CHARACTERIZATION OF AN Fc-RECEPTOR FOR HUMAN IgG IN THE TEGUMENT OF HUMAN CYTOMEGALOVIRUS

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Declaration

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SUMMARY

It is known that cells infected with human cytomegalovirus (HCMV) acquire the ability to bind human IgG by the Fc portion of the molecule, however, neither the functional role nor the physical nature of these receptors has been established. Electron microscopy using immunogold probes, has revealed the unexpected presence of an Fc receptor in the tegument of the HCMV particle itself. This study describes the isolation and characterization of that Fc receptor.

Pure Fc fragments of IgG were obtained by ion exchange chromatography and gel filtration of the products of concentrated IgG after hydrolysis with papain. Specific Fc probes were prepared by coupling the purified Fc fragments to colloidal gold particles. These were used for immuno-precipitation, as well as for visual identification of Fc reactions on Western blots or electron microscopy.

HCMV virions were obtained from the supernatant culture fluids of infected human embryonic fibroblasts (HEF) and concentrated by ultracentrifugation. Treatment with Triton X-100, caused disruption of the virion envelopes and allowed release of the tegument proteins into the Triton phase. The insoluble capsids did not bind the Fc-gold probes and were removed by centrifugation. Fc-binding proteins were immunoprecipitated from the Triton phase, using large colloidal gold particles coupled to the Fc portion of human IgG, and shown by SDS PAGE to consist of polypeptides of 70 and 33 kDa.

After electro-transfer to nitrocellulose membranes, the 33 kDa component retained the capacity to bind human Fc. In addition, the 33 kDa protein also reacted, on Western blots, with beta 2-microglobulin (β_2m). Previous work (Stannard, 1989) has shown by electron microscopy that β_2m, also binds to the tegument of the HCMV virion. In the present study, tegument proteins which reacted with β_2m yielded polypeptides identical in size to those which bind Fc. Identity was further confirmed by two procedures: firstly, proteins which had been precipitated by β_2m, could react, on Western blots, with Fc, (and vice versa); secondly, the Fc-binding protein which was extracted from a mixture of

viral proteins by affinity chromatography reacted equally strongly with both Fc and β_2m on dot blots.

These findings suggest that the Fc receptor in the tegument of the HCMV virion is the same as that which binds β_2m. The relevant protein appears to be a non-covalently linked hetero-dimer consisting of a 33 and a 70 kDa subunit; with the binding property for both Fc and β_2m residing in the 33 kDa subunit. This study has also demonstrated that the tegument Fc receptor of HCMV reacts not only with human IgG, but also with certain monoclonal antibodies, all of which belong to the IgG1 subclass of murine IgG.

Implications and relevance of the novel HCMV Fc receptor are discussed.

INTRODUCTION

HUMAN CYTOMEGALOVIRUS

Family: Herpesviridae

Subfamily: beta-herpesvirinae

Genus: cytomegalovirus

In the early twentieth century, the tissues of certain infants, dying of a systemic disease, were shown to contain enlarged or "cytomegalic" cells with characteristic intra-nuclear inclusions. The condition was termed Cytomegalic Inclusion Disease (CID). The pathology was initially attributed to a protozoon infection, but evidence for a viral aetiology for the disease was provided by Cole and Kuttner (1926) who were investigating a similar disease in guinea pigs. These workers showed that material from the salivary glands of guinea pigs, that had the typical histology of CID, could produce an infection (clinically and histologically similar to CID) after passage through a Berkfield N filter. This excluded the possibility that the infection was caused by a bacterium or protozoon.

In 1956, three different groups of investigators were successful in culturing the human virus *in vitro* in human embryonic fibroblasts (HEF). The laboratory-adapted strain, AD169 of human cytomegalovirus (HCMV), originates from virus isolated at that time from adenoidal tissue of healthy children (Rowe *et al.* 1956). Cytomegalovirus derives its name from the cytopathic effect that it causes in cell culture, namely the production of enlarged and refractile cells. Staining of these cells with haemotoxylin and eosin reveals the presence of prominent intranuclear inclusions which are identical to those seen in the tissues of infants dying of CID.

