 2500 rpm for 30 min in a Mistral 6L centrifuge (MSE), washed three times in 0.01M phosphate-buffered saline (PBS; prepared by mixing 28 ml 0.2M Na $H_2PO_4 . 2H_2O$ with 72 ml 0.2M Na_2HPO_4, dissolving 17g NaCl in this, adjusting the pH to 7.2 then making up to 2l with distilled water). After the final wash, cells were resuspended in glycine buffer, pH 9 (GB), 1 ml per contents of each Roux flask. GB was prepared on the day of use by

mixing three parts of 0.1M NaOH with five parts of 0.1M glycine. The cell suspension was then sonicated vigorously for 15s using an ultrasonic probe (MSE), allowed to stand at 4C for 4-6h then centrifuged at 3000 rpm for 15 min in a bench centrifuge. The suspension was aliquoted in 1 ml amounts and stored at -70C.

Polyethylene glycol (PEG)-precipitate antigen was prepared in a manner similar to that used by Hamelin & Lussier (1979). Cells were infected with virus as described above. 3-4 days prior to 100% CPE with monolayer disintegration, infected and control cell supernates were harvested and stored at 4C. Culture medium was replaced with 50 ml MM. This was repeated on each day following until the infected monolayer had collapsed. The pooled supernates from infected and control cultures were then clarified by centrifugation. PEG 6000 (Fisons) was then added to give a final concentration of 5%. Supernates were held overnight at 4C and the precipitate was pelleted by centrifugation at 5000 rpm for 30 min at 5C in a Sorvall RC2-B centrifuge. The pellet was resuspended in PBS, 1ml per contents of each Roux flask, and held at 4C for 4-6h to allow the precipitated virus to resuspend. The suspension was then stored in 1 ml aliquots at -70C.

Lymphocyte proliferation assays

Up to 12 ml heparinised peripheral venous blood was carefully layered on to 6 ml Ficoll-Hypaque lymphocyte separation medium (Flow Laboratories) and centrifuged at 2000

rpm for 15 min in a bench centrifuge. The uppermost layer of plasma from the centrifuged sample was harvested and used to supplement lymphocyte culture medium. The remainder was stored at -20C for serological analyses. The mononuclear leukocyte-rich layer from the Ficoll-Hypaque/plasma interface was harvested and washed three times in RPMI 1640 (Dutch Modification) medium (Flow Laboratories) by centrifugation as above. After the final wash the cell pellet was resuspended in lymphocyte culture medium which consisted of RPMI 1640 (Dutch Modification) medium supplemented with glutamine, penicillin and streptomycin at the same final concentrations as in GM and MM, and 15% autologous plasma. In some experiments as detailed under Results, autologous plasma was replaced by pooled homologous plasma. The cell density was adjusted to 10^6 viable mononuclear cells per ml with lymphocyte culture medium. Cell viability in most experiments was estimated at 95-100% on the basis of trypan blue exclusion. The resulting cell suspension was dispensed in 200 µl volumes into wells of U-bottomed tissue culture grade 96-well microtitre plates (Sterilin).

Stimulants used in the assay were phytohaemagglutinin (PHA; Miles-Yeda), concanavalin A (Con A; Sigma), pokeweed mitogen (PWM; Gibco) and CMV and control GE antigens. Dilutions of mitogens and antigens were added in 10 µl volumes to quadruplicate culture wells where lymphocyte yield permitted. In other cases stimulants were added to wells in triplicate or occasionally in duplicate. Some wells were left without stimulant as controls. Microtitre plates were covered

and incubated at 37C in a humidified atmosphere of 95% air and 5% CO_2. PHA-stimulated and respective control cultures were maintained for four days. Other mitogen- and antigen-stimulated and control cultures were maintained for six days. 24h before the end of the culture period, each culture well was pulsed with 5 μCi methyl-^{3}H thymidine (40-60 Ci/mmol; Amersham). At the end of the culture period, lymphocyte cultures were harvested on to glass-fibre filter paper (Whatman 934 AH) and washed 20x with tap water using a semi-automated cell harvester (Ilacon). Filter papers were dried at 37C. Individual filter paper discs were removed and placed in scintillation vials with 3 ml scintillation fluid which consisted of toluene ("Fisons AR") with 12.5% 2,5-diphenyl-oxazole (Fisons) and 0.5% 1,4-di(2-methlystyryl)-benzene (Fisons). Scintillation vials were counted for 60s in a Packard Tri-carb liquid scintillation spectrometer.

In calculating results the ^{3}H-thymidine incorporation for each treatment was taken as the mean cpm for all replicate cultures. Mitogen- or antigen-induced proliferation was determined by comparing the ^{3}H-thymidine incorporation of stimulated cultures with that of the appropriate control cultures (unstimulated and control antigen-stimulated cultures respectively). Results for mitogen-induced proliferation were recorded both as stimulation indices (SI = mean cpm of stimulated cultures / mean cpm of control cultures) and as net stimulation (the difference in mean cpm between stimulated and control cultures). Results for CMV-specific stimulation were recorded as SI values.

Virus isolation

To attempt virus isolation from clinical specimens, 0.2 ml of fresh urine or transport medium bathing throat swabs was inoculated into HEF tube cultures. Tubes were examined twice weekly for the development of CPE characteristic of CMV. Tubes were maintained for one month before being discarded.

SEROLOGICAL METHODS

Monoclonal antibodies

A range of CMV-reactive monoclonal antibody (McAb) preparations were gifted by Dr. H. Hart, Inveresk Research International Ltd, Musselburgh, Scotland (Hart & Wilkinson, unpublished). McAbs were provided as ascitic fluid, hybridoma culture supernatant or sodium sulphate-precipitated IgG (see below) and stored at -70C. McAbs were raised by immunising eight-week-old Balb/c mice intraperitoneally with HEF's infected with CMV Ad169 nine days previously in Freund's incomplete adjuvant. Immunised mice were boosted intravenously with sucrose gradient (20-70%) purified CMV virions four weeks later and spleen cells were removed three days later. Spleen cells were fused with cells of the mouse myeloma line P3 x 63.Ag8/NS-1 using PEG and grown in RPMI 1640 medium supplemented with glutamine, penicillin, streptomycin, 100 µg/ml pyruvate and 10% foetal calf serum (FCS). Hybrids were selected in HAT medium and culture supernatants were screened for CMV reactivity by indirect ELISA and indirect

immunofluorescence (IIF) as described below. Clones from colonies which gave CMV-IgG positive supernatants were selected by limiting dilution.

The following McAb preparations were received: HCMV 19, HCMV 23, HCMV 24, HCMV 25 and 96D3, all reactive with immediate early antigens (IEA) by IIF; HCMV 3, reactive with a nuclear early antigen (EA) by IIF; HCMV 1, HCMV 22, HCMV 29, AB3, 91D11, 92E9, 93F2 and 94H3, reactive with other CMV antigens of undetermined class.

McAbs CH 12, CH 16 and CH 28, reactive with late glycoprotein antigens, were gifted by Dr. L. Pereira, Viral and Rickettsial Disease Laboratory, Department of Health Services, Berkeley, California. Their production and characterisation has been described (Pereira et al., 1982b).

IgG was precipitated from ascitic fluid by adding 205 mg $Na_2SO_4 . 10 H_2O$ (finely ground for rapid dissolution) to 0.5 ml ascitic fluid. The suspension was incubated for 1h at room temperature (RT) with agitation. The precipitated IgG was then pelleted by centrifugation at 3000 rpm for 20 min in a bench centrifuge and washed twice with 18% Na_2SO_4 in 0.1M Na HCO_3, then dissolved in 0.1M Na HCO_3 and dialysed overnight against three changes of 0.1M Na HCO_3. The protein concentration was determined from the absorbance at 280 nm (based on an absorbance value of 13.8 for a 10 mg/ml IgG solution).

Rabbit antisera

Antisera 37 and 39, raised in rabbits against CMV virion

membrane and whole virion antigens respectively, were gifted by G. Farrar, Molecular Genetics Laboratory, PHLS, Porton. Virions were pelleted from 50 ml infected cell culture supernatant, purified on tartrate/glycerol gradients (Talbot & Almeida, 1977) and resuspended in 0.5 ml PBS. Virion membrane antigens were prepared from pelleted virions by resuspending in 1% Triton, incubating on ice for 20 min then centrifuging at 100 000 g for 2h at 1C to remove nucleocapsids. Rabbits were immunised with 0.5 ml doses of these preparations in Freund's complete adjuvant and boosted at three weekly intervals with the same preparation in Freund's incomplete adjuvant. Rabbits were bled periodically and serum was assayed for CMV-IgG.

CMV-specific IgG ELISA

Sera and plasmas were screened for CMV-specific IgG (CMV-IgG) by indirect ELISA. 96-well immunoplates (Nunclon) were coated overnight at 4C with GE test and control antigen diluted in coating buffer (1.50 g $Na_2CO_3 . 10 H_2O$, 2.93 g $Na HCO_3$, 0.2 g $Na N_3$ in 1l double distilled water, pH 9.6). All reagents were added in 300 µl volumes per well. An antigen dilution was chosen such that a reference positive control serum gave an ELISA reading within the range 0.90-1.00. Most batches of antigen were used at a dilution of 1:800-1:3200. Every third column of wells was coated with control antigen and the remainder were coated with test antigen. After coating plates were washed three times with washing fluid (PBS plus 0.1% bovine serum albumin (BSA;

Sigma), 0.05% Tween 20 (Biorad) and 0.02% Na N_3) using an automated plate washer (Organon-Teknika). Serum was diluted 1:100 in PBS containing 1% BSA and 0.05% Tween 20 (PBSAT). Diluted serum was added to triplicate wells (two wells coated with test antigen and one coated with control antigen). A control positive and negative serum was included on each plate. Plates were incubated for three hours at RT then washed as above. Alkaline phosphatase-conjugated swine anti-human IgG (Northumbria Biologicals Ltd.) diluted 1:400 in PBSAT was then added to each well and plates were incubated at RT for two hours, then washed as before. Alkaline phosphatase substrate solution was prepared by dissolving 5 mg substrate tablets (Sigma) in 10% diethanolamine-HCl buffer pH 9.8 to give a 1mg/ml solution. This was added to each well and plates were incubated at RT for 30 min. The enzyme reaction was stopped by adding 50 µl 3M NaOH to each well. Optical density (OD) measurements at 405 nm were determined for each well using an automated spectro-photometer (Dynatech MR 580). Sera were considered to be CMV-IgG positive when the mean OD reading of the two test antigen wells was greater than two standard deviations above the mean OD reading of test antigen wells of the negative control serum, unless nonspecificity was indicated by an equivalent OD reading in the control antigen well.

A similar protocol was used for testing rabbit antisera and McAb preparations for ELISA reactivity. A range of dilutions of antiserum, ascitic fluid, hybridoma culture supernatant or sodium sulphate-precipitated IgG were reacted with antigen-coated plates. Alkaline phosphatase-conjugated

goat anti-rabbit IgG (Sigma) or rabbit anti-mouse IgG (Miles) diluted 1:400 were then added as appropriate. The assay was the same in other respects.

IgM capture ELISA (MACELISA)

Biotin labelled McAbs were prepared according to Guedson et al. (1979). Sodium sulphate-precipitated IgG was mixed with varying amounts of 0.1M (34.1 mg/ml) N-hydroxysuccinimido (NHS)-biotin (Sigma) dissolved in dry dimethyl formamide (Pierce; silylation grade) such that the ratio of NHS-biotin : free IgG amino groups was either 1:1 or 4:1, based on a figure of 90 free amino groups per IgG molecule at pH values over 8.5 (Habeeb, 1966). The reaction was allowed to proceed for 1h at RT. The mixture was then made up to 0.5 ml with PBS and dialysed overnight at 4C against three changes of PBS. An equal volume of glycerol was then added and the label was stored at -20C. Biotinylation was found to proceed efficiently irrespective of the original IgG concentration or the volume of the reaction mixture. Biotin labels were assayed for CMV reactivity using a modification of the indirect ELISA in which 200 µl rather than 300 µl volumes of all reactants were used. Dilutions of biotinylated McAb were used in the first incubation and a 1:1600 dilution of avidin-peroxidase conjugate (Sigma) was used in the second incubation. The peroxidase substrate solution consisted of 0.4 mg/ml o-phenylenediamine (Sigma) in phosphate citrate buffer (22.5 g Na_2HPO_4 and 5.6 g citric acid in 1l distilled water, pH 6) with 0.2 µl/ml 30% hydrogen peroxide added

immediately prior to use. The substrate reaction was stopped with 100 µl 4M H_2SO_4 per well after 30 min. OD values were determined at 490 nm.

In the MACELISA 300 µl volumes of all reactants were added to each well when using McAbs or rabbit antisera as detector antibody, and in 200 µl volumes per well when using biotinylated McAbs as detector antibody.

96-well immunoplates were coated with various dilutions of a 1 mg/ml stock solution of affinity-purified goat anti-human µ chain antibody (TAGO, Burlingame, USA). Plates were coated overnight at RT and then washed three times as described for indirect ELISA. Triplicate wells were then treated with dilutions of test serum in PBSAT and incubated at 37C for 3h. After washing, test and control antigens, diluted in PBS plus 0.05% Tween 20 (PBST) and 5% CMV-seronegative human serum were added; test antigen to two of each triplicate set of wells and control antigen to the third well. Plates were incubated overnight at 4C then washed five times. To detect IgM-bound CMV antigen wells were then treated with dilutions of McAb preparations, rabbit antiserum or biotinylated McAb in PBSAT, incubated for 3h at RT and washed three times. The appropriate enzyme conjugate was then added to each well at the same respective dilutions as used above. Plates were incubated a further 2h and washed three times. Bound conjugate was detected with the appropriate substrate as detailed above. Modifications to this generalised protocol were introduced as described under Results.

The VirEnz M - CMV kit (Northumbria Biologicals Ltd.) was used according to the manufacturer's instructions. Test and control sera were diluted 1:50 in PBST and 100 µl volumes were added in duplicate to anti-human µ chain antibody coated wells. Plates were incubated for 1h at RT and then washed three times with rinse solution. Wells were then incubated overnight at RT with 100 µl diluted test antigen or antigen diluent (control), then washed as above. 100 µl peroxidase conjugated McAb was added to each well and incubated at RT for 3h. Plates were then washed and treated with 100 µl tetramethyl benzidine substrate solution for 30 min at RT. The enzyme reaction was stopped with 100 µl 5N H_2SO_4 and OD values were determined at 450 nm. Specimens which gave net OD readings (test antigen OD minus antigen diluent OD) greater than that of the weak positive control were considered positve. Those that gave net OD readings between 70% and 99% of that of the weak positive control were considered as doubtful positives. Other specimens were considered negative.

Radio-iodination of antibody preparations

a) Chloramine T method

20 µl of a 1 mg/ml solution of sodium sulphate-precipitated or affinity purified IgG was drawn into the tip of a glass pipette. 20 µl of a 2.5 mg/ml solution of chloramine T in double strength PBS was then drawn into the same pipette, leaving an air space between this solution and the IgG solution. The contents of the pipette were then expelled into a glass tube containing 200 µCi Na [^{125}I]

(Amersham). The mixture was incubated for 30s on ice then the reaction was stopped by adding 20 µl of a 6 mg/ml solution of sodium metabisulphite in PBS. After 2-3 min 0.5 ml carrier buffer (DM plus 0.5% BSA and 0.02% Na N_3) was added. The iodinated protein was separated from unbound isotope by fractionation on a 30 cm x 7mm diameter column of Sephadex G100 (Pharmacia) equilibrated with carrier buffer. 30x1 ml fractions were collected. 10 µl of each fraction was counted for 60s in an LKB Wallac 80 000 gamma counter. Fractions containing the first peak of radioactivity were pooled and stored at 4C for use in RIA. The percentage incorporation of iodine into antibody was estimated by comparing the sum of cpm of fractions containing the first peak of (incorporated) radioactivity with that of the second peak of (unincorporated) radioactivity.

b) Iodogen method

50 µl of a 1 mg/ml solution of sodium sulphate-precipitated or affinity purified IgG was mixed with 1 mCi Na [^{125}I] in an Iodogen-coated tube. These tubes were gifted by Dr. R.S. Tedder, Middlesex Hospital and were prepared by coating glass tubes with 50 µl of a 40 µg/ml solution of Iodogen (Pierce) in dichloromethane and drying at 50C in nitrogen. Tubes were stored at 4C. The reaction was allowed to proceed for 30 min at RT and was stopped by aspirating the reaction mixture from the Iodogen tube. Radiolabelled preparations were fractionated as described above.

Indirect RIA for CMV-IgM

The method of Kangro (1980) was used, except that radiolabel was prepared by the Iodogen method. PVC U-well microtitre plates (Dynatech) were coated with dilutions of GE test and control antigen in PBS. 50 µl volumes were dessicated on to the wells under a cold air stream and fixed with 10% formalin in PBS (pH 6.2), 150 µl/well for 20 min at RT. Plates were washed once with 0.2% gelatin in PBS, 200 µl/well and blocked overnight at 4C with 5% normal rabbit serum (NRS; Scottish Antibody Production Unit [SAPU]) in PBS, 100 µl/well. The blocking solution was then shaken off and plates were drained of excess moisture before use. 1:400 and 1:1600 dilutions of test and control sera in PBS plus 5% NRS and 1% Dextran T10 (Pharmacia) were tested on duplicate test and control antigen-coated wells. 50 µl of diluted serum was added to each well and plates were incubated for 3h at 37C. Some test and control antigen-coated wells were treated with serum diluent only, as controls. Plates were then washed three times with 0.2% gelatin and 50 µl of iodinated affinity-purified goat anti-human µ chain antibody was added to each well. Radiolabel was used four days to three weeks after preparation. Label diluent consisted of EMEM plus 3% NBCS, 0.5% lactalbumin hydrolysate (Sigma) and 10% NRS, adjusted to pH 7.2 with 8% Na HCO_3. Label was added to give 30 000 cpm per 50 µl. Plates were incubated for 2h at 37C, washed ten times with tap water and dried. Individual wells were cut from the plate and bound radioactivity was counted for 60s. Results were calculated by dividing the mean cpm of

test antigen wells treated with test or control serum by the mean cpm of test antigen wells treated with serum diluent, and repeatimg this for control antigen wells. The two values thus obtained were expressed as a ratio, with the test antigen value as the numerator (the specific binding index [SBI]). Sera which gave SBI values equal to or greater than two were considered positive.

All sera giving positive results were absorbed against IgG-coated latex to remove any false reactivity due to rheumatoid factor, and retested. To prepare IgG-coated latex, 0.5 ml of a 13% w/v suspension of latex beads (Yarsley Research Laboratories, The Street, Ashstead, Surrey) was added to 1.5 ml distilled water and filtered through ashless filter paper (Whatman No. 40). 0.5 ml filtered latex was added dropwise to 27.5 ml glycine buffer, pH 8.2 (5 g Na Cl, 3.7 g glycine and 1.2 g $Na\ N_3$ in 500 ml distilled water). 3 mg normal human IgG in the form of a 150 mg/ml solution (Scottish National Blood Transfusion Service) was then added dropwise to the latex suspension with constant agitation and the resulting suspension was agitated at RT for 20 min then centrifuged at 3500 rpm in a bench centrifuge for 20 min at 4C. The latex pellet was resuspended in 0.75 ml PBS and stored at 4C. To absorb test sera, 10 µl test serum was mixed with 25 µl IgG-coated latex and serum diluent was added to give the required test dilution. Sera were incubated at 37C for 1h and centrifuged as above. The supernantant was used for testing. A false positive-generating rheumatoid factor positive control serum was included in each series of

absorptions. The preparation of IgG-coated latex and the absorptions were all carried out in plastic containers.

Initial studies indicated that an antigen dilution of 1:20 was sufficient to give positive SBI values with all positive control sera. Higher dilutions than this often resulted in lower binding of radiolabel. This was probably due to overloading of the wells, resulting in uncoating of solid phase-bound antigen during the test serum incubation and competition between bound and unbound antigen for IgM.

Indirect RIA with monoclonal antibodies

This assay was a modification of the indirect RIA for CMV-IgM. Test serum dilutions were replaced with dilutions of McAb preparations and the radiolabel was rabbit anti-mouse IgG (Miles) labelled by the Iodogen method. Serum diluent control wells were omitted in this assay and results were calculated by dividing the mean cpm of test antigen-coated wells by that of control antigen-coated wells.

IgM antibody capture RIA (MACRIA)

Anti-human μ chain antibody-coated etched polystyrene beads (Northumbria Biologicals Ltd.) were incubated with test serum dilutions in PBSAT for 3h at 37C. Dilutions of serum and all other reactants were added in 200 μl volumes. Beads were then washed three times with PBST using a Pentawash bead washing device (Abbot). Beads were then incubated overnight at 4C with dilutions of GE test and control antigen in PBST. Beads were washed five times as above and then incubated for

2h at 37C with radiolabelled McAb diluted in PBSAT plus 15% NBCS and 5% CMV-seronegative human serum. Beads were then washed five times and bound radioactivity was counted for 60s. Modifications to this basic procedure were used as described under Results.

Indirect immunofluorescence for CMV-IgM (CMV-IgM IIF)

Slides for IIF were prepared from HEF's infected with CMV as described previously. When infected cells showed 100% CPE, but before the monolayer had collapsed, infected and control cells were harvested by brief trypsinisation. An equal volume of NBCS was then added to quench trypsin activity. Cells were washed three times in PBS then resuspended in PBS at a concentration of 10^6 cells/ml. 10 µl aliquots were placed on the wells of PTFE-coated multispot slides (Hendley Essex; 6 mm diameter wells) such that each slide consisted of a row of infected cell spots opposite a row of control cell spots. Slides were dried at RT in a moving air stream, fixed in acetone for 5 min then stored at -20C.

In the test, sera were diluted 1:10 in DM plus 2% NBCS. 40 µl was spotted on to an infected cell and a control cell spot. Slides were incubated in a humid atmosphere for 3h at 37C. Excess serum dilution was then shaken off and the slides were washed twice, each time for 10 min, in PBS stirred continuously by a magnetic stirrer. Slides were then allowed to dry at room temperature before applying 30 µl fluorescein isothiocyanate (FITC)-conjugated sheep anti-human IgM (SAPU)

diluted 1:20 in DM plus 2% NBCS. Slides were incubated as above but for 1h only, then washed and dried as above. Slides were mounted in 33% glycerol in 0.1M Na HCO_3 (pH 8.2) and examined at a magnification of 400 X with a Leitz Ortholux ultraviolet microscope. IgM-positive sera resulted in diffuse membrane and cytoplasmic fluorescence in infected cells only. All positive sera were retested after absorption with IgG-coated latex or sucrose density centrifugation. Only sera which remained positive after either of these steps were considered IgM positive.

Sucrose density gradient centrifugation was performed on 5 ml preformed and equilibrated 12.5-37% sucrose gradients. 0.9 ml volumes of 37%, 31%, 25%, 18.75% and 12.5% sucrose in DM plus 0.02% Na N_3 were layered successively into 2.5 in x 0.5 in diameter polyallomer centrifuge tubes (Beckman) and allowed to stand for at least 4h at 4C before use. 150 µl test serum mixed with an equal volume of DM was then layered on top of the gradient. Gradients were centrifuged at 35 000 rpm for 18h at 4C in a Beckman L8-55 ultracentrifuge using an SW 50 rotor. 0.4 ml fractions were collected from the bottom of the gradient by piercing the base of the tube and fractions were tested for the presence of IgG and IgM by Ouchterlony gel diffusion in 0.9% agarose (Miles) against sheep anti-human IgG and IgM (SAPU). IgM containing fractions (usually fractions 2-5) were tested by IIF.

Indirect immunofluorescence for IgG to CMV early immediate early antigens (CMV IEA-IgG IIF)

Cells were infected with CMV as described previously. 1h after the replacement of culture medium following the adsorption period, infected and control cells were harvested and used to make CMV IEA slides for IIF in an identical manner to that described above for CMV-IgM IIF. In the test, sera were diluted 1:10, spotted on to prepared slides and incubated as in the CMV-IgM IIF, but for 45 min only. Slides were then washed, dried and treated with 30 µl FITC-conjugated sheep anti-human IgG (SAPU) diluted 1:80 in DM plus 2% NBCS. Slides were incubated a further 45 min as above, then were washed and prepared for examination in the same way as in the CMV-IgM IIF. Slides were examined at a magnification of 630 X. This facilitated detection of CMV IEA fluorescence which was present as discrete intranuclear inclusion fluorescence (Plate 1) and/or fine speckled cytoplasmic fluorescence which did not give good photographic reproduction.

Indirect immunofluorescence for IgG to CMV early antigens (CMV EA-IgG IIF)

HEF's were infected with CMV as described previously. After the absorption period, infected and control cell cultures were incubated for three days in MM containing 80 µg/ml cytosine arabinoside (Ara C; Sigma), then harvested as described above. The preparation of slides and the IIF procedure was the same as described for CMV IEA-IgG IIF,

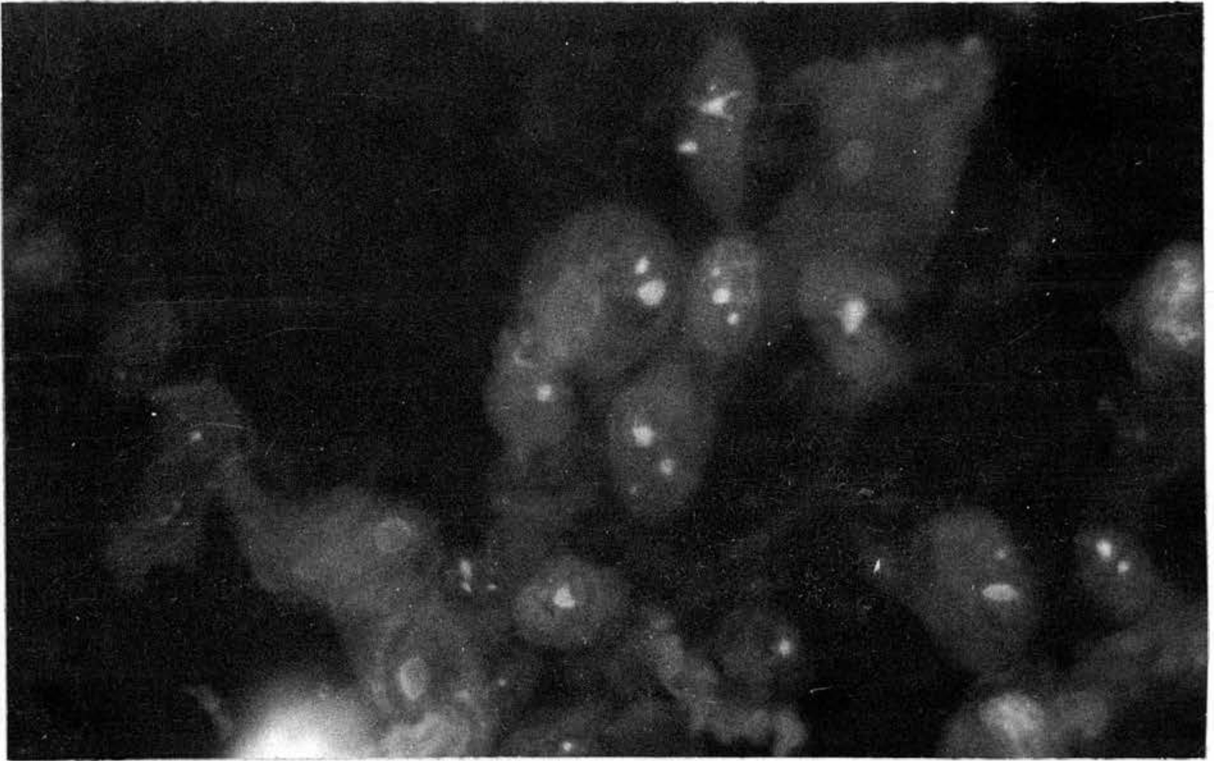


Plate 1: CMV immediate early antigens stained by indirect immunofluorescence with monoclonal antibody 96D3.

except that sera were tested at dilutions of 1:16 and 1:32. CMV EA fluorescence was present as discrete inclusion fluorescence (Plate 2) and/or fine speckled nuclear fluorescence which did not give good photographic reproduction.

Indirect immunofluorescence with McAbs

Slides prepared for the detection of CMV-IgM, CMV IEA-IgG and CMV EA-IgG were also used in the characterisation of McAbs. Incubation conditions used were as described for the CMV IEA-IIF. A range of McAb dilutions were used in the first incubation. FITC-conjugated rabbit anti-mouse IgG (Miles) diluted 1:100 in DM plus 2% NBCS was used in the second incubation. McAbs characterised by Dr. Hart were also tested on slides of unfixed cells.

Indirect immunofluorescence for EBV-IgM and EBV-IgG

Slides of acetone-fixed EB3 cells were gifted by Dr. E. Edmond, Bacteriology Department, Edinburgh University. The IIF procedures used were the same as IgM and IgG IIF procedures described above, using 1:10 dilutions of test sera. EBV-IgM and -IgG fluorescence was present as diffuse membrane and cytoplasmic fluorescence.

Indirect immunofluorescence for HTLV III-IgG (HTLV III-IgG IIF)

Multispot slides (8 mm spots) of acetone-fixed HTLV III-infected H9 cells were gifted by Dr. J.F. Peutherer. The IIF

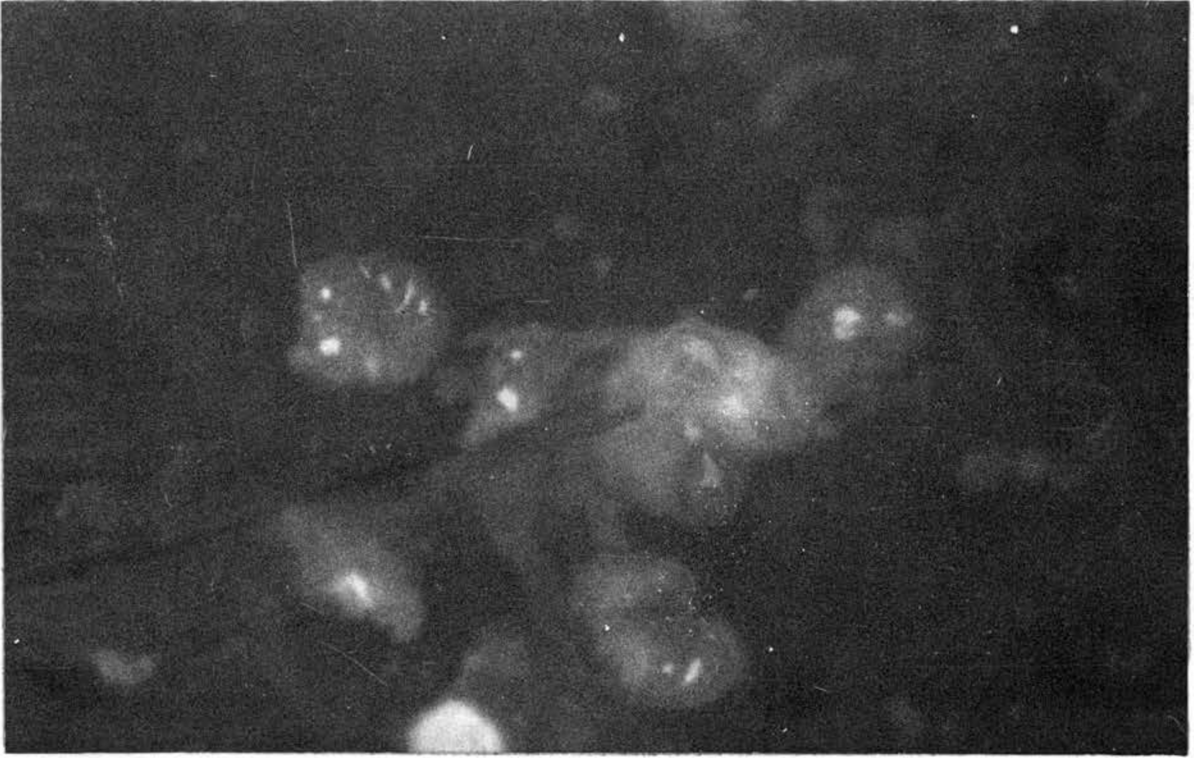


Plate 2: CMV early antigens stained by indirect immunofluorescence with plasma from laboratory personnel WN.

procedure was similar to that of other IgG IIF assays described above. 100 µl of test serum diluted 1:10 was used in the first incubation and 50 µl of conjugate dilution was used in the second incubation. IgG positive specimens resulted in diffuse membrane fluorescence.

Indirect ELISA for HTLV III-IgG (HTLV III-IgG ELISA)

The Abbott HTLV III EIA kit was used according to the manufacturer's instructions. Antigen-coated beads were incubated with 210 µl test or control serum diluted 1:440 in specimen diluent for 1h at 40C. Beads were then washed three times with distilled water and incubated with 200 µl anti-human IgG peroxidase conjugate preparation for 2h at 40C. Beads were washed as above and transferred to assay tubes. 300 µl o-phenylene diamime substrate solution was then dispensed into two empty assay tubes and to each bead-containing tube. Tubes were incubated at RT for 30 min before stopping the enzyme reaction with 1 ml 0.5M H_2SO_4. OD values at 492 nm were determined in a dual wavelength analyser (Abbott) using one of the control assay tubes as a blank. The cutoff was determined as the mean OD value of two negative control specimens plus one tenth of the mean OD value of three positive control specimens. The results were only accepted if the difference between positive and negative control mean OD values was equal to or greater than 0.4.

Western blot immunostaining for HTLV III-IgG

Polyacrylamide gel electrophoresis and electrophoretic

transfer of HTLV III polypeptides on to nitrocellulose was performed by Dr. P. Simmonds, Bacteriology Department, Edinburgh University.

Sodium dodecyl sulphate (SDS)-solublised HTLV III proteins were separated by discontinuous polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (1970). A 12% acrylamide separating gel was prepared by mixing 20.25 ml stock acrylamide solution (26.65% acrylamide [BDH] with 0.72% N, N'-methylene bisacrylamide [acrylamide cross-linking agent; BDH] in double distilled water) with 22.5 ml separating gel buffer (0.75M Tris-HCl, pH 8.8 plus 0.2% SDS [BDH]) and degassing the mixture under vacuum for 10 min. A 15 mg/ml solution of ammonium persulphate (BDH) was prepared freshly and 2.25 ml of this plus 62.5 µl NNN "N" tetramethylene diamine (TEMED; BDH) were then added to the acrylamide solution (polymerisation catalysts). A vertical slab gel was prepared by pouring this solution between two glass plates, one of which was 20 x 21 cm and the other 20 x 23 cm. The glass plates were separated by 2 mm spacers on three sides, held together with bulldog clips and sealed with molten Vaseline. The gel was covered with a layer of butanol and allowed to polymerise for 45 min.

A 5.3% acrylamide stacking gel was prepared by mixing 2 ml stock acrylamide solution with 5 ml stacking gel buffer (0.25M Tris-HCl, pH 6.8 plus 0.2% SDS) and 2.5 ml distilled water, and degassing as above. 0.5 ml ammonium persulphate solution and 20 µl TEMED were then added. The butanol layer was aspirated from the top of the polymerised separating gel

and the stacking gel was layered on top to give a depth of 1 cm, layered over with butanol and allowed to polymerise for 45 min. The butanol layer was then aspirated, the bottom spacer removed and the gel was mounted with bulldog clips in a homemade perspex gel tank with platinum electrodes. The bottom reservoir was filled with 200 ml electrode buffer (0.025M Tris plus 0.192M glycine and 0.1% SDS, pH 8.3), excluding air spaces from the bottom of the gel.

The antigen preparation was a 1% Triton extract of HTLV III-infected H9 cells containing 1 mg/ml protein and was gifted by Dr. Peutherer. The antigen was prepared for PAGE by boiling a 200 µl sample mixed with 200 µl sample buffer (0.125M Tris-HCl, pH 6.8 plus 4% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.002 % bromophenol blue) for 4 min. The sample was layered evenly on top of the stacking gel and the upper reservoir was filled with 200 ml electrode buffer. The tank was connected to a VOKAM V500-500 power pack (lower reservoir to positive terminal). The gel and gel tank, prepared for electrophoresis, are illustrated in Figure 1. The gel was subjected to 60V at 10mA for approximately 1.5h (until the dye front reached the stacking gel/separating gel interface), and then to 160V at 25mA for approximately 4h (until the dye front had travelled 10 cm through the separating gel. The power was disconnected and the gel removed from the tank and from between the glass plates. The stacking gel and the remainder of the separating gel below the dye front were removed.

The electrophoretically separated polypeptides were blotted on to nitrocellulose according to the method of

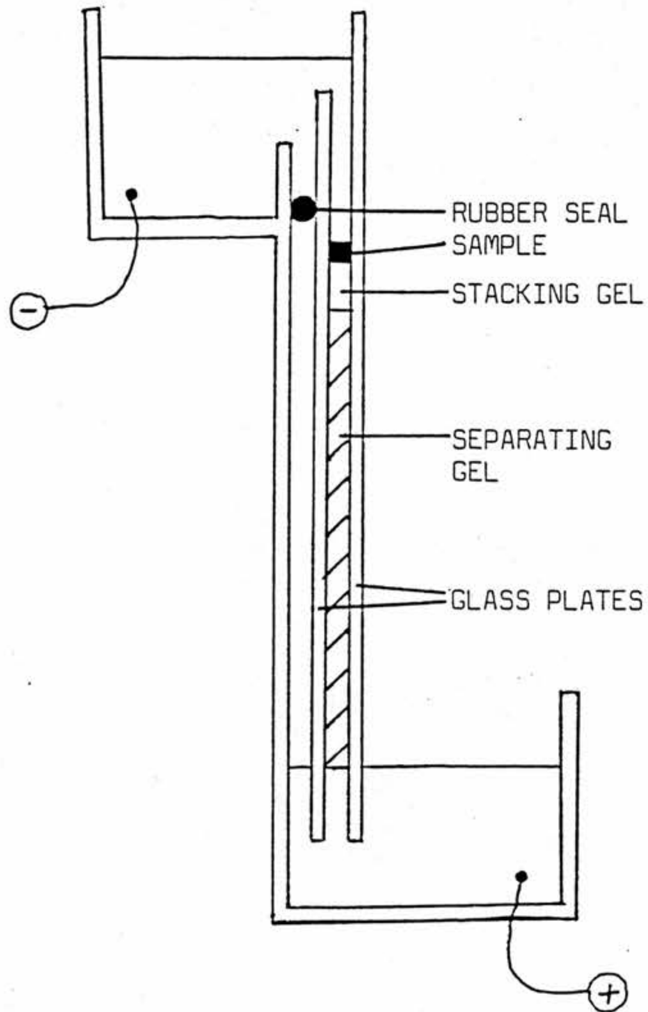


Figure 1: Cross-sectional view of gel tank used for polyacrylamide gel electrophoresis.

Towbin et al. (1979). A 16 x 10 cm sheet of nitrocellulose (0.45 µm pore size; Schleicher & Schull) was placed over the gel, excluding air bubbles. The gel and nitrocellulose were placed between two sheets of Whatman 3M filter paper and then between two sheets of Scotchbrite. This was placed in a notched perspex cassette and placed in a 20 x 15 x 15 cm homemade perspex blotting tank with stainless steel grid electrodes (20 x 15 cm) such that the nitrocellulose sheet was nearer the positive electrode. The tank was filled with 5 l 0.025M Tris buffer plus 0.192M glycine and 20% methanol, pH 8.3. The gel was blotted overnight at 4C under 22V at 40mA using a homemade 0-25V, 500mA power pack. The nitrocellulose was then removed and immersed in blocking buffer which consisted of Tris-buffered saline (TBS; 20mM Tris, 0.5M NaCl, brought to pH 7.5 with 1M HCl) plus 3% gelatin (Sigma). The nitrocellulose was incubated in blocking buffer for 30 min at RT with agitation, then drained and stored at -20C.

For immunostaining of PAGE-separated, nitrocellulose-bound antigens, 3mm strips were cut from the nitrocellulose sheet and used as the solid phase in an indirect ELISA. Strips were incubated in stoppered test tubes containing 2 ml test or control serum diluted 1:50 in TBS plus 0.025% Tween 20 (TBST) and 1% gelatin. Test tubes were incubated for 2h at RT with agitation. Strips were then rinsed with distilled water and washed twice with TBST, each time for 5 min, with agitation. Strips were then treated with peroxidase-conjugated goat anti-human IgG (Sigma) diluted 1:250 in TBST plus 1% gelatin, 8 ml per test tube with up to seven strips per test tube, and incubated for 2h as above. Strips were

then rinsed and washed three times. To develop the strips 10 ml ice cold methanol containing 3 mg/ml 4-chloro-1-naphthol-containing substrate (Biorad) was mixed with 50 ml TBS containing 30 µl of 30% hydrogen peroxide immediately before use. Strips were incubated in this mixture for 20 min. The enzyme reaction was stopped by washing the strips several times in tap water. Strips were then dried and examined for staining of HTLV III antigens. Staining of the p25 and gp41 bands (Sarngadharan et al., 1984) was most commonly observed and sera which resulted in staining of either or both of these bands were considered positive.