EPIDEMIOLOGY

Cytomegaloviruses are strictly species specific and humans are the only reservoir for HCMV. The virus may be detected in various body fluids such as saliva, urine, cervical or vaginal secretions, semen, breast milk, tears, faeces or blood. Transmission occurs with close person to person contact. After primary infection, the virus establishes a persistent infection in its host who may excrete

virus for prolonged periods. Virus may also be shed intermittently throughout life, which greatly increases the opportunity for horizontal transmission of infection to susceptible individuals. Crowding and lack of hygiene is associated with higher rates of infection. Thus in the third world, the majority of children have been infected by the age of puberty. Primary infection usually occurs in the first few years of life. One percent of infants are congenitally infected; others may be perinatally infected by maternal genital secretions during birth or, during the first few months of life, from virus excreted in breast milk. Transmission between toddlers and young children is common, although the precise route of infection is not known. In first world countries, only 40% of individuals have serological evidence of exposure to HCMV by the age of puberty. Thereafter, the percentage of infected individuals increases gradually until middle age. Oral or venereal contact probably accounts for transmission post puberty. Other potential sources of infection include blood transfusions and organ grafts (Alford and Britt, 1990; Griffiths, 1990).

CLINICAL MANIFESTATIONS

When infection occurs in childhood, symptoms are usually mild or inapparent. Older children and adults may suffer an infectious mononucleosis-like illness. Primary or recurrent infection with HCMV during pregnancy may result in congenital infection of the foetus, sometimes with serious consequences for the newborn. It is as yet unclear what factors determine both the transmission of virus in utero and the severity of disease in the foetus. Approximately five percent of congenitally-infected infants are born with clinical evidence of disease, which in severe cases may present as growth retardation, jaundice, hepato-splenomegaly, thrombocytopenia, encephalitis or microcephaly. In these cases the prognosis is poor. The infected infants who appear normal at birth (95% of all congenital infections) may later develop sequelae such as mental retardation, blindness, deafness and learning disorders (Alford and Britt, 1990; Griffiths, 1990).

Immunosuppression usually exacerbates the severity of infection caused by HCMV. Immunocompromised patients may develop pyrexia, pneumonitis, hepatitis, retinitis, leukopenia or thrombocytopenia. Disease is likely to be most severe in the case of a primary infection, but reactivation of latent virus or

reinfection by another strain of HCMV (which can occur despite the presence of both neutralizing antibody and specific cell-mediated immunity) can also cause significant pathology (Griffiths, 1990). A study by Grundy *et al.* (1988) involving sero-positive renal allograft recipients, showed that symptoms are more commonly associated with reinfection of the host with the donor strain of HCMV, than with reactivation of endogenous virus. In transplant recipients, infection with HCMV is also associated with an increased rate of graft rejection (Waltzer *et al.*, 1987). Whether this is due to decreased immunosuppression, or to an alteration in the graft tissue, is not clear. It may be relevant that infection of human fibroblasts with HCMV in vitro, is known to increase the expression of class I HLA antigens, the major target recognised by immune cells during graft rejection (Grundy *et al.*, 1988).

A report by Waltzer *et al.* (1987), which examined rejection rates in renal allograft recipients, indicated that the beneficial effects of matching donor and recipient HLA antigens was nullified when infection with HCMV supervened. In a similar study of liver transplant patients, who became infected with HCMV, O'Grady *et al.*(1988) found that, contrary to expectation, the risk of chronic rejection was greater in those cases where the donor and recipient tissues were well matched with respect to the DR (HLA class II) antigens, than in those cases where the tissues were poorly matched. Chronic graft verses host disease has also been significantly associated with HCMV infection in bone marrow transplant patients (Lonqvist *et al.*, 1984). Clearly, the effect of HCMV infection in graft recipients is more complex than that of an opportunistic infection in an immunocompromised host.

VIRUS MORPHOLOGY AND MORPHOGENESIS

The morphology of HCMV virions is typical of that of all herpesviruses. Particles, approximately 150-200 nm in diameter, have a membranous envelope surrounding a 100 nm icosahedral capsid which contains the dsDNA genome. The envelope contains a variety of virus-encoded glycoprotein projections. Between the capsid and the envelope is a proteinacious layer termed the tegument or matrix. An additional particle, known as a "dense body", is formed in HCMV infected cells. These large dense particles consist of predominantly tegument proteins, surrounded by a membrane containing viral

glycoproteins, but lack a capsid and nucleic acid and are therefore non-infectious.