Safety precautions

The risk of HTLV III infection to health care and laboratory personnel appears to be low (Hirsch et al., 1984). This is dependent on the maintainance of recognised safety regulations however. Moreover the risk of infection from laboratory specimens was unknown at the outset of this work. All specimens from homosexual and heterosexual volunteers were therefore handled as recommended by the National Insitutes of Health (1982). Disposable gloves were worn when handling specimens and all manipulations were performed in still-air cabinets, except the harvesting of lymphocyte cultures. The cell harvester, centrifuge buckets and all other laboratory apparatus used was rinsed with activated glutaraldehyde solution after use, which has been shown to inactive HTLV III efficiently (Spire et al.,1984). All sera were inactivated at 56C for 30 min when it became known that

this too eliminated infectivity. Disposable plastics were used for all manipulations and containments. These were disposed of by chloros treatment followed by autoclaving. These precautions were approved by Edinburgh University Safety Committee.

Statistical analysis

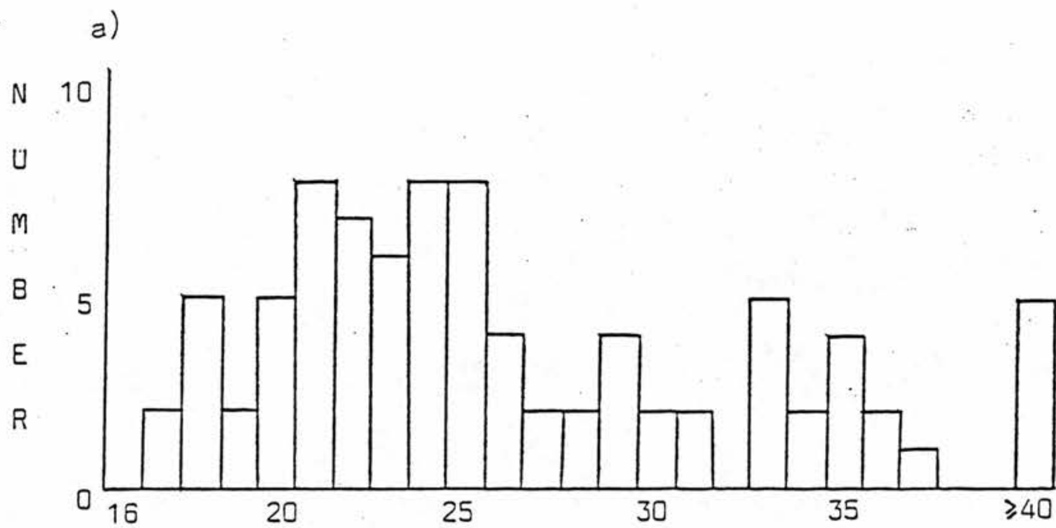
Population means were compared using the simple significance test based on the normal distribution when numbers were sufficient (both populations >30). Student's *t*-test was used in other cases. Differences in the frequency of a given event between populations were compared using the χ^2 formula with Yates correction when numbers were sufficient (when the occurrence of each possible event was >4). In other cases the exact test for 2 x 2 contingency tables was used. Probability (P) values $\leqslant 0.05$ were considered to be statistically significant.

A summary of demographic features and results of virological and serological investigation in homosexuals and heterosexuals is given in the Appendix.

Demographic features of study populations

Specimens were received from a total of 84 male homosexuals and from 140 male heterosexuals. Information regarding age, sexual preference and any current infections was obtained for all volunteers. Information on the reported number of sexual partners in the previous three months was also available for most volunteers. Information on the reported duration of sexual activity was available for most homosexuals but only for a limited number of heterosexuals.

The age distribution of these two study populations is shown in Figure 2. The mean age of the homosexuals was 26.2 years, and the age range was 17-45. The mean age of the heterosexuals was 22.4 years with an age range of 16-48. The difference in mean age was highly significant ($p < 0.001$). Homosexuals also reported longer histories of sexual activity and greater numbers of sexual partners than heterosexuals. The former reported a mean duration of sexual activity of 7.6 years and a mean partner number of 2.9 in the previous three months. For the heterosexual group the mean duration of sexual activity was 3.2 years and the mean partner number was 1.25 ($p < 0.001$ for both differences). 73% of homosexuals and only 23% of heterosexuals reported more than one sexual



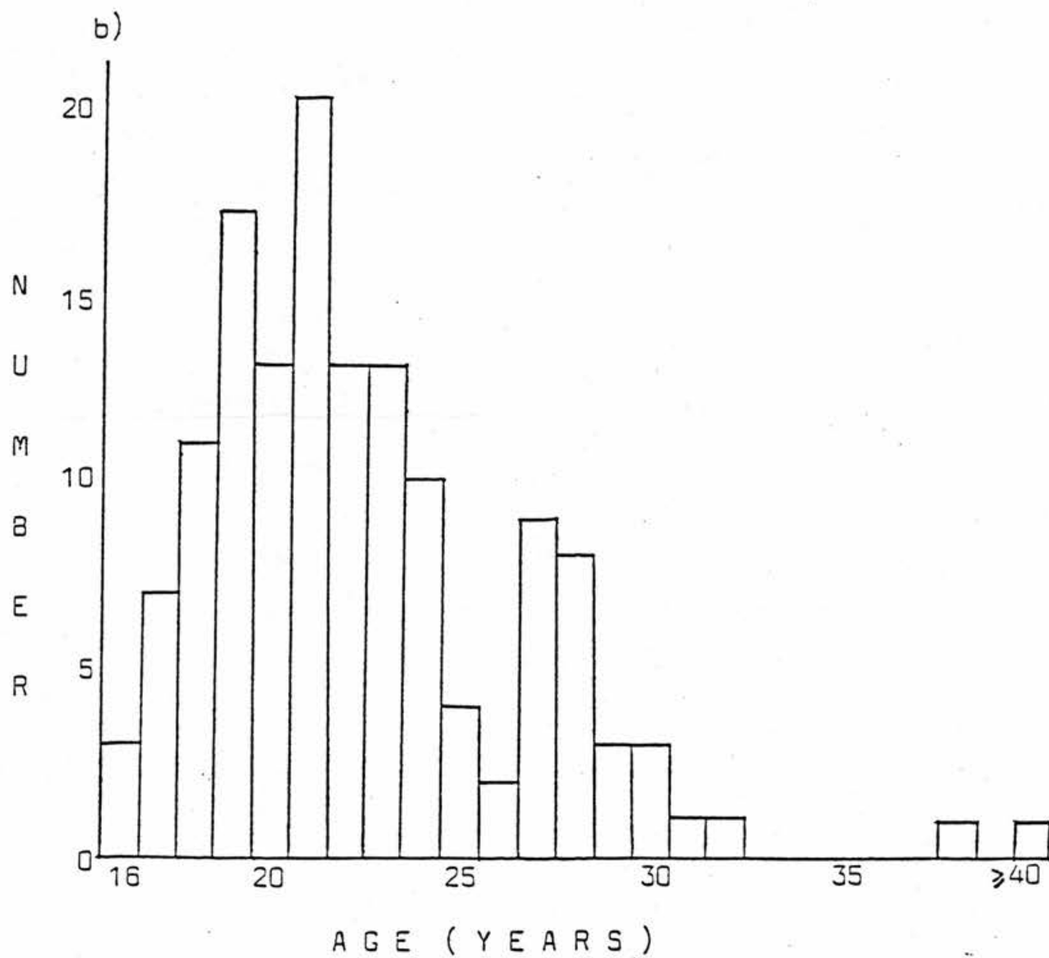


FIGURE 2: AGE DISTRIBUTION OF a) HOMOSEXUAL AND b) HETEROSEXUAL VOLUNTEERS UNDER STUDY.

partner in the previous quarter ($p < 0.001$).

Current infection was diagnosed in 55% of the homosexuals and in 64% of the heterosexuals (not significant [NS]). Infections most commonly diagnosed were gonorrhoea (in 15% of homosexuals and 20% of heterosexuals), non-gonococcal urethritis (NGU; in 11% of homosexuals and 24% of heterosexuals), and warts (in 13% of homosexuals and 17% of heterosexuals). Only the difference in incidence of NGU between the two study groups was significant ($p < 0.05$). Gonococcal infections were predominantly rectal in homosexuals and predominantly urethral in heterosexuals. Warts were frequently anal in homosexuals and usually penile in heterosexuals. Infections other than these were diagnosed more commonly in homosexuals (15% compared to 4%; $p < 0.01$) and included HSV, scabies, giardiasis, amoebiasis, molluscum contagiosum, enterobiasis and chlamydial infection. Rectal or anal infections (gonorrhoea, warts or HSV) were diagnosed in 19% of homosexuals.

CMV-IgG antibody prevalence in study groups

Antibody to CMV was detected by indirect ELISA in 63/84 homosexuals (75%) and in 36/140 heterosexuals (26%). This difference is extremely significant ($p << 0.001$; $\chi^2 = 49.7$). To account for the possible influence of age on this difference, age-matched sub-groups of the two populations were compared for the prevalence of CMV-IgG (Table 1). The rate of seropositivity among homosexuals under 26 years of age is significantly higher than that of age-matched

Age (years)	Number seropositive / Total number (%)		p value
	Homosexuals	Heterosexuals	
16-19	6/9 (73%)	5/38 (13%)	p = 0.002
20-22	16/20 (80%)	13/46 (28%)	p = 0.0001
23-25	15/21 (71%)	4/23 (17%)	p = 0.0001
>25	32/44 (73%)	20/36 (56%)	NS

Table 1: The CMV-IgG antibody prevalence of male homosexuals and heterosexuals of different ages.

heterosexuals. In older age groups the prevalence of antibody in heterosexuals approaches that of homosexuals.

Comparison of sub-groups of the two populations after matching for the number of recent sexual partners reported similarly reveals a greater antibody prevalence among homosexuals in both sub-groups where numbers were sufficient for comparison (Table 2).

Finally, comparison of subgroups matched for reported duration of sexual activity indicates that significantly more homosexuals with less than five years of sexual experience are seropositve than are heterosexuals with a similar history of sexual activity. Heterosexuals with longer sexual experience than this however are seropositive as frequently as homosexuals (Table 3).

Within the homosexual cohort antibody was detected in 22/29 (76%) of those under 23 years old, in 19/29 (66%) of those from 23-28 years old and in 22/26 (85%) of those over 28 years old. None of these differences were statistically significant. Of those reporting only one recent partner, 14/22 (64%) were seropositive, compared to 48/60 (80%) of those who reported more than one recent partner and 20/23 (87%) of those who reported more than two recent partners. None of these differences acheived statistical significance. A small but significant difference in antibody prevalence was observed in homosexuals with a history of sexual activity greater than seven years compared to other homosexuals (29/33 [88%] compared to 29/43 [67%] respectively; $p = 0.026$) however no other subdivision of this group with respect to sexual

No. of Partners (last 3 mo.)	Number seropositive / Total (%)		p value
	Homosexuals	Heterosexuals	
1	14/22 (64%)	26/104 (25%)	$p < 0.01$
2	28/37 (76%)	7/30 (23%)	$p < 0.001$

Table 2: The CMV-IgG antibody prevalence of male homosexuals and heterosexuals with different numbers of recent partners.

Sexual history (years)	Number seropositive / Total (%)		p value
	Homosexuals	Heterosexuals	
<3	8/11 (73%)	3/23 (13%)	$p = 10^{-19}$
3-4	13/19 (68%)	2/12 (17%)	$p = 0.006$
>4	37/46 (80%)	4/5 (80%)	NS

Table 3: The CMV-IgG antibody prevalence of male homosexuals and heterosexuals of different sexual histories.

history revealed significant differences.

Within the heterosexual cohort, 19/97 (20%) of those under 24 years of age, 9/33 (27%) of those between 24 and 28 years old inclusive, and 8/10 (80%) of those over 28 years old were seropositive. Antibody prevalence was significantly higher in the oldest age group (p = 0.0003). Antibody was present in 26/104 (25%) of those who reported only one recent partner and in the same proportion (8/32) of those reporting more partners. Those who had been sexually active for longer than four years were seropositive significantly more frequently than those with shorter a history of sexual activity (4/5 [80%] compared to 5/35 [14%]; p = 0.006).

Virus excretion in study populations

Results of virus excretion were obtained for urine specimens from 48 homosexuals and from 91 heterosexuals. Specimens from the remaining volunteers were either not obtained or were unsuitable for virus isolation due to microbial contamination or cytotoxicity in cell culture. Throat swab specimens from 62 homosexuals and 114 heterosexuals were also successfully cultured however CMV was isolated from these specimens in only one case (homosexual volunteer P7473). The corresponding urine specimen from this volunteer also yielded CMV, therefore only urinary virus excretion is considered in this analysis. Viruria was detected in 7/48 (15%) of homosexuals and in 1/91 (1%) of heterosexuals. This difference was significant (p = 0.002) however when specimens from only those who were seropositive were included in the

analysis, the difference was not significant, probably due to lack of numbers. (7/37 [19%] of seropositive homosexuals and 1/20 [5%] of seropositive heterosexuals were viruric; $p = 0.125$.) No CMV excretion in seronegative individuals was observed.

Within the homosexual cohort viruria was predominantly confined to those under 22 years old (6/12 [50%] compared to 1/35 [3%] of the older age group; $p = 0.0005$) and was confined entirely to those under 26 years old (7/28 [25%] compared to 0/19; $p = 0.019$). Viruria was also confined to those with a history of sexual activity of less than eight years (7/23 [30%] compared to 0/19 of those with a longer history; $p = 0.009$). Viruria was highly prevalent in those with a history of sexual activity of one year or less; three of five were excreting, a significantly greater proportion compaired to the remainder of the cohort (4/36 [11%]; $p = 0.028$). The two virus negative specimens from this group were from seronegative individuals. Viruria was also confined to those who reported fewer than three recent sexual partners (7/24 [29%] compared to 0/8) however this association was not significant ($p = 0.105$). This possibly reflected an association between recent partner number and the duration of sexual activity.

Repeat isolation specimens were available for three of those who were viruric on initial testing. One homosexual (P7473) was still viruric (but was no longer excreting virus in saliva) three weeks after initial testing. Two homosexuals (P4331 and P7894) were no longer viruric nine and six weeks

respectively after initial testing.

CMV-IgM antibodies

a) Development of a CMV-IgM assay

A CMV-IgM capture assay was developed for the purpose of screening plasmas from the study populations for the presence of IgM antibodies. The usefulness of the assay as an aid to diagnosis of CMV infection in other groups was also assessed. The principle of the IgM-capture assay is illustrated in Figure 3. In developing a CMV-IgM capture assay, a range of reagents and methodologies were assessed. Radioimmunoassay (RIA) techniques were employed initially, using radiolabelled CMV-reactive murine monoclonal antibody (McAb) preparations as detector antibodies. Subsequently, enzyme-linked immunosorbent assay (ELISA) techniques were assessed with both McAbs and polyclonal antisera raised against CMV as detector antibodies. The development of the assay is described below.

IgM antibody capture by RIA (MACRIA)

McAb preparations were assessed for their reactivity with CMV GE antigen by testing twofold dilutions in an indirect RIA. The results are shown in Table 4. McAb preparations HCMV 1, HCMV 23 and 96D3 showed greatest reactivity. Of these HCMV 23 showed strongest reactivity on dilution and was selected for use in the MACRIA. Precipitated IgG was iodinated by the Iodogen method, giving a 90% incorporation of radioisotope. This label was diluted to give 40 000 cpm in 200 µl for use in the MACRIA. To determine

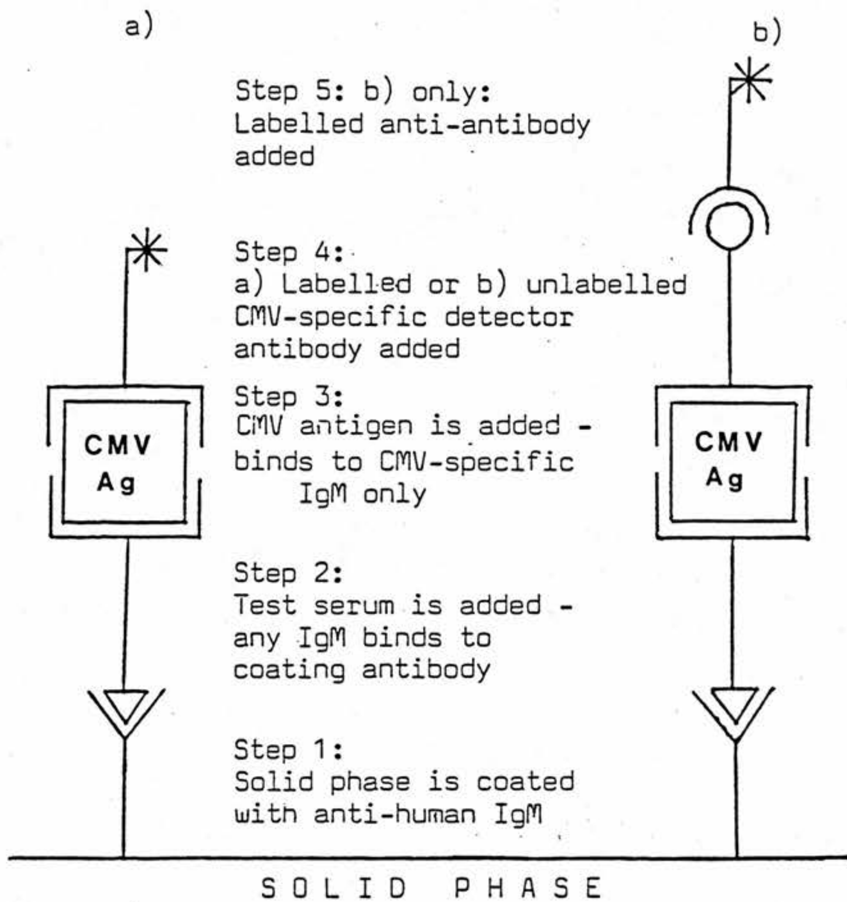


Figure 3: Principle of the IgM-capture assay using a) labelled or b) unlabelled detector antibody

McAb	Dilution of McAb					
	1:25	1:50	1:100	1:200	1:400	1:800
HCMV 1 (1)	14.7	6.2	3.4	2.3	NT	NT
HCMV 3 (2)	3.1	4.1	4.5	5.8	4.1	2.9
HCMV 23 (3)	8.8	11.5	8.9	11.1	10.9	12.3
HCMV 25 (3)	3.7	2.4	2.8	2.0	2.3	1.4
AB3 (1)	2.6	4.4	5.3	5.2	NT	NT
91D11 (1)	1.3	1.5	1.3	1.7	NT	NT
92E9 (1)	2.1	2.7	4.3	4.5	NT	NT
93F2 (1)	2.5	3.7	4.9	4.0	NT	NT
94H3 (1)	1.5	2.3	1.9	1.7	NT	NT
96D3 (1)	5.0	8.2	9.0	6.0	NT	NT

1; Hybridoma culture supernatant
2; Ascitic fluid
3; Sodium sulphate precipitated IgG
NT = not tested

Table 4: Positive : negative binding ratios of McAbs by indirect RIA

optimal reactant concentrations a range of dilutions of CMV-IgM positive and negative control sera were tested against a range of antigen dilutions by chessboard titration. The results are shown in Table 5. As the radiolabel gave no significant reaction with control antigen, it would appear that non-specific binding of test antigen to the solid phase resulted in high binding of radiolabel with both IgM positive and IgM negative sera. This non-specific binding of antigen was not mediated by non-specifically bound CMV-IgG as CMV-IgG negative sera gave similar results (not shown).

In an attempt to reduce this non-specific antigen binding, anti μ chain antibody-coated beads were quenched overnight at room temperature with 0.2% Tween 20 in PBS prior to use (Table 6). In addition, test serum diluent was modified to contain 2% CMV-seronegative human serum to reduce any nonspecific reaction of the radiolabel with test antigen (Table 7). Both these modifications resulted in some reduction of non - specific binding although the effect of the former was minor.

At this point it became necessary to prepare a fresh radiolabel. It was considered that a label containing a wider range of antigenic reactivities may be a more sensitive detector of bound antigen. A pool of equal concentrations of McAbs HCMV 1, HCMV 23 and HCMV 24 (also shown to be strongly reactive to CMV by Dr. Hart) was therefore radiolabelled. This radiolabel was prepared by Dr. Hart using the chloramine T method and was also used at a dilution of 40 000 cpm in 200 μl. This was used in a chessboard titration of varying test serum and antigen dilutions. This titration was designed to

Antigen dilution	a) CMV-IgM positive serum dilution				
	1:50	1:200	1:800	1:3200	1:12800
1:10	3.4	3.9	3.4	4.2	4.3
1:20	4.8	3.5	3.0	4.6	4.8
1:40	3.7	3.8	4.0	4.3	4.5
1:80	4.5	2.3	3.2	8.6	3.2
1:160	4.0	4.0	7.0	5.2	8.6

Antigen dilution	b) CMV-IgM negative serum dilution				
	1:50	1:200	1:800	1:3200	1:12800
1:10	2.1	2.2	3.0	1.1	4.9
1:20	3.6	1.9	5.0	3.1	3.9
1:40	9.0	2.8	4.4	2.7	4.9
1:80	3.5	3.9	5.2	5.1	5.4
1:160	3.9	5.1	7.6	5.3	4.2

Table 5: Positive : negative antigen ratios obtained by MACRIA on chess board titration of antigen and a) IgM positive control or b) IgM negative control serum

Control serum	Serum dilution	Antigen dilution	Positive:negative antigen ratio	
			Quenched beads	Unquenched beads
IgM	1:50	1:20	14.9	12.1
+ve	1:50	1:80	8.4	11.5
	1:200	1:20	14.1	12.0
	1:200	1:80	12.4	7.5
IgM	1:50	1:20	4.2	5.1
-ve	1:50	1:80	3.0	5.1
	1:200	1:20	6.0	8.4
	1:200	1:80	3.5	5.7

Table 6: The effect of pre-quenching anti-μ coated beads with 0.2% Tween 20 on the MACRIA reactivity of IgM+ve and -ve sera.

Control serum	Serum dilution	Antigen dilution	Positive:negative antigen ratio	
			Modified serum diluent	Unmodified serum diluent
IgM +ve	1:50	1:20	12.1	21.4
	1:50	1:80	5.3	5.0
	1:200	1:20	47.7	11.9
	1:200	1:80	4.0	13.7
IgM -ve	1:50	1:20	6.9	41.9
	1:50	1:80	7.0	11.3
	1:200	1:20	5.5	5.1
	1:200	1:80	8.5	15.4

Table 7: The effect of modifying serum diluent to contain 2% CMV-seronegative serum on the MACRIA reactivity of IgM+ve and -ve sera.

investigate the relative merits of an assay based on low serum and antigen dilutions, and an assay based on high dilutions of both serum and antigen. The effect of adding 20% NBCS to the antigen diluent as a more effective means of blocking non-specific antigen binding was also assessed (Table 8). From these results it is clear that the addition of NBCS to the antigen diluent had no discernable effect when reactants were used at low dilution and was too inhibitory at higher dilutions of reactants. The problem of non-specificity persisted. This experiment was repeated using intermediate dilutions of serum and antigen, with NBCS added to the antigen diluent (Table 9). This resulted in no improvement; reactivity decreased rapidly with increasing antigen dilution in the presence of NBCS.

An assay system using test serum diluted 1:50 and antigen diluted 1:10 was capable of distinguishing a panel of control IgM-positive sera from a panel of IgM-negative sera (Table 10). Test resolution was low however and reproducibility proved to be poor. In view of these shortcomings, an alternative assay system was sought.

IgM antibody capture by ELISA (MACELISA)

The McAb preparations used previously in RIA were assessed for their suitability as detector antibodies in a MACELISA. Further McAb preparations were also available for testing at this stage and two polyclonal rabbit antisera raised against CMV whole virions and virion envelopes were also tested. These antisera were shown to be CMV-reactive by indirect ELISA, although considerable reaction with control

Control serum	Serum dilution	Antigen dilution	NBCS added to antigen diluent	Positive:negative antigen ratio
IgM	1:50	1:10	yes	11.8
+ve	1:50	1:10	no	11.1
	1:1000	1:100	yes	3.4
	1:1000	1:100	no	NT
	1:10000	1:100	yes	2.8
	1:10000	1:100	no	9.9
	1:100000	1:100	yes	3.5
	1:100000	1:100	no	7.1
IgM	1:50	1:10	yes	2.4
-ve	1:50	1:10	no	2.8
	1:1000	1:100	yes	1.8
	1:1000	1:100	no	4.2
	1:10000	1:100	yes	1.3
	1:10000	1:100	no	3.1
	1:100000	1:100	yes	1.7
	1:100000	1:100	no	3.1

Table 8: Titration of IgM positive and negative control serum by MACRIA using pooled McAb radiolabel: investigation of the effect of addition of 20% NBCS to antigen diluent.

Control serum	Serum dilution	Antigen dilution			
		1:10	1:20	1:50	1:100
IgM +ve	1:50	15.9	7.3	4.4	3.5
	1:100	17.0	9.6	5.4	3.4
	1:500	21.0	8.8	5.4	3.0
	1:1000	14.1	9.1	6.1	3.4
IgM -ve	1:50	4.1	2.4	2.8	1.7
	1:100	4.9	3.7	1.5	1.6
	1:500	7.6	4.2	2.6	2.0
	1:1000	7.2	4.4	2.1	1.5

Table 9: Positive : negative antigen ratios obtained on chess-board titration of IgM positive and negative serum and antigen.

Positive : negative Ag ratios	
CMV-IgM +ve control sera	CMV-IgM -ve control sera
12.0	4.1
14.3	5.0
8.9	6.3
15.9	6.7

Table 10: MACRIA reactivity of a panel of CMV-IgM +ve and -ve sera.

antigen was observed at low dilution (Table 11). CMV-IgM positive and negative control sera were used at a dilution of 1:50 and reacted with microtitre plate wells coated with anti-human μ chain antibody at a 1:100 dilution of stock solution (1mg/ml protein concentration). CMV GE and also PEG precipitate antigen were used at a dilution of 1:100. (Only test antigens were used in this initial experiment.) Ascitic fluids and antisera were tested at three twofold dilutions and bound antibody was detected with the appropriate anti-species IgG enzyme conjugate. The results are shown in Table 12.

While some McAbs distinguished between the positive and negative control sera, the problem of high reactivity with IgM negative serum encountered in the MACRIA remained. Although this was also true to some extent of the rabbit antisera, this reactivity was greatly reduced at higher antiserum dilutions. Antiserum 39 in particular showed strong reactivity and good specificity and was selected for further experiments. PEG precipitate antigen was of inferior reactivity in the MACELISA and was no longer used.

This assay system was tested with a range of IgM positive and negative control sera. The rabbit antiserum was also tested at higher dilutions as was the coating antibody. (Table 13). It was decided to use the antiserum at a dilution of 1:1600 as this gave low readings with IgM negative sera but was still capable of identifying all IgM positive sera. A coating antibody dilution of 1:200 was chosen as this gave little difference in results compared to the lower dilutions but gave somewhat higher results than the higher dilution with

Antiserum dilution	OD (405 nm) readings		
	Test antigen (mean)	Control antigen	Net
Antiserum 37			
1:400	1.411	1.142	0.269
1:800	1.048	0.707	0.341
1:1600	0.805	0.441	0.364
1:3200	0.582	0.251	0.331
1:6400	0.409	0.140	0.269
1:12800	0.275	0.061	0.214
1:25600	0.160	0.029	0.131
1:51200	0.090	0.010	0.080
Antiserum 39			
1:400	>1.500	0.892	-
1:800	>1.500	0.511	-
1:1600	1.161	0.318	0.843
1:3200	0.878	0.164	0.714
1:6400	0.605	0.078	0.527
1:12800	0.403	0.029	0.374
1:25600	0.247	0.029	0.218
1:51200	0.144	0,004	0.140

Table 11: Reactivity of rabbit antisera 37 and 39 by indirect ELISA with CMV GE antigen.

Detector antibody	Dilution	GE Ag		PEG precipitate Ag	
		IgM +ve serum	IgM -ve serum	IgM +ve serum	IgM -ve serum
HCMV 1 (1)	1:50	1.027	0.917	0.902	0.876
	1:100	0.876	0.832	0.907	0.748
	1:200	1.157	0.895	0.910	0.845
HCMV 3 (2)	1:50	0.851	0.890	0.841	0.984
	1:100	1.002	1.087	1.047	0.935
	1:200	0.939	0.917	0.939	0.880
HCMV 19 (3)	1:1000	>1.5	1.157	0.914	0.848
	1:2000	>1.5	1.151	0.970	1.027
	1:4000	1.489	1.131	0.862	0.862
HCMV 22 (3)	1:50	>1.5	>1.5	1.318	1.445
	1:100	>1.5	>1.5	1.245	1.359
	1:200	1.395	1.398	1.111	1.131
HCMV 23 (3)	1:50	>1.5	1.369	1.234	1.252
	1:100	>1.5	1.359	1.245	1.359
	1:200	>1.5	1.268	1.179	1.027
HCMV 24 (1)	1:50	1.327	1.060	0.923	0.947
	1:100	1.336	1.145	0.960	0.855
	1:200	1.280	1.007	0.960	0.802
HCMV 25 (3)	1:50	1.350	1.038	0.978	1.043
	1:100	1.318	1.060	0.978	0.998
	1:200	1.268	0.984	0.926	0.779
HCMV 29 (3)	1:50	1.202	1.245	1.095	1.157
	1:100	1.106	1.031	1.047	0.956
	1:200	0.970	0.917	0.978	1.007
AB3 (2)	1:50	1.007	0.992	0.952	0.943
	1:100	1.111	0.926	0.966	0.956
	1:200	0.923	0.926	0.930	0.858

Table 12

Continued overleaf

Detector antibody	Dilution	GE Ag		PEG precipitate Ag	
		IgM +ve serum	IgM -ve serum	IgM +ve serum	IgM -ve serum
91D11 (2)	1:50	0.876	0.923	0.952	1.069
	1:100	1.043	1.131	0.974	0.984
	1:200	0.865	0.890	0.926	0.910
92E9 (2)	1:50	1.196	1.406	1.260	1.406
	1:100	1.151	1.209	1.173	1.350
	1:200	1.139	1.082	0.926	1.082
93F2 (2)	1:50	0.966	0.988	0.978	0.884
	1:100	1.082	0.984	1.027	0.930
	1:200	1.189	1.119	1.119	0.952
94H3 (2)	1:50	0.966	1.022	0.907	0.978
	1:100	1.125	0.923	0.917	0.966
	1:200	0.907	1.047	0.926	0.884
96D3 (2)	1:50	1.433	1.119	1.031	0.822
	1:100	1.219	1.007	0.923	0.974
	1:200	1.369	1.055	0.884	1.002
37 (4)	1:50	0.572	0.409	0.541	0.508
	1:100	0.393	0.157	0.211	0.244
	1:200	0.133	-0.083	-0.044	0.004
39 (4)	1:50	1.457	0.712	0.855	0.566
	1:100	1.359	0.401	0.598	0.383
	1:200	1.234	0.186	0.511	0.109

1: Hybridoma culture supernate
2: Ascitic fluid
3: Sodium sulphate precipitated IgG
(1–3: Murine McAb)
4: Rabbit antiserum

Antigens were diluted 1:100 and control sera 1:50

Table 12: OD readings obtained in the MACELISA using various detector antibodies.

a) IgM +ve serum S6824

Anti-serum diln.	Anti-human μ chain coating Ab diln.		
	1:100	1:200	1:400
1:200	>1.5	>1.5	>1.5
1:400	>1.5	>1.5	>1.5
1:800	>1.5	>1.5	>1.5
1:1600	>1.5	>1.5	1.482

b) IgM +ve serum S5150

Anti-serum diln.	Anti-human μ chain coating Ab diln.		
	1:100	1:200	1:400
1:200	1.249	1.249	1.186
1:400	0.974	0.952	0.923
1:800	0.742	0.704	0.698
1:1600	0.519	0.500	0.498

c) IgM +ve serum SB14318

Anti-serum diln.	Anti-human μ chain coating Ab diln.		
	1:100	1:200	1:400
1:200	>1.5	>1.5	>1.5
1:400	>1.5	>1.5	>1.5
1:800	>1.5	>1.5	1.327
1:1600	1.374	1.256	1.090

b) IgM +ve serum 1937

Anti-serum diln.	Anti-human μ chain coating Ab diln.		
	1:100	1:200	1:400
1:200	>1.5	>1.5	>1.5
1:400	>1.5	1.340	1.395
1:800	1.327	1.280	1.179
1:1600	1.111	1.050	0.990

e) IgM -ve serum PM

Anti-serum diln.	Anti-human μ chain coating Ab diln.		
	1:100	1:200	1:400
1:200	0.577	0.627	0.585
1:400	0.362	0.375	0.337
1:800	0.209	0.204	0.188
1:1600	0.085	0.088	0.077

f) IgM -ve serum PW

Anti-serum diln.	Anti-human μ chain coating Ab diln.		
	1:100	1:200	1:400
1:200	0.407	0.410	0.433
1:400	0.223	0.236	0.235
1:800	0.088	0.100	0.077
1:1600	0.015	0.000	0.003

GE antigen was diluted 1:100 and control sera 1:50.

Table 13: The MACELISA - Titration of coating antibody and detector antibody using CMV-IgM +ve (a-d) and -ve (e-f) sera.

at least two of the four IgM positive sera tested.

This test system was used to determine the optimun test serum concentration. At this point control antigen was included in the assay (Table 14). Positive and negative control sera were readily distinguished at all serum dilutions tested. OD readings for control antigen wells and for test antigen wells with IgM negative sera generally increased with increasing serum dilution. This may be due to increased non-specific binding of rabbit antibody or anti-rabbit conjugate to the solid phase at higher test serum dilutions. Greater non-specific binding of antigen may also occur under these conditions and may result in increased background readings in view of the reactivity of the rabbit antiserum with control antigen. A test serum dilution of 1:50 was retained in further tests.

In order to determine a cutoff point sera were assigned an IgM value which was calculated by the equation:

$$\text{IgM value} = \frac{\text{NET OD}^{1}\ \text{(TEST SERUM)} - \text{NET OD (-VE CONTROL SERUM)}}{\text{NET OD (+VE CONTROL)}^{2} - \text{NET OD (-VE CONTROL SERUM)}}$$

MULTIPLIED TIMES 100

1. NET OD = mean OD reading of two test antigen wells minus OD reading of one control antigen well.

2. A strongly positive serum was used as positive control.

Test serum	OD readings (Test antigen/control antigen)			
	Serum dilution			
	1:50	1:200	1:500	1:1000
IgM +ve				
S6824	1.364/0.000	1.276/0.003	1.084/0.045	1.056/0.073
S5150	0.456/0.000	0.416/0.000	0.422/0.011	0.416/0.011
1937	1.078/0.061	0.780/0.091	0.820/0.100	0.710/0.214
1861	1.004/0.006	0.926/0.012	0.806/0.037	0.730/0.060
S6004	1.179/0.000	1.174/0.000	1.157/0.020	1.174/0.034
SB14318	1.217/0.005	1.179/0.048	1.116/0.057	1.122/0.056
IgM -ve				
PM	0.217/0.053	0.198/0.008	0.264/0.103	0.271/0.120
PW	0.164/0.008	0.160/0.040	0.202/0.033	0.222/0.101
RF+ve 1	0.195/0.100	0.239/0.057	0.255/0.058	0.272/0.102
RF+ve 2	0.179/0.007	0.079/0.000	0.091/0.000	0.072/0.000

Table 14: The effect of dilution on the MACELISA reactivity of CMV-IgM +ve and -ve control sera.

The assay was then used to screen a panel of sera from patients with proven or suspected primary CMV infection and a panel of potential false positive generating sera. The results are shown in Table 15. As can be seen seen few sera of the latter group gave IgM values greater than 20. The one specimen which did was a rheumatoid factor positive serum which remained positive after sucrose density gradient fractionation. (See Figure 4) This specimen was therefore considered to be genuinely CMV-IgM positive. Most of the sera from proven primary CMV infections gave IgM values much greater than 20, although some from asymptomatically infected individuals with unknown types of infection gave low values around 20. An IgM value of 20 was therefore taken as the cutoff value. The IgM status of sera giving values under but close to the cutoff may be considered as doubtful. In view of the fact that several EBV-IgM positive sera gave IgM values which were close to the cutoff it was considered prudent to test sera giving positive results for the presence of EBV-IgM by IIF.

Subsequently it became apparent that incusion of a weak CMV-IgM positive sample with an IgM value close to cutoff in each run was necessary. This was because inter-test fluctuations in the OD readings of weakly positive sera were greater than those of strongly positive sera, where IgM is likely to be present in excess. IgM values were then calculated using a projected net OD reading equivalent to an IgM value of 100 which was extrapolated from the net OD readings of the weak positive (IgM value = 21) and negative (IgM value = 0) control samples.

The sensitivity and specificity of the assay were then

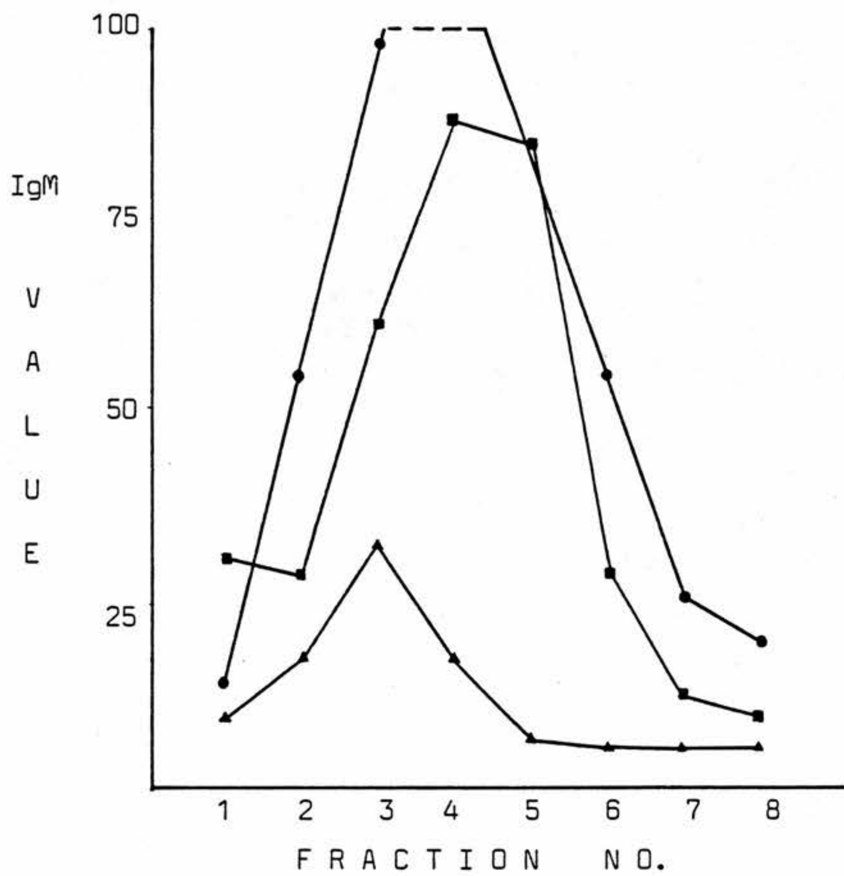
Serum	Diagnosis	IgM value
S10622	CMV-mononucleosis	>100
S10623	Paired serum	>100
S12564	CMV hepatitis	108
S11982	Rash, hepatomegaly	0
S11983	Paired serum	0
S11486	Acute renal failure	4
S11494	Sequential serum	3
S12080	Sequential serum	19
S12551	Sequential serum	24
S11398	Jaundice	5
S11399	Paired serum	6
S12101	Abnormal liver function tests	0
CS 763	Renal transplant patient,	2
CS 779	CMV seroconversion	13
CS 795	Sequential sera	52
CS 496	Renal transplant patient,	6
CS 518	CMV seroconversion	40
CS 528	Sequential sera	50
CS 444	Bone marrow transplant patient,	1
CS 477	CMV seroconversion	4
CS 503		6
CS 516	Sequential sera	12
RF 17	Rheumatoid factor positive	34
S3697	Monospot positive	6
S5696	Monospot positive	9
S7615	Monospot positive	18
S7639	Monospot positive	18
S8112	Monospot positive	17
S6737	Monospot positive	2
21481	Rubella-IgM positive	2
423	Rubella-IgM positive	0
238	EBV-IgM positive	18
403	Hepatitis A-IgM positive	7
1192	Hepatitis A-IgM positive	7
1169	VZV-IgM positive	1
S2519	HSV-IgM positive	11
SB5058	HSV-IgM positive	3
1709	Mycoplasma CF titre >256	1
797	VZV CF titre 1024	0
31170	HSV seroconversion	0
298	Psittacosis CF titre 512	12
23974	Influenza B CF titre >256	0

Table 15: MACELISA IgM values of sera from various acute infections.

tested further. To verify that MACELISA reactivity was in fact due to the presence of IgM in test sera several IgM positive sera were fractionated by sucrose density centrifugation and individual fractions were tested in the MACELISA. Results of representative sera are shown in Figure 4. Maximum IgM values were obtained with fractions containing most IgM. The positive results obtained with higher fractions of strongly positive sera are probably due to carry-over of IgM during fraction collection.

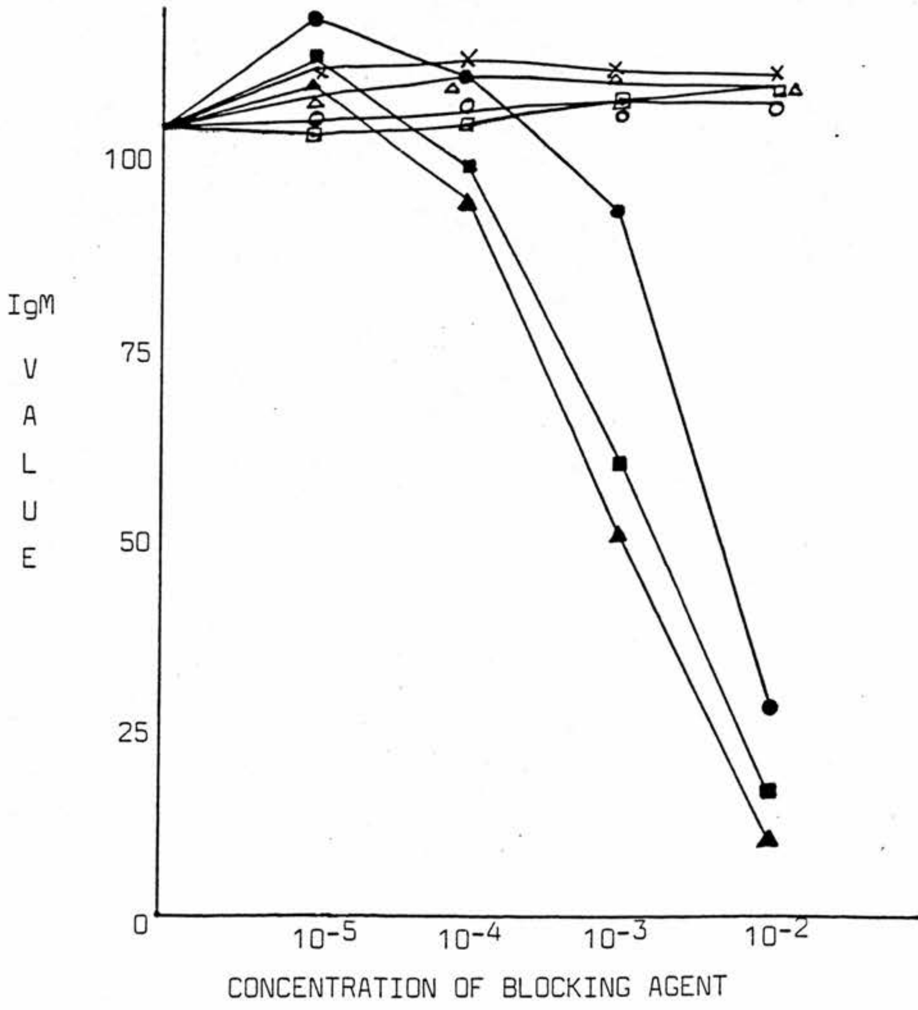
The IgM-specificity of the assay was further confirmed by inhibition experiments with myeloma sera. When added to CMV-IgM positive sera, IgM myeloma sera consistently inhibited the capture of specific IgM by the coating antibody in a dose-dependent fashion. In contrast, an IgG myeloma serum, an IgA myeloma serum and a gamma globulin preparation did not inhibit specific IgM binding any more than observed with CMV-seronegative serum. Representative results of this experiment using a strong CMV-IgM positive serum are shown in Figure 5.

The sensitivity of the MACELISA was assessed by testing a panel of sera which had previously been tested for CMV-IgM by indirect RIA. Of 173 sera tested, three were CMV-IgM positive by indirect RIA. These three sera were also strongly positive by MACELISA (IgM values were 63-78). A further three sera which were negative by indirect RIA gave IgM values of 20 by MACELISA. It could not be determined whether this slight discordance was due to greater specificity of indirect RIA or greater sensitivity of the MACELISA.



● Serum S14318
■ Plasma P5612
▲ Serum RF 17

Figure 4: MACELISA reactivity of sucrose density gradient fractions of CMV-IgM positive specimens.



KEY

- ● IgM myeloma 1 (16 mg/ml IgM)
- ■ IgM myeloma 2 (192 mg/ml IgM)
- ▲ IgM myeloma 3 (87 mg/ml IgM)
- □ IgA myeloma (57 mg/ml IgA)
- ○ IgG myeloma (50 mg/ml IgG)
- △ Pooled normal gamma globulin (150 mg/ml protein)
- × CMV-seronegative human serum

Figure 5: Inhibition of CMV-IgM capture by different antibody classes.

The assay was then used to screen plasma samples from the homosexual and heterosexual volunteers. These results are discussed below. It became apparent during further use that the MACELISA may be of selective sensitivity. For example, several plasmas from individuals who were viruric at the time of study were found to give IgM values close to but consistently below the cutoff. This may simply reflect a waning of IgM titres following acute infection, however it was also considered possible that the MACELISA detected IgM specificities which are not representative of those in some specimens.

In view of this possibility it was decided to develop an alternative MACELISA system using McAbs directed against glycoprotein antigens. It was considered that biotinylation of McAbs may permit their use at higher dilutions than those used previously in the MACRIA and MACELISA using unlabelled McAbs, possibly overcoming the problems of nonspecificity encountered in these assays. McAb HCMV 24, directed against an immediate early glycoprotein which is also present at late stages of infection was selected for biotinylation. Three McAbs, CH 12, CH 16 and CH 28, reactive with late glycoproteins were also available at this stage. Of these three, CH 16 showed greatest reactivity against CMV GE antigen by indirect ELISA (Table 16) and was also selected for biotinylation. McAb HCMV 3, reactive with an early nuclear antigen was also biotinylated for comparison. Each McAb was biotinylated at two different biotin : free IgG amino group ratios as detailed under Materials, Methods and Study Populations. The biotin labels

McAb	Dilution	OD readings		
		Mean test antigen	Control antigen	Net
CH 12	1:200	0.104	0.001	0.103
	1:400	0.124	0.044	0.080
	1:800	0.115	0.049	0.066
	1:1600	0.130	0.063	0.067
	1:3200	0.097	0.038	0.059
	1:6400	0.066	0.028	0.038
	1:12800	0.076	0.010	0.066
	1:25600	0.017	0.000	0.017
CH 16	1:200	0.849	0.041	0.808
	1:400	0.824	0.008	0.816
	1:800	0.832	-0.010	0.842
	1:1600	0.904	0.045	0.859
	1:3200	0.852	0.004	0.848
	1:6400	0.838	0.091	0.747
	1:12800	0.851	0.017	0.834
	1:25600	0.720	0.035	0.685
CH 28	1:200	0.142	0.050	0.092
	1:400	0.069	0.028	0.041
	1:800	0.040	0.043	<0
	1:1600	0.034	0.028	0.006
	1:3200	0.036	0.080	<0
	1:6400	0.018	0.008	0.010
	1:12800	-0.044	-0.046	0.002
	1:25600	-0.085	-0.054	<0

Table 16: The reactivity of McAbs from L. Pereira with CMV GE by indirect ELISA.

were then tested for their CMV GE reactivity (Table 17). Those showing greatest specificity (HCMV 24 and CH 16 labelled at a 4:1 molar ratio and HCMV 3 labelled at a 1:1 molar ratio) were selected for use in the MACELISA.

Initially, biotinylated McAb HCMV 24 (B-HCMV 24) was used. On testing a selected panel of sera and plasmas it was evident that this assay detected CMV-IgM relatively more efficiently in some specimens and less efficiently in others compared to the MACELISA using rabbit antiserum as detector antibody (Table 18). Plasma P7473 was initially weakly positive in the original MACELISA test but had become negative on repeat testing, probably due to multiple thawing and refreezing which is known to inactivate IgM. This specimen was still readily identifiable as IgM positive in the modified MACELISA however. In contrast the positive serum S12564 which was strongly positive in the original MACELISA gave lower IgM values in the modified test. The MACELISA using B-HCMV 24 was used to test a wider selection of sera (Table 19). The biotin label was used at a dilution of 1:10 000 as this gave maximal distinction between the positive and negative control sera. The results of this experiment reinforced the conclusion of the initial test, namely that the two MACELISA tests detected IgM in different sera to different relative extents. It was also noticed that some sera from patients with other acute infections gave high background IgM values. An IgM value of 25 was therefore taken as the cutoff.

A MACELISA using B-CH 16 gave similar results to that using B-HCMV 24. When B-HCMV 3 was used however, only sera

Biotin label (molar ratio)	Dilution	OD readings		
		Mean test antigen	Control antigen	Net
B-HCMV 24 (4:1)	1:5120	0.889	0.166	0.723
	1:20480	0.860	0.109	0.751
	1:81920	0.559	0.046	0.513
	1:327680	0.328	0.102	0.226
B-HCMV 24 (1:1)	1:5120	<0.900	0.807	-
	1:20480	<0.900	0.569	-
	1:81920	0.783	0.283	0.500
	1:327680	0.495	0.181	0.314
B-HCMV 3 (4:1)	1:5120	0.783	0.314	0.469
	1:20480	0.434	0.133	0.070
	1:81920	0.173	0.070	0.103
	1:327680	0.156	0.169	<0
B-HCMV 3 (1:1)	1:5120	0.406	0.058	0.348
	1:20480	0.211	0.005	0.206
	1:81920	0.084	0.002	0.082
	1:327680	NT	NT	NT
B-CH 16 (4:1)	1:10000	1.450	0.179	1.271
	1:20000	1.131	0.119	1.012
	1:40000	0.737	0.085	0.652
	1:80000	0.512	0.087	0.425
B-CH 16 (1:1)	1:10000	0.748	0.189	0.559
	1:20000	0.550	0.158	0.392
	1:40000	0.423	0.181	0.242
	1:80000	0.394	0.183	0.211

NT = not tested

Table 17: ELISA reactivity of biotinylated McAbs

a) Detector antibody = rabbit antiserum (1:1600)				
	OD readings			IgM
Serum	Mean test antigen	control antigen	Net	value
wk +ve	0.322	0.043	0.279	21
-ve	0.033	0.001	0.032	0
S12564				
(1:200)	1.187	0.141	1.046	86
(1:400)	0.904	0.127	0.777	63
(1:800)	0.566	0.124	0.442	35
NP	0.180	0.118	0.062	3
PM	0.355	0.233	0.133	9
P7473	0.452	0.220	0.232	17

b) Detector antibody = B-HCMV 24 (1:5000)				
	OD readings			IgM
Serum	Mean test antigen	control antigen	Net	value
wk +ve	0.426	0.190	0.236	21
-ve	0.245	0.264	<0	0
S12564				
(1:200)	0.794	0.209	0.585	52
(1:400)	0.629	0.239	0.390	35
(1:800)	0.493	0.230	0.263	23
NP	0.363	0.200	0.163	15
PM	0.445	0.243	0.202	18
P7473	0.928	0.380	0.548	49

Table 18

Continued overleaf

c) Detector antibody = B-HCMV 24 (1:10000)				
	OD readings			IgM
Serum	Mean test antigen	control antigen	Net	value
wk +ve	0.286	0.075	0.211	21
-ve	0.124	0.055	0.069	0
S12564				
(1:200)	0.583	0.092	0.491	62
(1:400)	0.425	0.089	0.336	39
(1:800)	0.311	0.091	0.220	22
NP	0.216	0.088	0.128	9
PM	0.271	0.095	0.176	16
P7473	0.645	0.147	0.498	63

d) Detector antibody = B-HCMV 24 (1:20000)				
	OD readings			IgM
Serum	Mean test antigen	control antigen	Net	value
wk +ve	0.188	0.004	0.184	21
-ve	0.071	0.017	0.054	0
S12564				
(1:200)	0.405	0.048	0.357	49
(1:400)	0.291	0.050	0.241	30
(1:800)	0.209	0.053	0.156	16
NP	0.103	0.054	0.049	0
PM	0.164	0.043	0.121	11
P7473	0.452	0.123	0.329	44

CMV-IgM +ve: S12564, P7473
CMV-IgM -ve: NP, PM
S12564 diluted in NP.

Table 18: Comparison of a) rabbit antiserum and b)-d) B-HCMV 24 as detector antibodies in the MACELISA.

Serum	Diagnosis	IgM value
S10622	CMV-mononucleosis	92
S10623	Paired serum	73
S14273	Bone marrow transplant recipient, CMV CF titre 512	41
CS 175	Renal transplant recipient,	9
CS 197	CMV seroconversion	108
CS 212	Sequential sera	84
S3697	Monospot positive	0
S5696	Monospot positive	0
S7615	Monospot positive	11
S8112	Monospot positive	4
21481	Rubella-IgM positive	23
423	Rubella-IgM positive	12
238	EBV-IgM positive	0
285	EBV-IgM positive	25
1192	Hepatitis A-IgM positive	17
1169	VZV-IgM positive	20
1709	Mycoplasma CF titre >256	12
797	VZV CF titre 1024	17
31170	HSV seroconversion	14
298	Psittacosis CF titre 512	8
23974	Influenza B CF titre >256	19
	Plasmas positive in original MACELISA	
P4710	Original IgM value = 25	39
P7663	23	29
P7473	26	42
P4331	25	17
P7202	32	37
P7775	24	28
P7138	27	18

Table 19: Modified MACELISA: IgM values of sera from various acute infections and from plasmas characterised in the original MACELISA.

which were strongly positive by other assays gave detectably positive results (Table 20). It was initially intended to employ a MACELISA using a mixture of B-HCMV 24 and B-CH 16 as detector antibody, however it became necessary to stop using B-CH 16 as high background IgM values were observed with many sera. This was found to be due to label deterioration. The final test therefore used B-HCMV 24 only.

The reproducibility of both MACELISA tests proved to be reasonable. Strongly positive sera gave positive results on all occasions. Fluctuations in the IgM values of weak positive specimens were in the order of 0-9 IgM units. This occasionally resulted in fluctuations around the cutoff value. All positive or doubtful specimens were therefore confirmed by repeat testing. After multiple testing several positive sera gave consistently negative results which was probably due to multiple freeze-thawing.

During the course of these developments a CMV-IgM capture assay using peroxidase-labelled McAb as detector antibody became available commercially. The VirEnz - M CMV kit was tested with sera and plasmas which had been characterised by MACELISA. Only results obtained with the modified MACELISA were compared as considerable deterioration of MACELISA reactivity had occurred with several specimens since testing with the original MACELISA. The results are shown in Table 21. The two tests are comparable although there are some discrepancies. Some sera which were weakly positive originally by MACELISA were still positive in the VirEnz kit. Some EBV-IgM positive sera were also positive or

Serum	Detector antibody		
	B-HCMV 24 (1:10000)	B-CH 16 (1:10000)	B-HCMV 3 (1:2000)
S12564			
(1:200)	0.776	0.541	0.162
(1:400)	0.337	0.245	0.078
-ve	0.092	0.171	0.022
CS 1057	0.093	0.095	0.029
CS 1075	0.435	0.381	0.095
CS 1087	0.474	0.383	0.089
CS 1101	0.281	0.253	0.084
S3697	0.000	0.000	0.084
S5696	0.000	0.000	0.038
S7629	0.819	1.418	0.164
PM	0.124	0.136	0.035
FC	0.105	0.136	0.026
P8392	0.210	0.157	0.037
S10623	1.031	0.966	0.243
P4710	0.409	0.450	0.113

Specimens CS 1057 - CS 1101 were sequential sera from a bone marrow transplant recipient showing CMV seroconversion. S3697-S7629 were Monospot positive specimens. S12564, S10623, P8392 and P4710 were CMV-IgM positive in the original MACELISA. Other specimens were CMV-IgM negative.

Table 20: Net OD readings of selected sera in the modified MACELISA using different detector antibodies.

Serum	IgM value	VirEnz result
P7775	14	-ve
M9937	40	+ve
S12564	100	+ve
P4710	14	+ve
P7138	4	-ve
P6592	3	-ve
P7894	0	-ve
P8392	6	-ve
P4331	6	-ve
S10622	92	+ve
P5997	5	-ve
P7473	6	-ve
S13945	21	+ve
P7663	14	-ve
P5612	8	-ve
S3697	0	doubtful +ve
S7629	45	+ve

P6592, P7894, P4331, P5997 and P7473 were from viruric homosexuals. P7775, S3697 and S7629 were EBV-IgM positive. Other specimens were CMV-IgM positive in the original MACELISA.

N.B. Discrepancies between IgM values quoted in this table and those of other tables are probably due to deterioration of MACELISA reactivity on storage.

Table 21: Comparison of the modified MACELISA with the VirEnz - M CMV kit.

doubtful in the latter test but negative by MACELISA. It is likely that both assays could be used on a diagnostic basis provided EBV-IgM positive sera were excluded.

b) CMV-IgM antibodies in the study populations

All plasmas from the two study populations were tested for CMV-IgM using the original MACELISA with rabbit antiserum as detector antibody (MACELISA 1) and with the modified assay using B-HCMV 24 as detector antibody (MACELISA 2), with a few exceptions where a specimen had been exhausted. A random selection was also tested by IIF for CMV-IgM. All other specimens which were positive in one or both MACELISA's were also tested by IIF. All specimens which were positive in any of these three tests were also tested by IIF for EBV-IgM and -IgG. Any which were EBV-IgM positive were regarded as CMV-IgM negative.

114 specimens were tested by IIF for CMV-IgM at the same time as specimens were tested by MACELISA 1. Correlation between these two sets of results was 98%; 107 were negative in both tests and five were positive in both tests. Two specimens were positive by IIF only. One of these specimens was from an individual who was excreting virus at the time (P7895). The other specimen was from an individual who had no other evidence of active infection (M2866).

In all, 200 plasmas from the two study populations were tested by both MACELISA 1 and MACELISA 2. Correlation between the results obtained was 98%; 190 were negative in both tests and six were positive in both tests. There were three which

were positive only by MACELISA 1 and one which was positive only by MACELISA 2. It should be noted however that some sera which were originally positive by MACELISA 1 were no longer positive in this test at the time of testing by MACELISA 2.

In addition, 17 specimens were tested by MACELISA 1 only, one of which was positive, and six specimens were tested by MACELISA 2 only, none of which were positive. The results obtained for sera which were positive by either or both MACELISA's are shown in Table 22. The single specimen which was EBV-IgM positive was considered CMV-IgM negative in the analysis that follows. The remainder were considered CMV-IgM positive.

7/84 (8.3%) of the homosexual group and 3/140 (2.1%) of the heterosexual group were CMV-IgM positive. This difference is statistically significant ($p = 0.028$). If only seropositive individuals are considered in the analysis no significant difference is detectable. (7/63 [11.1%] of seropositive homosexuals and 3/34 [8.8%] of seropositive heterosexuals were CMV-IgM positive.) No seronegative individuals were CMV-IgM positive.

Within the homosexual group, CMV-IgM was only found in those under 25 years of age; 7/43 (16%) of this age group and 0/41 of the older age group were IgM positive ($p = 10^{-6}$). CMV-IgM was also restricted to those with under six years of sexual experience. (7/39 [18%] of this group and 0/37 of the more sexually experienced group were CMV-IgM positive; $p = 0.0007$.) There was no significant association between the presence of CMV-IgM and the number of recent partners.

Serum	IgM value		CMV-IgM IIF[1]	EBV-IgM IIF[1]
	MACELISA 1	MACELISA 2		
M9937	16	40	+	-
P4331	25	17	+	-
P4710	25	39	+	-
P5612	94	17	-	-
P6867	31	NT	NT[2]	NT
P7138	27	18	-	-
P7202	32	37	+	-
P7473	26	42	+	-
P7663	23	23	+	-
P7775	24	28	+	+
P8392	22	43	+	-

[1]IIF results recorded are those determined after absorption against latex-bound IgG.
[2]NT = not tested

Table 22: MACELISA positive specimens from study populations;

IgM values and IIF results.

Numbers were insufficient for a similar analysis within the heterosexual group.

Retrospective specimens were available for four homosexuals who were CMV-IgM positive at the time of testing. One individual (P4331) was IgG negative 17 months prior to testing. Another (P4710) was IgG negative 13 days prior to testing. One (M9937) was IgG and IgM positive five months prior to testing and one (P5612) was IgG positive 7.5 months prior to testing. The IgM status of this retrospective specimen was not determined.

CMV IEA-IgG antibodies in the study populations

CMV IEA-IgG antibodies were only detected in plasmas which were CMV-IgG positive by indirect ELISA. This type of antibody was detected in 46/63 (73%) of seropositive homosexuals and in only 16/34 (47%) of seropositive heterosexuals, a significantly lower prevalence ($p < 0.05$). Within the seropositive homosexual cohort, no significant trends were apparent when individuals were grouped according to age (32/41 [78%] of those under 29 years of age and 14/22 [64%] of the older age group were CMV IEA-IgG positive; p = NS), duration of sexual activity (24/29 [83%] of those with a history of sexual activity shorter than eight years and 18/29 [62%] of those with a longer history of sexual activity were CMV IEA-IgG positive; p = NS) or recent partner number (23/42 [55%] of those reporting fewer than three recent partners and 11/20 [55%] of those reporting a higher number of recent partners were CMV IEA-IgG seropositive). No other breakdown of these figures showed any significant

differences.

CMV IEA-IgG was present in five of the seven homosexuals who were excreting virus and in all seven of those who were CMV-IgM positive. Neither of these associations were statistically significant when compared to the remainder of the seropositive homosexual group.

Numbers were insufficient for a similar breakdown within the heterosexual group.

CMV EA-IgG antibodies in the study populations

Like CMV IEA-IgG, CMV EA-IgG antibodies were only detected in plasmas which were CMV-IgG positive by indirect ELISA. Specimens which were positive at either of the two screening dilutions were regarded as positive.

CMV EA-IgG was detected in 35/63 (56%) of seropositive homosexuals and in 8/34 (24%) of seropositive heterosexuals. This difference is more striking than that observed with CMV IEA-IgG ($p < 0.01$). Within the seropositive homosexual group, the presence of CMV EA-IgG did not correlate with age (22/41 [54%] of those under 28 years of age and 13/22 [59%] of the older age group were positive), duration of sexual activity (16/29 [55%] of those with a history of sexual activity less than eight years and identical numbers of those with a longer history were positive) or the number of recent partners (6/14 [43%] of those reporting one recent partner, 17/28 [61%] of those reporting two partners and 11/20 [55%] of those reporting more than two recent partners were positive). None of these differences were significant.

CMV EA-IgG was present in five of the seven homosexuals who were viruric at the time of testing and in six of the seven who were CMV-IgM positive. These associations are not significant.

The presence of CMV EA-IgG correlated to some extent with the presence of CMV IEA-IgG ($p < 0.05$) although the former was detected less frequently. 34/43 (79%) of CMV EA-IgG positive specimens were also CMV IEA-IgG positive.

Persistence of CMV IEA-IgG and CMV EA-IgG

To determine whether or not the presence of these antibody reactivities fluctuated with time in individuals of long-standing immunity, repeat plasma specimens from seropositive, healthy laboratory personnel, collected over periods ranging from 5-16 months were tested for CMV IEA-IgG and CMV EA-IgG. The results are shown in Figure 6. As can be seen, the presence or absence of CMV IEA-IgG was constant in all individuals. Two individuals however showed a change in CMV EA-IgG status.

Lymphocyte proliferative responses to mitogens

Initial tests were carried out using lymphocytes from laboratory personnel. Maximal proliferation in response to phytohaemagglutinin (PHA) occurred when stock PHA solution was added to cultures to give a final dilution of either 1:400 or 1:800. Responses to pokeweed mitogen (PWM) were more variable, and frequently the dose-response curve gave two peaks, possibly reflecting stimulation of different

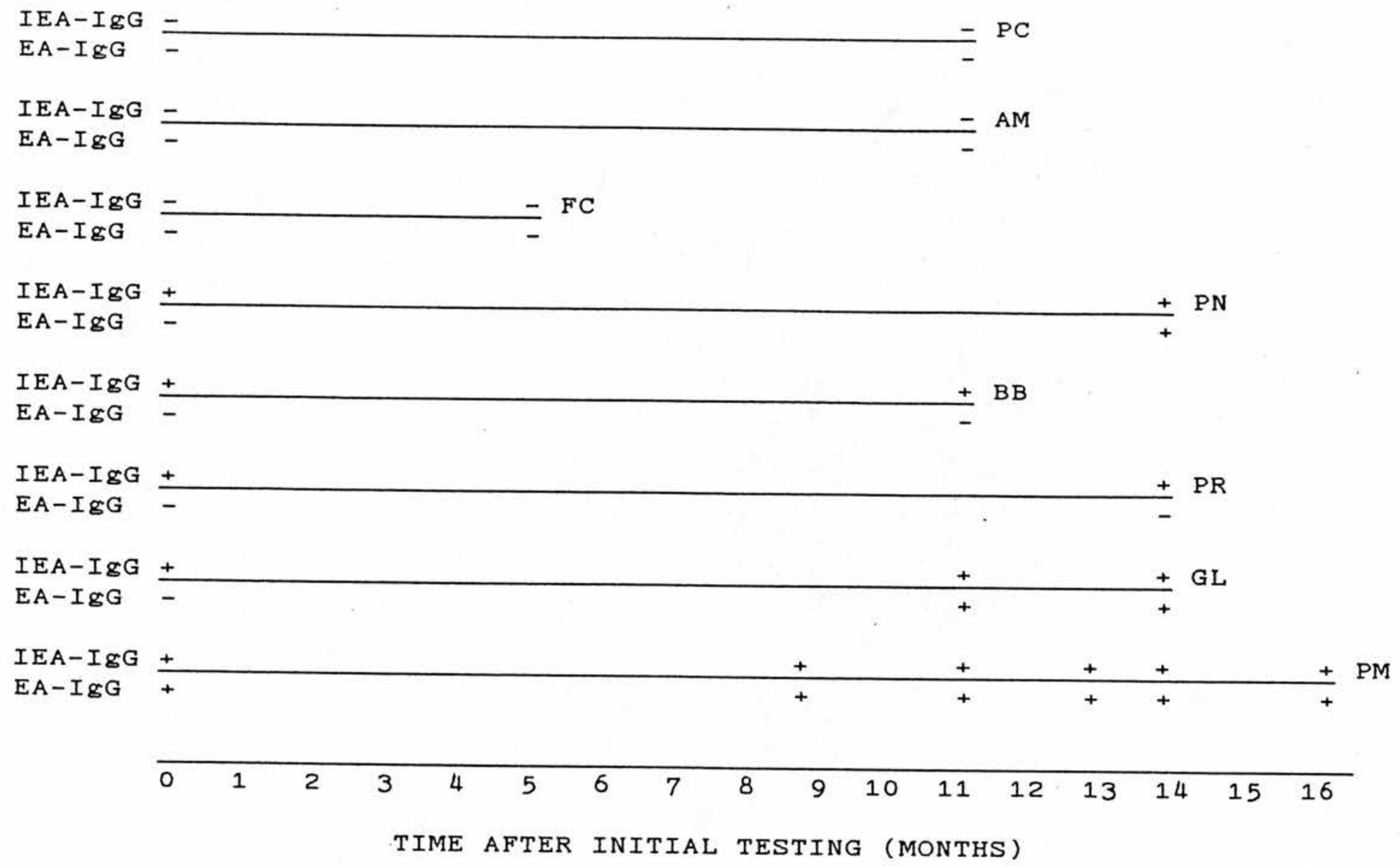


Figure 6: Time-dependent variation in the CMV IEA-IgG and CMV EA-IgG status of seropositive laboratory personnel.

lymphocyte populations. In other cases a plateau effect was observed. Lymphocytes from most individuals gave good responses when the final dilution of stock PWM solution was 1:200-1:800. PHA and PWM were therefore used at dilutions of 1:200, 1:400 and 1:800 wherever lymphocyte yield permitted. Concanavalin A (Con A) was found to give maximal stimulation when a 10 mg/ml stock solution was added to give a final dilution of 1:40 or 1:80. Con A was often inhibitory at higher concentrations and was therefore used at dilutions of 1:40, 1:80 and 1:160 wherever possible. The dilution which resulted in maximal proliferation was used for statistical analysis.

Lymphocyte proliferation studies with the above mitogens were attempted successfully on specimens from 138 study volunteers, although lymphocyte yield did not permit investigation with all three mitogens on each specimen. The test failed on a number of occasions; this was usually recognised by the absence of significant proliferation in all cultures initiated at the same time. Test failure appeared to be due to fluctuations in CO_2 concentration in most cases.

Results of mitogen-induced proliferation of lymphocytes from homosexuals and heterosexuals are shown in Table 23. None of the differences in mean proliferation between the two study populations were significant. Lymphocytes from individuals with evidence of current or recent CMV infection (those who were viruric and/or CMV-IgM positive) did not show any apparent trends towards lower or higher mitogen-induced lymphocyte proliferative responses.

	Homosexuals	Heterosexuals
<u>PHA responses</u>		
Number tested	21	21
Mean net cpm	42977	45764
Standard deviation	+28573	+23282
Net cpm range	3757-102970	3194-83582
Mean SI[1]	76	86
Standard deviation	+42	+49
Mean SI range	18- 165	10-173
<u>PWM responses</u>		
Number tested	42	62
Mean net cpm	18716	14635
Standard deviation	+15584	+12101
Net cpm range	1906-71739	3116-46538
Mean SI	24	21
Standard deviation	+42	+49
Mean SI range	5-78	4-84
<u>Con A responses</u>		
Number tested	21	47
Mean net cpm	13167	10729
Standard deviation	+9224	+7843
Net cpm range	1079-25803	1913-29741
Mean SI	25	22
Standard deviation	+20	+14
Mean SI range	4-85	5-55

[1] SI = stimulation index

<u>Table 23: Mitogen-induced lymphocyte proliferative responses of homosexuals and heterosexuals.</u>

Lymphocyte proliferative responses to CMV antigens

CMV-specific proliferative responses were assayed in parallel with mitogen-induced responses and results were discarded when the latter tests failed. This test was also initially characterised using lymphocytes from laboratory personnel. CMV GE antigen was added to cultures to give a range of final dilutions while control antigen was used at a single dilution of 1:80. The results are shown in Table 24. The antigen dilution which resulted in the maximum stimulation index (SI) was used in all evaluations. Clearly not all seropositive individuals gave significant CMV-specific proliferative responses in this assay. No seronegative individual gave a response with an SI greater than two. This value was therefore taken as the cutoff.

CMV-specific lymphocyte proliferation was successfully studied in 133 homosexual and heterosexual volunteers. These assays were carried out before the CMV serostatus of the volunteers was determined. 77 of those studied were CMV-seronegative. In only four of these cases (5%) were SI values above the cutoff observed. These values were all low (2.0-2.7). Of the 56 seropositive volunteers studied 37 were homosexuals and 19 were heterosexuals. The results of these assays are shown in Table 25. There was no significant difference between the study groups, either in the proportion who gave a positive response or in the mean SI of those who did respond.

There was no correlation between lymphocyte

Seropositive laboratory personnel	SI	Seronegative laboratory personnel	SI
GL (F)	23.1	IR (F)	1.2
PM (M)	20.8	PW (F)	0.9
AG (F)	6.2	IC (F)	0.9
PR (F)	4.4	GO (M)	1.1
OC (M)	0.8	MA (F)	1.0
BB (F)	1.8	MB (F)	1.1
PN (M)	1.8		
WN (M)	4.1		
PC (M)	2.8		
AM (M)	0.8		
EE (F)	4.7		

Table 24: CMV-specific lymphocyte proliferative responses of seropositive and seronegative laboratory personnel.

Homosexuals	SI	Heterosexuals	SI
K5914	6.7	H9499	2.1
K6561	2.0	M6615	3.4
K7967	1.6	P6685	3.0
M653	1.2	P6824	17.3
M946	1.4	P6867	1.2
M1968	34.3	P6910	1.5
M2244	0.9	P7004	13.5
M2688	3.0	P7072	1.8
M4158	2.7	P7097	2.0
M5058	1.3	P7138	0.9
M5928	1.4	P7181	1.5
M6845	6.3	P7224	5.7
M8018	1.3	P7262	2.0
M8092	2.9	P7356	1.2
M9937	2.5	P7469	2.3
P341	1.9	P7775	3.9
P706	4.1	P7824	2.4
P2182	2.7	P7829	0.8
P3007	1.9	P7886	1.2
P3881	3.0		
P4541	1.4		
P4604	3.9		
P4710	2.3		
P4780	4.5		
P5612	2.1		
P5997	1.7		
P6592	1.3		
P6821	1.3		
P7145	5.9		
P7202	1.3		
P7473	1.8		
P7757	4.5		
P7760	2.3		
P7842	1.2		
P7895	1.2		
P7923	4.3		
Proportion responding (SI $\geq$2) = 20/37 (54%)		Proportion responding (SI $\geq$2) = 11/19 (58%)	

Table 25: CMV-specific lymphocyte proliferative responses of seropositive homosexuals and heterosexuals.

responsiveness to CMV antigens and the presence of CMV IEA-IgG or CMV EA-IgG. 21/29 (72%) of responders and 13/23 (52%) of non-responders were CMV IEA-IgG positive. 16/31 (52%) of responders and 11/25 (44%) of non-responders were CMV EA-IgG positive.

To determine if unresponsiveness to CMV antigens in seropositive individuals was due to the presence of soluble inhibitory factors, or to the absence of necessary growth factors in autologous plasma, lymphocytes from some volunteers were tested in the presence of pooled homologous plasma as well as in autologous plasma. Three plasma pools were used in these studies. Pool 1 consisted of equal volumes of plasma from eight seropositive individuals whose lymphocytes had shown good responsiveness to CMV antigens (SI = 4.1-34.3). Pool 2 consisted of equal volumes of plasma from eight seronegative individuals. Pool 3 consisted of equal volumes of plasma from eight seropositive individuals whose lymphocytes had been unresponsive to CMV antigens. The maximal SI values obtained with lymphocytes cultured in these three pools, and in autologous plasma are shown in Table 26. No clear pattern emerges from these results. Of the nine seropositive individuals whose lymphocytes were tested in this series, some enhancement was observed in four when lymphocytes were cultured in pooled plasma, but this was only significant in three cases. Of these three, one (P5997) was excreting CMV at the time of study and one (P7775) was EBV-IgM positive. The use of these plasma pools, particularly those of seropositive plasmas was found to increase

Code no.	Plasma supplement			
	Autologous	Pool 1	Pool 2	Pool 3
P3007 (sero+ve)	1.9	1.2	1.9	1.0
P3455 (sero-ve)	1.7	0.6	1.6	1.5
P3881 (sero+ve)	3.0	5.2	4.2	7.6
P4153 (sero-ve)	2.1	1.9	2.0	3.5
P5278 (sero-ve)	0.9	2.5	1.2	1.4
P5997 (sero+ve)	1.7	3.3	4.6	4.0
P6097 (sero-ve)	0.9	1.0	1.2	0.6
P6615 (sero-ve)	0.7	2.2	1.8	NT
P7680 (sero-ve)	0.9	1.0	1.2	0.6
P7693 (sero-ve)	1.3	0.9	1.0	1.6
P7757 (sero+ve)	4.5	6.7	5.0	6.6
P7760 (sero+ve)	2.3	5.9	2.3	2.7
P7775 (sero+ve)	3.9	10.7	5.0	7.3
P7779 (sero-ve)	1.4	0.8	0.7	1.2
P7821 (sero-ve)	1.1	0.9	1.1	2.0
P7824 (sero+ve)	2.4	1.9	1.4	1.2
P7829 (sero+ve)	0.8	1.3	1.7	1.4
P7830 (sero-ve)	2.1	0.7	1.1	0.7
P7832 (sero-ve)	0.7	1.7	1.3	1.1
P7842 (sero+ve)	1.2	NT	NT	1.4
P7888 (sero-ve)	0.9	1.4	NT	2.4
P7890 (sero-ve)	1.1	1.6	1.3	0.7
P7892 (sero-ve)	1.3	1.2	0.8	1.4

Pool 1: Pooled homologous plasma from seropositive individuals whose lymphocytes responded to CMV antigen.

Pool 2: Pooled homologous plasma from seronegative individuals.

Pool 3: Pooled homologous plasma from seropositive individuals whose lymphocytes were unresponsive to CMV antigen.

NT = not tested

Table 26: Stimulation indices of lymphocytes cultured in plasma from different sources.

non-specific stimulation in some assays using lymphocytes from seronegative individuals.

To determine if the presence or absence of the CMV-specific proliferative response in seropositive individuals was constant, or fluctuated with time, a number of seropositive laboratory personnel were tested on a number of occasions. The results are shown in Figure 7. It is clear that not only did the presence or absence of the response vary with time in most individuals, but that the magnitude of the response when present was variable in all individuals.

Antibodies to HTLV III

Plasmas from the homosexual cohort were screened for HTLV III by indirect ELISA. A high proportion of specimens (73%) gave ELISA readings above cutoff, however this was considered to be largely due to non-specific reactions perhaps related to the fact that specimens had been thawed and refrozen frequently. Most specimens gave absorbance values less than 0.2. (The mean positive control absorbance value was 0.531 and the mean negative control value was 0.027, giving a recommended cutoff value of 0.080.) Those specimens which gave absorbance values greater than 0.2 were retested. All remained positive on retesting and were therefore tested for HTLV III-IgG by IIF and by Western blot immunostaining. The results for these specimens are shown in Table 27. Two specimens which were positive in both confirmatory tests were regarded as HTLV III-IgG positive. One which was positive only by IIF was regarded as doubtful positive.

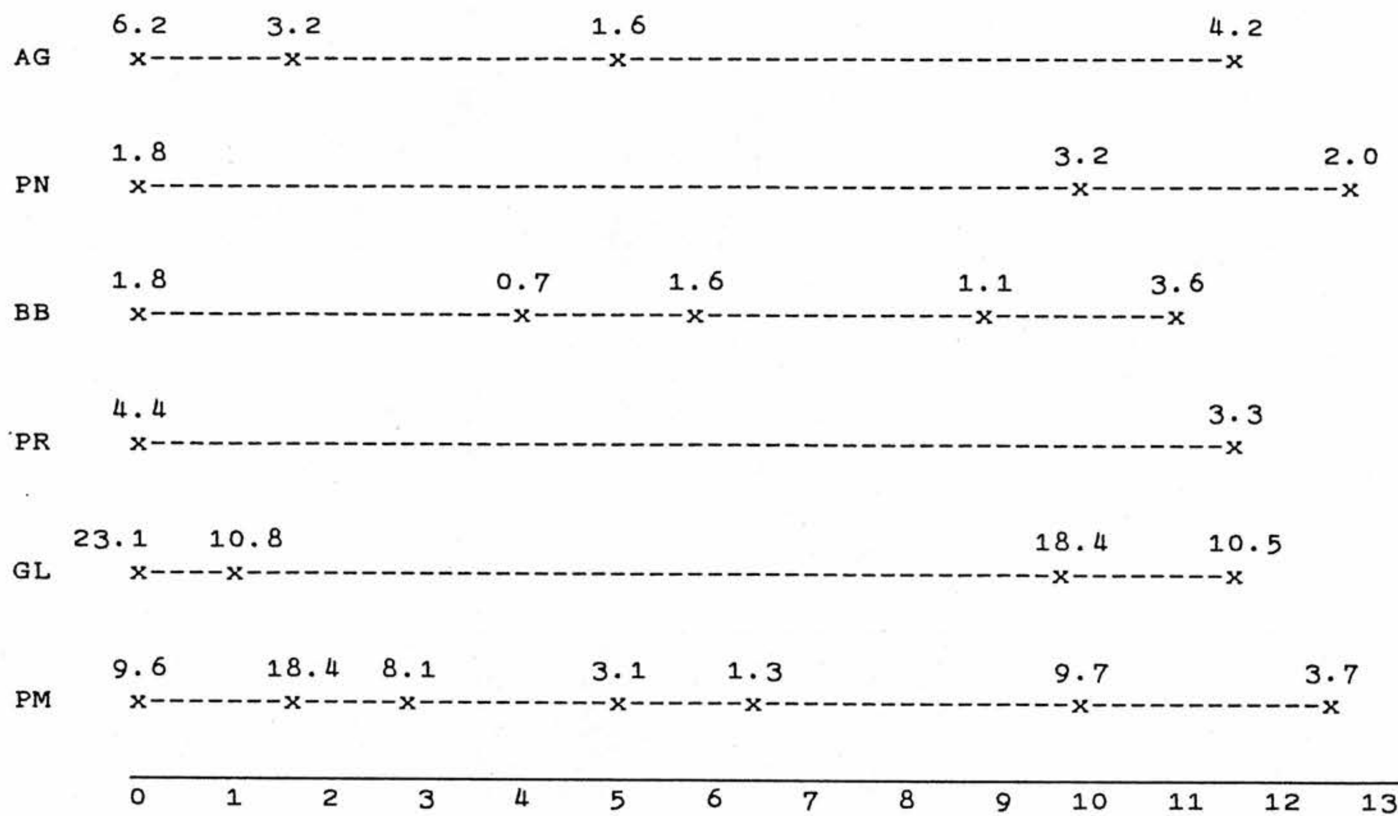


Figure 7: CMV-specific lymphocyte stimulation indices of seropositive laboratory personnel; fluctuation of the response with time.

Code no.	HTLV III-IgG ELISA OD reading	HTLV III-IgG IIF	HTLV III-IgG Western blot
K9214	0.201	-ve	-ve
M6847	0.230	-ve	-ve
P706	0.218	-ve	-ve
P1122	0.367	-ve	-ve
P2827	0.596	+ve	+ve
P4331	0.266	-ve	-ve
P4780	0.210	-ve	-ve
P5612	0.597	+ve	+ve
P6671	0.380	+ve	-ve
P7473	0.232	-ve	-ve

Positive control OD = 0.531; negative control OD = 0.027.

Table 27: HTLV III-IgG IIF and Western blot immunostaining results of specimens considered positive by ELISA.

Exposure to CMV infection in male homosexuals

The detection of CMV-specific serum antibodies is the most accessible means of determining the extent of past CMV infection in a given population. This method has been successfully applied in the present project to compare exposure to infection in male homosexuals and heterosexuals. The results clearly show a large, very significant difference in exposure, CMV infection being approximately three times more common in the homosexual group. This is in general agreement with the findings of others (Drew et al., 1981; Goldmeier et al., 1893; Mindel & Sutherland, 1984). Antibody prevalence among homosexuals was 75%. This is similar to that found by others in the UK; Goldmeier et al. (1983) reported a figure of 76%. Mindel & Sutherland (1984) however found that 92% were seropositive in one study. This is more akin to prevalences reported from the USA where up to 94% of homosexuals are seropositive (Drew et al., 1981). Antibody prevalence in the heterosexual group under study was 24%, a figure much lower than those reported by the above groups where 50% of heterosexuals have been found to be seropositive.

The rate of exposure to CMV infection is known to increase in an age-dependent fashion in the general population; the proportion who are seropositive has been estimated to increase by 2% for each successive year of life between the ages of 20 and 50 years (Kane et al., 1975)

although the rate may be higher than this in the younger end of this range due to the onset of sexual activity. The homosexual group under study was on average four years older than the heterosexual group. This then cannot explain the large difference in antibody prevalence, even assuming a higher than average seroconversion rate in this age group. Additionally, comparison between age-matched subgroups still reveals striking differences in antibody prevalence, except in older age groups where exposure to CMV infection in heterosexuals approaches that observed in homosexuals.

Comparison of antibody prevalence within each cohort reveals a slight age-dependent increase among homosexuals. This increase is much lower than might be expected, considering the age range of the group and suggests that the influence of homosexuality on antibody prevalence largely overrides that of age, a conclusion also reached by Mindel & Sutherland (1984). The heterosexual group in contrast showed a striking age-dependent increase in antibody prevalence, particularly during the late twenties. This increase is statistically significant despite the small numbers involved in the older age group. This probably explains the low antibody prevalence in this group compared to heterosexual groups studied by others, who were on average ten years older.

The high prevalence of CMV infection in homosexuals is frequently attributed to the fact that homosexuals are generally more promiscuous than heterosexuals. This may explain the greater level of exposure in homosexuals from the USA over that in homosexuals from the UK as many of the

former group are known to be extremely promiscuous, often reporting several hundred lifetime partners. The present results indicate that this explanation in itself is insufficient to account for the high rate of seropositivity in the homosexuals studied in this project. Using the reported number of sexual partners in the previous three months as an indicator of promiscuity, there was little indication of a higher antibody prevalence among more promiscuous individuals within either group although numbers were insufficient for meaningful comparison in the heterosexual group. On matching subgroups of the two cohorts for recent partner number significantly more homosexuals were seropositive. If exposure to CMV was related to the extent of sexual activity then differences may be expected on comparing those who have recently become sexually active with those with a longer history of sexual activity. This was true of the heterosexuals studied; the increase in antibody prevalence with increasing length of sexual history was large and significant, again despite small numbers. The same was also true to a lesser extent of the homosexuals studied. Significant differences were observed. However the magnitude of this difference between the more sexually experienced and the less so was much smaller.

The most plausible conclusion is that CMV-seroconversion usually occurs rapidly following the onset of homosexual activity. As this did not seem to be related to greater promiscuity in homosexuals compared to heterosexuals, it would appear that some other aspect of homosexuality

predisposes to CMV infection. It is possible that the transmission route(s) associated with homosexual contact are more efficient than those associated with heterosexual contact. Oro-anal and ano-genital contact are often practised by homosexuals and can be presumed to be common in the group under study on the basis of the high frequency of anal and rectal infections. As discussed in Chapter 4, anal intercourse may allow infectious agents to enter directly into the circulation, and so to avoid the initial defence mechanisms of innate immunity at mucosal membranes. An alternative possibility is that the immune control of CMV infection in seropositive homosexuals is defective. This would result in increased excretion of virus due to reactivated or persistent infections and consequently more efficient transmission to seronegative partners. A consideration of other aspects of CMV infection and immunity in homosexuals may allow these possible explanations for CMV susceptibility in this group to be evaluated further.

Active CMV infection in male homosexuals

Active CMV infection can be diagnosed by demonstrating replicating virus in biopsy or autopsy tissue, by demonstrating virus excretion, or by detecting virus-specific serum IgM antibody. The last two of these methods were employed in this study.

a) CMV excretion

Virus excretion was detected in a significant proportion

of homosexuals, but only in one heterosexual. The proportion in the homosexual group who were viruric was 8.3%, a figure similar to that of 7-8% reported by Drew et al. (1981). In contrast, Goldmeier et al. (1983) found no instances of viruria in homosexuals. Drew et al. (1981) found that viruria and virusemenia in homosexuals were confined to those under 30 years old. Biggar et al. (1983) also found that virusemenia was largely confined to younger men, except in those with evidence of immune deficiency. The present findings are consistent with these results, as no instances of viruria were detected in homosexuals over 25 years old, and most excretors were in the range of 18-21 years of age. This probably explains the absence of viruria in the study of Goldmeier et al. (1983) as the mean age of those studied was 33 years. Based on the findings of others who have studied both viruria and virusemenia in homosexuals (Chapter 3) it would be anticipated that the rate of virusemenia would be higher than that of viruria in the present study group. This parameter was not assessed in this study as isolation specimens are not readily available.

The age distribution of viruric homosexuals suggests that much of this excretion is related to recent primary infection. In the present study this is also borne out by the srong inverse correlation between viruria and the duration of homosexual activity, in particular, the strikingly high incidence of viruria in those with less than two years of homosexual experience. This evidence does not rule out the possibility that reactivations and/or reinfections may occur

in older homosexuals, but suggests that these infections are not detected using normal virus isolation procedures. Virus excretion following primary infection is often prolonged, at least in those with CMV-mononucleosis (Chapter 3). It is therefore possible that homosexuals who have recently seroconverted are a significant source of infection to seronegative partners. Follow up of contacts may have clarified this but was not possible. The main argument against this sequence of events being responsible for the proposed high seroconversion rate among inexperienced homosexuals is that it is dependent upon a certain level of promiscuity for its perpetuation whereas viruria in this group was entirely confined to those reporting only one recent sexual partner. It is still possible that infection in these individuals may have followed contact with a promiscuous homosexual who was actively infected at the time, however the absence of detectable viruria in the promiscuous group and the lack of correlation between the number of recent partners and antibody prevalence suggest that other factors are responsible for the high exposure to CMV in homosexuals. Insufficent data was available to determine the duration of excretion in the present study.

b) CMV-IgM detection in male homosexuals

CMV-specific IgM is a useful diagnostic marker for CMV infection and in non-immunocompromised individuals is indicative of primary infection. The standard detection method is indirect immunofluorescence, however this has been

shown to be both insensitive and non-specific (Griffiths et al. (1982b). In accounting for the possibilities that the prevalence of CMV-IgM may be low in the groups under study and that IgM titres may be low in asymptomatically infected individuals, it was considered important to use an alternative CMV-IgM detection assay in order to obtain an indication of its prevalence which was as accurate as possible. An indirect RIA (Kangro, 1980) was considered but was found to be time-consuming and very demanding on the quality of antigen used; most batches were found to be unsuitable for use in this assay. It was therefore decided to develop an IgM-capture assay. This type of assay has at least two theoretical advantages over indirect IgM assays. Firstly the presence of specific IgG antibody at high titre does not inhibit the detection of IgM as the initial test serum reaction is entirely IgM specific. In indirect assays it is necessary to use a large excess of antigen or to selectively remove IgG from test specimens to overcome this problem. Secondly the presence of IgM rheumatoid factor (antibody which binds to the Fc component of IgG when the latter is bound to antigen) should not give false positive results, except perhaps in sera with antigen-antibody complexes or denatured antibody, as IgG is removed by washing prior to the addition of antigen.

Initial investigation was aimed towards developing an IgM-capture RIA (MACRIA) using radiolabelled monoclonal antibody (McAb) to detect IgM-bound antigen. The major problem encountered appeared to be the non-specific binding

of antigen to the solid phase in the absence of solid phase-bound specific IgM. As this resulted in poor differentiation between the strong positive and negative control sera, the test was considered unsuitable for the detection of low titre IgM antibody. Initial experiments using a MACELISA system indicated that this problem persisted when McAbs were used as detector antibody but was much less noticeable when a rabbit antiserum was used. This may indicate that the McAbs used were reactive with antigens which tended to bind to the solid phase non-specifically whereas the rabbit antiserum reacted with antigens which did not. The McAbs were directed against a variety of antigens however and it is also possible that a component of the test antigen which bound to the solid phase non-specifically also reacted with mouse IgG in a non-specific way.

It was decided to develop the MACELISA with rabbit antiserum as detector antibody (MACELISA 1) using unlabelled rabbit antibody followed by enzyme-labelled anti-rabbit IgG rather than use enzyme-labelled rabbit antibody, as it was considered that the extra amplification step may give greater sensitivity. The assay was found to be sensitive when compared to the results of previous testing by indirect RIA, but the suspicion that this sensitivity was somewhat selective was justified in the light of results from the modified assay using biotinylated McAb as detector antibody (MACELISA 2). This assay was of comparable sensitivity to the VirEnz - M CMV kit but was also found to be selective in its detection of positive sera. This is perhaps a disadvantage of

the IgM-capture principle compared to the indirect system where IgM reactive with all components of the antigen is detected. The former assay type does however offer the possiblity of measuring IgM responses to individual antigens which may be of value in distinguishing between acute and convalescent phases of infection, or between primary and non-primary infection in immunocompromised patients. It is probable that a MACELISA of broader sensitivity could be acheived by using a pool of biotinylated McAbs selected for their antigenic reactivity as detector antibody. In the absence of such an assay the results of the two MACELISA tests were combined for the purposes of this project.

Some sera from patients with infectious mononucleosis (IM) were found to give high IgM values in these assays, particulary in MACELISA 2. These sera do not appear to be reactive in indirect RIA tests for CMV-IgM (Kangro, 1980; Rasmussen et al., 1982) but are known to be reactive in the CMV-IgM IIF (Hanshaw et al., 1972), in a CMV MACRIA using radiolabelled human immune serum as detector antibody (Sutherland & Briggs, 1983) and in a rubella MACRIA using radiolabelled McAb as detector antibody (Morgan-Capner et al., 1983). In view of this last observation in particular, it is likely that this reactivity is due to the polyclonal IgM stimulation that occurs in IM (Chapter 3) and is not due to cross-reactivity between EBV-IgM and other viral antigens. In the development of MACELISA 2 it was noticed that one serum from an IM case gave positive results when three different McAbs were used as detector antibody; one of

which reacted with an immediate early glycoprotein, one with an early nuclear antigen and one with a late glycoprotein. It is unlikely that EBV-IgM antibodies would be cross-reactive with three different CMV antigens and this too points to polyclonal IgM stimulation as the source of this reactivity. Testing of MACELISA positive specimens by EBV-IgM IIF is a straightforward means of identifying such reactivity as the EBV-CMV cross-reactivity is one way (Horwitz et al., 1977) probably due to the lower level of polyclonal IgM stimulation that occurs in CMV infection.

Some sera from patients with other acute infections gave high background IgM values. This may have been due to increased hydrophobic interactions between glycoproteins and the solid phase in the presence of solid phase-bound non-specific IgM.

Application of these MACELISA tests in the screening of plasmas from the homosexual and heterosexual study groups revealed a significantly higher prevalence of IgM antibodies in the homosexual group, which was confined to the lower age groups and to those with a shorter history of sexual activity. Although statistical analysis was not possible within the heterosexual cohort it was noticed that the three IgM positive individuals were not restricted to the lower age range. These results supplement those of virus isolation and provide further evidence for the hypothesis that primary CMV infection occurs commonly in young homosexuals. The less than complete concordance between viruria and the presence of CMV-IgM probably reflects the fact that the phase of virus

excretion only partially overlaps with the period when IgM is present (Chapter 3).

These results are in contrast to those reported by Mintz et al. (1983) who found that CMV-IgM was present in 95% of homosexuals on at least one occasion and was present in 66% of all specimens from homosexual men. IgM was unrelated to virus excretion. This suggests that immune function was deranged in this American group of homosexuals. This may have resulted from excessive immune stimulation from multiple antigenic exposures encountered as a result of highly promiscuous behaviour. It is also possible that polyclonal stimulation of immunoglobulin synthesis associated with asymptomatic stages of HTLV III infection was responsible. Certainly this is not a constant feature of all homosexual groups in the light of the present study.

There was some indication from the testing of retrospective sera that persistence or reactivation of CMV-IgM occurred in the homosexuals under study here also. Prospective study is required to establish how frequently this occurs and to elucidate its causes.

Antibodies to CMV IEA and CMV EA in male homosexuals

Antibodies to CMV IEA and EA are present in a proportion of seropositive individuals. As discussed in Chapter 3, their significance, if any, is not known. Antibody to CMV EA has been found with greater frequency in transplant patients (The et al., 1977) who are known to be susceptible to serious CMV infections, and have been found at high titre in patients with

early rheumatoid arthritis (Male et al., 1982). CMV excretion has been observed in these patients both prior to and during corticosteroid therapy (Dowling et al., 1976). This indicates that the development or persistence of CMV EA-IgG occurs more readily when CMV infection takes place in certain clinical settings. The significantly increased prevalence of CMV EA-IgG in seropositive homosexuals compared to heterosexuals also indicates that their presence is of some significance.

Screening for the presence of CMV IEA-IgG has not been used in seroepidemiological surveys of this nature before, although the time course of their development following infection or vaccination has been studied in some groups. The present results demonstrate that their presence may also be of significance. The difference in prevalence between the two study groups is less striking however than that of CMV EA-IgG, largely due to the greater background prevalence of CMV IEA-IgG in the heterosexual group.

A number of explanations may be offerred to account for this difference in the prevalence rates of these antibody reactivities. Their greater prevalence in seropositive homosexuals may be due to the fact that primary CMV infection has been a more recent occurrence in a greater proportion of this group, since these antibodies may decline in titre with time in a proportion of cases (The et al., 1984). Several observations argue against this. Firstly, CMV EA-IgG was not observed any more frequently in those who were viruric or CMV-IgM positive compared to those who were not (although CMV IEA-IgG was universal in those who were IgM positive.)

Secondly, the presence of these antibodies in seropositive homosexuals did not correlate in any way with age or duration of sexual activity. More of the younger and less sexually experienced would be expected to be CMV IEA- and CMV EA-IgG positive if their presence was related to recent infection. Thirdly, the limited study in which repeat specimens from seropositive laboratory personnel were tested indicates that the presence or absence of these antibodies, particularly CMV IEA-IgG is a relatively constant feature in many individuals. In no case was a decline in their titre observed in this study. Their presence does not therefore seem to be related in a temporal fashion to CMV infection.

A second possibility is that the presence of CMV IEA- and CMV EA-IgG is a reflection of the frequency of reactivation and/or reinfection, or possibly of the level at which immune control of "latent" infection operates. To test this would require the development of more sensitive means of detecting CMV infection. If true however this may indicate a reduced capacity to contain latent infection or to resist reinfection in homosexuals. Yao et al. (1985) have shown that the EBV carrier state in seropositive individuals may be maintained at different levels, reflected in the extent of oral excretion of virus. In no way, however was the nature of the carrier state related to the serological profile.

A third possibility is that the development and persistence of CMV IEA- and CMV EA-IgG is determined by the extent of viral replication during the initial infection. Where this is considerable the increased antigenic load may

result in greater immunisation to antigens which are of low immunogenicity in other infections. The extent to which such antigens are presented to the immune system may determine whether the antibody response is detectable or not and, if so, whether it is transient or persistent. Increased viral replication may occur if the local immune response at the site of initial infection is deficient or is bypassed. The importance of local immune functions has often been overlooked. However it is becoming apparent that they may significantly influence events at later stages of infection (Dhar & Ogra, 1985). CMV infection probably begins at the oral or genital mucosal membranes in most individuals, where local immune functions are highly active. In homosexuals, CMV infection may also be encountered via the rectal mucosa. The possibility that this structure may be breached, allowing infectious agents to avoid the local immune response has already been considered. The presence of immunomodulating agents in semen may also inhibit local immune responses at this site. This particular infection route may then not only result in efficient transmission of CMV infection, but may also favour more extensive viral infection resulting in greater stimulation of humoral immunity at later stages of infection.

Cellular immunity in male homosexuals

In view of the prevalence of AIDS in male homosexuals the functional capacity of immune responses in this group prior to disease has been the subject of frequent speculation. This

study provides no evidence of this in male homosexuals, as determined by CMV-specific and mitogen-induced lymphocyte proliferation responses. It could be argued that this assessment of cellular immunity is unsuitable for detecting all but gross abnormalities in immunity; the standard deviations observed in mitogen responses were large and would probably mask slight impairments in the response. Others however have observed lowered responses in healthy homosexuals (Chapter 4). This may be caused by the early stages of AIDS-related immunodeficiency in some as Krohn et al. (1985) found that serological analysis for HTLV III infection distinguished those who had reduced mitogenic responses from those who did not. That healthy, HTLV III infection-free homosexuals are immunocompromised to any significant extent therefore remains to be established. It is possible that assessment of other aspects of acquired and innate immunity which have been shown to be deficient in AIDS such as NK and CTL functions may reveal differences in healthy homosexuals.

Absence of CMV-specific lymphocyte proliferation responses in healthy seropositive individuals was frequently recorded in this study and has been reported by others (Chapter 3). The reason(s) for this are not known, however sequential investigation of healthy laboratory personnel indicated fluctuations in responsiveness in some individuals. The relevance of this assay as an assessment of CMV-specific cellular immunity, then is uncertain. The presence of serum inhibitory factors did not appear to be responsible for unresponsiveness in this study, except perhaps in a few cases

which are interesting because they include two individuals who had evidence of CMV and EBV infection.

Proliferative responses revealed no consistent immune deficiency in those with current or recent active CMV infection. This may indicate that asymptomatic CMV infection is not associated with the immunological effects observed in CMV-mononucleosis and other CMV syndromes. Clearly further work is required in this area.

Antibodies to HTLV III in the homosexual study group

The results of HTLV III serological analysis clearly show that the homosexual population under study was largely free of infection with this virus as had originally been assumed. Two or possibly three of the 84 studied were seropositive. It could not be decided if the discrepancy between Western blot immunostaining and IIF was due to insensitivity of the former or nonspecificity of the latter, however this combination of tests is the best indication of seropositivity that is currently available. One of those who was seropositive was one of the individuals with evidence of a persistent or reactivated IgM response to CMV. This individual may have experienced an unusually vigourous non-primary CMV infection in the setting of HTLV III infection. It is also possible however and perhaps more likely that the CMV-IgM detected was the result of polyclonal stimulation of immunoglobulin synthesis associated with HTLV III infection. This might explain the unusual IgM profile in this case as indicated by the striking differential reactivity in the two MACELISA tests. Other than this, no

abnormalities were noticed among the HTLV III seropositive individuals although the numbers are clearly too small for meaningful comparison.

Conclusion

This project clearly demonstrates a high prevalence of CMV infection in healthy male homosexuals who are not in incubation or prodromal phases of AIDS. This infection appears to be acquired readily through homosexual contact and this is probably related to the nature of this contact rather than to its frequency. The profile of the humoral immune response to CMV suggests that CMV stimulates immune responses to a greater extent than usual in many homosexuals. This may be due to more frequent reactivation or reinfection, which may point to defective immune control of the virus carrier state. Alternatively it may indicate greater viral replication during a past infection. This might also indicate impaired immunity, or it may be related to the route of infection. These possibilities of course are not mutually exclusive. Control of infection appears to be achieved eventually as older homosexuals have no evidence of active infection. Transient or low grade virus excretion in such individuals may nevertheless be a significant source of infection for other homosexuals.

The present results do not indicate any suppression of cellular immunity during active CMV infection in individuals who remain asymptomatic. However this remains an open question and requires testing using more sensitive assays than

lymphocyte proliferation responses proved to be in this project. Studies of cellular immunity in other CMV-infected patients show that cytotoxic responses are more relevant to protection and recovery from CMV infection (Chapter 3) and investigation of these responses in the group under study would be of value.

Although active but subclinical CMV infection may not normally cause significant disturbances in immunity, this may not be the case if other infections such as HTLV III infection concur. It is possible that HTLV III infection occurring in a young homosexual may result in exacerbation of low grade CMV infection which had not yet been eliminated following primary infection. CMV infection may then cause further cycles of immune suppression. Similar events may also occur in older, more CMV-immune individuals although a more vigourous initial immunosuppressive event may be required to lower immune functions below the threshold at which reactivation of CMV infection occurs. Such cycles may be predicted to result in a more severe immune dysfunction and clinical manifestations of AIDS after a shorter incubation period than would be expected in the absence of concurrent CMV infection.

Little is known concerning events in the incubation phase of AIDS in different groups and their possible influence on the length of this period. Determination of the incubation period is difficult as the original exposure which resulted in infection usually cannot be pinpointed accurately in time. There is some limited data avaiable however. The time of

exposure can be identified reasonably confidently when transfusion-associated infection occurs in otherwise low-risk individuals and Jaffe et al. (1984) determined a mean incubation period of 24.6 months in those patients who had developed AIDS by the time of assessment. Hanrahan et al. (1984) studied intravenous drug users who developed AIDS following imprisonment. Taking the time of imprisonment as the most recent possible time of exposure, a minimum average incubation period of 22.6 months was arrived at. In contrast Auerbach et al. (1984) in studying male homosexuals with AIDS in whom the probable time of exposure could be estimated by follow-up of contacts, calculated a mean incubation period of 10.5 months.

This remarkably shorter incubation period may be due to the occurrence of KS in many of these homosexuals. KS may occur in less severely immunosuppressed patients, but as its occurrence results in fulfillment of the criteria by which AIDS is diagnosed, an apparently shorter incubation phase may be observed. In view of the possible association between CMV infection and KS, the development of the latter in homosexuals with AIDS may support the concept that HTLV III infection results in increased or altered expression of CMV infection. A greater frequency of interactions of this nature in homosexuals may be due to the higher rate of CMV infection and its more active state in a proportion. A more extensive replication of HTLV III during the initial acute infection may also be responsible. Since HTLV III infection may be acquired by the same route as CMV infection in homosexuals, what has

been discussed already with regard to the possible consequences of acquisition of CMV infection via this route may also apply to HTLV III infection. This may result in more efficient reactivation of other infections such as CMV or EBV. These infections may then contribute both to the progressive ablation of immune function and also to the appearance of malignant disease.

The other group of AIDS patients in which KS is more common are cases of African origin. CMV infection is also highly prevalent in this population and usually occurs in infancy (Chapter 2). Infection at this age has been suggested to result later in life in an increased propensity to transmit CMV infection to the foetus during non-primary maternal infection (Chapter 3) and may also predispose to the development of KS in the setting of HTLV III infection.

Further studies

The work presented in this thesis should be considered as the first part of an ongoing study. The issues raised regarding the effects of CMV infection in male homosexuals, its possible differences from infections in other settings and its possible interactions with other infections, in particular with HTLV III infection all warrant further investigation. These issues are important not only in the immediate context of the pathogenesis of CMV infection, HTLV III infection and of AIDS, but also in the wider context of viral pathogenesis in general.

The conclusions that have been reached are based on epidemiological evidence from a cross-sectional survey of homosexuals and heterosexuals. This should ideally be followed up by a longitudinal survey in smaller numbers which would enable the persistence of viral excretion and of IgM responses to be determined. This would also allow the time course of CMV IEA-IgG and CMV EA-IgG responses to be determined and their possible significance to be evaluated further. It is possible that application of Western blot immunostaining technology or of RIA/ELISA techniques using antigens captured by solid phase-bound McAbs may permit further breakdown of the humoral response to CMV infection and this may reveal further differences between the study populations. Longitudinal study may also allow the temporal sequence of events associated with HTLV III infection to be elucidated. This type of study would be expected to yield results reasonably quickly if conducted among young, inexperienced male homosexuals in view of their high susceptibility to CMV infection.

Other aspects of CMV-specific and general immunity could be assessed in a cross-sectional survey such as that of the present project. Determination of CMV-IgG titres in addition to merely screening for their presence may be of value. It was anticipated that this would provide little additional information in this study; as CMV antigen preparations used for serological methods consist of immediate early, early and late antigens the magnitude of CMV-IgG titres would probably be influenced to a greater or lesser extent by the presence

or absence of CMV IEA-IgG and CMV EA-IgG. Detection of CMV-IgA antibodies may have provided additional information regarding the prevalence of current or recent CMV infection. This would probably be most readily accomplished using an IgA capture assay analogous to the MACELISA.

Cellular immune parameters are also worthy of study on a longitudinal basis. This may indicate fluctuations in response to infection which would not be identified from single determinations.

Future studies might also investigate the prevalence and effects of other infections in homosexuals such as EBV. Significant associations may be less readily demonstrable in this case however in view of the generally higher prevalence of past infection in the general population. In addition, detection of EBV excretion is not a standard procedure in most laboratories.

Finally, the rationale and techniques employed in this project may be suitable for the characterisation of CMV infection in other populations or patient groups where an abnormal pathogenesis is suspected. Where associations exist, they may be even more striking than those in homosexuals as the departures from normal may be subtle in many of the latter group. Clearly much remains to be clarified regarding CMV infection and its "protean clinical manifestations" (Weller, 1971). Epidemiological analysis will be an important aspect of future endeavours to this end.

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ABBREVIATIONS

ADCC	Antibody dependent cellular cytotoxicity
AIDS	Aquired immune deficiency syndrome
ARC	AIDS-related complex
ATG	Anti-thymocyte globulin
BCGF	B cell growth factor
B-(McAb)	Biotinylated McAb
BSA	Bovine serum albumin
CF	Complement fixing
CMV	Cytomegalovirus
Con A	Concanavalin A
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
DM	Dulbecco Medium
DTH	Delayed-type hypersensitivity
EA	Early antigen(s)
EBV	Epstein-Barr virus
ELISA	Enzyme-limked immunosorbent assay
EMEM	Eagles minimal essential medium
FCS	Foetal calf serum
FeLV	Feline leukaemia virus
FITC	Fluorescein isothyocyanate
GE	Glycine extract
GM	Growth medium
HBsAg	HBV surface antigen
HBV	Hepatitis B virus
HEF	Human embryo fibroblasts

HLA	Human leukocyte antigen
HSV	Herpes simplex virus
HTLV	Human T cell leukaemia-lymphoma virus or human T lymphotropic virus
IE	Immediate early
IEA	IE antigen(s)
IFN	Interferon
IIF	Indirect immunofluorescence
IL	Interleukin
IM	Infectious mononucleosis
K	Killer
KS	Kaposi's sarcoma
MACELISA	M antiboby capture ELISA
MACRIA	M antibody capture RIA
McAb	Monoclonal antibody
MHC	Major histocompatibility complex
MM	Maintenance medium
moi	multiplicity of infection
NBCS	New-born calf serum
NGU	Non-gonococcal urethritis
NK	Natural killer
NRS	Normal rabbit serum
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBSAT	PBS plus 1% BSA and 0.05% Tween 20
PBST	PBS plus 0.05% Tween 20
PEG	Polyethylene glycol

PHA	Phytohaemagglutinin
PMNL	Polymorphonuclear leukocyte
PWM	Pokeweed mitogen
RIA	Radioimmunoassay
RIP	Radioimmunoprecipitation
RT	Room temperature
SAPU	Scottish Antibody Production Unit
SBI	Specific binding index
SI	Stimulation index
TBS	Tris-buffered saline
TBST	Tris-buffered saline plus 0.025% Tween 20
T4+	T cells of helper/inducer phenotype
T8+	T cells of suppressor/cytotoxic phenotype
VZV	Varicella zoster virus
vDNA	Viral DNA
vmRNA	Viral mRNA

APPENDIX

A SUMMARY OF DEMOGRAPHIC DETAILS AND RESULTS OF SEROLOGICAL AND VIROLOGICAL INVESTIGATION OF MALE HOMOSEXUALS AND HETEROSEXUALS.

KEY TO APPENDIX

Hom = male homosexual

Het = male heterosexual

Recent partner number = number of different sexual partners reported in the preceding three months.

U = unknown

CMV-IgG = IgG antibodies to CMV measured by indirect ELISA.

CMV IEA-IgG = IgG antibodies to CMV immediate early antigens detected by indirect immunofluorescence (IIF).

CMV EA-IgG = IgG antibodies to CMV early antigens detected by IIF.

CMV-IgM = IgM antibodies to CMV measured by IgM capture ELISA.

+ = parameter in question was detected.

- = parameter was not detected.

NT = not tested.

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
H9499	Het	30	16	1	+	-	+	-	-
K890	Hom	44	26	2	+	+	+	-	NT
K5914	Hom	37	15	2	+	+	-	-	NT
K6561	Hom	26	10	3	+	+	-	-	-
K7457	Het	26	U	1	-	-	-	-	-
K7512	Hom	36	16	2	+	+	-	-	-
K7967	Hom	29	11	2	+	+	+	-	NT
K9214	Hom	41	21	2	+	+	+	-	-
M653	Hom	26	8	8	+	-	+	-	NT
M946	Hom	24	8	3	+	-	+	-	-
M1968	Hom	31	10	1	+	+	-	-	-
M2244	Hom	43	U	1	+	+	-	-	-
M2866	Het	34	14	1	+	+	+	-	-
M3042	Hom	24	8	1	+	+	+	-	NT
M3060	Het	28	U	1	-	-	-	-	-

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
M4158	Hom	33	15	3	+	-	+	-	NT
M4394	Hom	33	10	2	+	-	+	-	-
M4812	Hom	25	8	2	-	-	-	-	NT
M5058	Hom	21	7	1	+	-	-	-	+
M5928	Hom	31	15	6	+	-	-	-	NT
M6266	Hom	30	11	2	-	-	-	-	-
M6615	Het	25	U	1	+	NT	-	-	-
M6845	Hom	42	22	2	+	+	+	-	NT
M6847	Hom	24	10	12	+	+	+	-	NT
M7228	Het	28	U	2	-	-	-	-	-
M8018	Hom	21	3	1	+	+	+	-	NT
M8092	Hom	21	8	10	+	+	+	-	-
M8661	Het	22	3	2	-	-	-	-	NT
M8723	Hom	23	6	3	+	-	-	-	-
M9415	Hom	24	8	4	+	-	-	-	-

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
M9516	Het	27	U	2	-	-	-	-	-
M9937	Hom	18	4	2	+	+	+	+	NT
P118	Het	21	2	1	-	-	-	-	NT
P341	Hom	23	4	10	+	+	+	-	NT
P564	Hom	24	6	1	-	-	-	-	NT
P706	Hom	45	25	>20	+	-	+	-	NT
P1122	Hom	25	9	2	+	-	-	-	-
P1410	Hom	35	16	12	-	-	-	-	-
P1805	Het	21	U	1	-	-	-	-	-
P2039	Hom	33	15	8	+	+	+	-	-
P2152	Het	22	3	1	-	-	-	-	-
P2182	Hom	22	3	2	+	+	-	-	-
P2768	Hom	36	15	1	+	-	-	-	-
P2827	Hom	22	6	3	+	+	-	-	NT
P3007	Hom	20	3	3	+	+	+	-	NT

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P3455	Het	22	3	2	-	NT	-	-	-
P3688	Het	20	2	1	-	-	-	-	-
P3713	Hom	23	6	1	-	-	-	-	-
P3848	Hom	23	U	1	+	+	+	-	-
P3881	Hom	33	16	1	+	+	+	-	-
P4001	Hom	25	3	6	+	+	-	-	-
P4153	Hom	25	4	1	-	-	-	-	-
P4331	Hom	18	3	2	+	+	+	+	+
P4351	Hom	33	16	1	+	+	+	-	-
P4541	Hom	29	11	2	+	-	-	-	NT
P4604	Hom	23	3	1	+	+	+	-	-
P4624	Hom	22	3	2	-	-	-	-	NT
P4683	Het	19	U	1	+	+	-	-	-
P4710	Hom	20	6	2	+	+	+	+	NT
P4780	Hom	35	U	2	+	-	-	-	NT

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P5056	Hom	19	1	1	-	-	-	-	-
P5058	Hom	21	4	2	+	-	+	-	NT
P5278	Het	20	2	1	-	-	-	-	NT
P5586	Het	19	2	1	-	-	-	-	NT
P5612	Hom	22	5	6	+	+	+	+	NT
P5666	Hom	21	3	2	-	-	-	-	MT
P5832	Hom	29	8	2	+	-	-	-	NT
P5997	Hom	20	1	2	+	+	+	-	+
P6069	Het	21	U	1	+	-	-	-	NT
P6074	Hom	25	6	2	-	-	-	-	NT
P6091	Het	18	3	4	-	-	-	-	-
P6097	Het	22	U	1	-	-	-	-	NT
P6147	Het	27	U	2	-	-	-	-	NT
P6155	Hom	29	U	U	-	-	-	-	NT
P6224	Het	24	U	1	-	NT	-	-	NT

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P6239	Het	25	U	1	+	-	-	-	NT
P6335	Het	21	U	1	-	-	-	-	NT
P6470	Het	23	U	1	-	-	-	-	-
P6592	Hom	19	1	1	+	-	-	-	+
P6671	Hom	27	10	1	+	+	-	-	NT
P6674	Het	18	U	1	-	NT	-	-	-
P6685	Het	28	U	2	+	-	-	-	-
P6687	Het	27	U	1	-	NT	-	-	-
P6821	Hom	20	U	2	+	+	+	-	-
P6824	Het	30	U	1	+	-	-	-	NT
P6860	Het	27	U	U	-	NT	-	-	-
P6867	Het	22	U	1	+	NT	+	+	NT
P6872	Het	21	U	1	-	-	-	-	NT
P6882	Het	23	U	1	-	-	-	-	NT
P6897	Het	16	0.5	1	-	NT	-	-	-

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P6898	Hom	34	10	1	-	-	-	-	-
P6907	Hom	22	3	1	-	-	-	-	NT
P6910	Het	21	U	1	+	-	-	-	NT
P6935	Het	18	U	1	-	-	-	-	-
P6976	Het	20	3	2	-	-	-	-	NT
P6977	Het	23	4	1	-	-	-	-	NT
P6980	Hom	22	4	3	-	-	-	-	NT
P6987	Hom	21	2	2	+	+	-	-	NT
P7004	Het	48	1	1	+	+	+	-	NT
P7045	Het	22	U	2	-	-	-	-	NT
P7046	Het	19	U	2	-	-	-	-	NT
P7072	Het	28	U	1	+	NT	-	-	NT
P7097	Het	22	U	1	+	NT	-	-	NT
P7102	Het	20	U	2	-	-	-	-	NT
P7105	Het	23	U	1	-	-	-	-	NT

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P7125	Het	28	U	2	+	-	-	-	NT
P7129	Het	24	U	1	-	-	-	-	-
P7138	Het	30	U	2	+	+	-	+	-
P7141	Het	18	1	1	-	-	-	-	NT
P7145	Hom	30	8	3	+	+	+	-	-
P7181	Het	21	U	2	+	-	+	-	NT
P7202	Hom	24	5	8	+	+	-	+	NT
P7224	Het	17	U	1	+	-	-	-	-
P7226	Het	24	U	1	-	-	-	-	-
P7247	Het	27	U	2	-	-	-	-	-
P7252	Het	24	U	2	-	-	-	-	NT
P7254	Het	17	1	1	-	-	-	-	-
P7262	Het	22	U	2	+	+	-	-	-
P7280	Het	18	0.5	1	-	-	-	-	NT
P7286	Het	27	U	1	-	-	-	-	NT

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P7300	Het	18	U	1	-	-	-	-	-
P7304	Het	20	U	1	-	-	-	-	-
P7305	Het	24	U	1	-	-	-	-	-
P7314	Het	18	1	1	-	-	-	-	NT
P7315	Het	23	U	1	-	NT	-	-	-
P7319	Het	28	U	1	-	-	-	-	-
P7321	Hom	17	0.25	2	-	-	-	-	NT
P7334	Het	24	U	1	-	-	-	-	NT
P7337	Het	21	U	2	-	-	-	-	-
P7348	Het	20	U	1	-	-	-	-	-
P7356	Het	29	U	1	+	-	-	-	-
P7367	Het	25	U	2	-	-	-	-	-
P7370	Het	19	U	1	-	-	-	-	-
P7372	Het	19	U	1	-	-	-	-	-
P7376	Het	27	U	U	-	-	-	-	-

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA-IgG	CMV EA-IgG	CMV-IgM	Viruria
P7379	Het	25	U	5	-	-	-	-	-
P7388	Het	16	0.25	2	-	-	-	-	NT
P7392	Het	23	U	1	-	-	-	-	-
P7398	Het	20	U	1	-	-	-	-	NT
P7435	Het	27	U	1	-	-	-	-	NT
P7438	Het	21	U	1	-	-	-	-	-
P7450	Het	29	U	2	-	-	-	-	-
P7454	Het	23	U	U	-	-	-	-	-
P7466	Het	19	U	1	-	-	-	-	NT
P7473	Hom	21	3	2	+	+	+	+	+
P7476	Het	22	U	1	-	-	-	-	NT
P7479	Het	19	U	2	-	-	-	-	NT
P7642	Het	22	U	1	-	-	-	-	NT
P7654	Het	18	U	2	-	-	-	-	-
P7655	Hom	28	U	2	-	-	-	-	-

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P7656	Het	24	U	2	-	-	-	-	-
P7658	Het	19	U	1	-	-	-	-	-
P7661	Het	23	U	1	-	-	-	-	-
P7663	Hom	20	3	3	+	+	+	+	-
P7680	Het	17	U	1	-	-	-	-	-
P7693	Het	17	0.25	1	-	-	-	-	-
P7757	Hom	25	5	2	+	+	+	-	-
P7759	Het	21	3	2	-	-	-	-	-
P7760	Hom	21	4	3	+	+	-	-	NT
P7775	Het	26	4	3	+	+	+	-	NT
P7779	Het	21	2	1	-	-	-	-	-
P7802	Het	20	U	1	+	+	+	-	-
P7806	Het	18	1	1	-	-	-	-	NT
P7807	Het	19	U	1	-	-	-	-	-
P7819	Hom	27	6	2	-	-	-	-	-

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P7821	Het	20	3	1	-	-	-	-	-
P7824	Het	23	4	1	+	+	-	-	+
P7829	Het	20	2	1	+	-	-	-	NT
P7832	Het	18	U	1	-	-	-	-	-
P7842	Hom	21	3	1	+	+	-	-	NT
P7854	Het	24	U	1	-	-	-	-	-
P7886	Het	37	17	1	+	+	-	-	-
P7888	Het	20	1	1	-	-	-	-	-
P7890	Het	22	3	2	-	-	-	-	-
P7892	Hom	24	4	6	-	-	-	-	-
P7894	Hom	25	6	2	+	+	+	-	+
P7895	Hom	18	1	2	+	+	+	-	+
P7910	Het	22	U	1	+	+	-	-	-
P7914	Hom	35	15	2	+	+	+	-	-
P7922	Het	19	1	1	-	-	-	-	NT

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P7923	Hom	28	8	1	+	+	+	-	-
P7932	Hom	18	U	1	+	+	-	-	-
P7938	Het	19	U	1	-	-	-	-	-
P7941	Het	24	6	1	-	-	-	-	-
P7992	Het	23	1	1	-	-	-	-	-
P7997	Het	21	4	1	-	-	-	-	-
P7999	Hom	26	U	1	-	-	-	-	-
P8040	Hom	26	7	1	-	NT	-	-	NT
P8053	Het	22	1	1	-	-	-	-	-
P8060	Het	21	2	1	-	-	-	-	-
P8201	Het	28	U	1	+	+	-	-	-
P8215	Het	32	U	2	-	-	-	-	-
P8242	Het	19	1	1	-	-	-	-	NT
P8260	Het	19	U	1	-	-	-	-	-
P8264	Het	23	4	1	-	-	-	-	-

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P8269	Het	24	U	1	+	+	–	–	–
P8284	Het	19	U	1	–	–	–	–	NT
P8286	Het	23	U	1	–	–	–	–	–
P8295	Het	31	U	2	+	+	–	–	NT
P8303	Het	17	6	1	+	+	+	–	–
P8374	Het	19	U	1	–	–	–	–	NT
P8385	Het	19	U	2	–	–	–	–	–
P8391	Het	28	U	1	–	–	–	–	–
P8392	Het	27	U	2	+	+	–	+	–
P8407	Het	20	U	1	–	–	–	–	NT
P8417	Hom	18	2	2	+	+	+	–	–
P8424	Het	23	U	1	–	–	–	–	–
P8427	Hom	23	2	2	+	+	–	–	–
P8429	Het	19	U	1	–	–	–	–	–
P8430	Het	22	U	1	–	–	–	–	NT

Code no.	Sexual preference	Age	Sexual history (yrs)	Recent partner no.	CMV-IgG	CMV IEA -IgG	CMV EA -IgG	CMV-IgM	Viruria
P8453	Hom	17	1	2	-	-	-	-	-
P8454	Hom	22	2	1	+	-	-	-	-
P8455	Het	21	U	1	-	-	-	-	-
P8462	Het	21	U	1	-	-	-	-	-
P8469	Het	17	1	1	+	+	-	-	-
P8478	Het	21	U	1	+	-	-	-	-
P8578	Het	27	U	1	-	-	-	-	-
P8591	Het	16	0.02	1	-	-	-	-	-
P8594	Het	21	U	2	-	-	-	-	-
P8599	Het	18	2	1	+	-	-	-	-