In vivo, HCMV appears to replicate predominantly in cells of epithelial origin. *In vitro,* however, only human fibroblasts are fully permissive to productive infection by HCMV. The virus is also capable of adsorbing to, and penetrating a number of other cell types *in vitro*, but replication does not advance beyond the immediate early phase. The block to complete viral replication in these cells has not yet been elucidated (Sissons *et al.*, 1986).

The major receptor for HCMV on human fibroblasts is a 30 kDa membrane glycoprotein (Taylor and Cooper, 1990). After adsorption of the virus to its specific cell surface receptor, entry into the cell occurs by membrane fusion of the virion envelope with the plasma membrane. Little is known about the early events in the replication of HCMV, but it is assumed to be similar to that of herpes simplex virus (HSV). Virus is uncoated in the cytoplasm and naked capsids are transported to the nuclear pores where viral DNA is released into the nucleus.

Replication of HCMV proceeds under the control of sequentially activated viral genes. The first proteins to be synthesized are encoded by the immediate early or alpha genes. These are required for the transcription of mRNA for the second group of proteins, the beta or early genes. The β proteins are required for the replication of the viral genome. After multiple copies of viral DNA have been synthesized, the gamma or late genes (which code for structural virion proteins) are transcribed, and the proteins synthesized. This cascade synthesis of viral proteins is similar to that of HSV, except that in the case of HCMV, the period between DNA replication and the synthesis of the γ proteins, is delayed, resulting in a marked prolongation of the replication cycle.

Capsid assembly occurs in the nucleus and the virus acquires its envelope by budding through the inner nuclear membrane into the perinuclear space. From there, virions probably enter the endoplasmic reticulum and are transported to the golgi apparatus. Glycosylation and maturation of the virion envelope glycoproteins probably occurs in these organelles. Mature virions are transported in vesicles to the plasma membrane and are released from the cell.

VIRUS STRUCTURAL PROTEINS

Two proteins make up the capsid of the virion; a 150 kDa protein, known as the major capsid protein and a 34 kDa protein, known as the minor capsid protein. The minor capsid protein probably plays a role in capsid assembly (Stinski, 1990).

Proteins of the envelope are more complex. Three immunologically distinct, covalently-linked glycoprotein complexes have been separated from membrane preparations of HCMV after solubilization with Triton X-100. These complexes have been designated gcI, gcII and gcIII (Gretch *et al.*, 1988a):

The gcI complex consists of three protein species which, after reduction have a molecular weight of 95, 130 and 55 kDa (Farrar and Greenaway 1986). The 55 kDa protein has strong homology with the glycoprotein gB of HSV, Epstein-Barr virus (EBV) and Varicella-Zoster virus (VZV) (Cranage *et al.*, 1986). Morphologically, this protein appears as 12-14 nm protein spikes, occurring in clusters on the virion envelope (Stannard *et al.*, 1989).

The gcII complex is probably a multimeric form of a single protein which has a (reduced) molecular weight of 47-52 kDa (Gretch *et al.*, 1988b). This protein appears to be coded for by a number of homologous genes. It has not been shown to have homology to any other herpesvirus glycoprotein.

The gcIII complex is a heterodimer, consisting of subunits of 86 and 145 kDa (Gretch *et al.*, 1988). The 86 kDa protein appears to have some homology to the glycoprotein gH of HSV, particularly in the C-terminal half of the protein (Cranage *et al.*, 1988).

Although little is known about the role of the proteins that comprise the tegument of the virion, five genes have been identified which code for known tegument proteins (Stinski, 1990), all of which are phosphorylated proteins (pp):

The 71 kDa "upper matrix" protein (pp71) which is present in the virion, but not in dense bodies (Rueger *et al.*, 1987; Stinski, 1990). The function of this protein is not known.

A 65 kDa protein, known as the "lower matrix" protein, or pp65, is found in both virions and, abundantly, in dense bodies. This protein appears to be present in larger amounts in laboratory adapted strains than in wild type strains. It has protein kinase activity, phosphorylating serine and threonine residues (Rueger *et al.*, 1987; Stinski, 1990).

A matrix protein of 67 kDa (pp67), like pp65, also appears to have protein kinase activity (Davis and Huang, 1985). The function of kinase activity associated with tegument proteins is not known, although it has been suggested that they may play a role in regulating transcription, viral DNA synthesis or virus assembly (Stinski, 1990).

The tegument protein pp150, which occurs only in association with the virion and is absent from dense bodies, is highly immunogenic (Stinski, 1990). It has an unusual form of glycosylation, where N-acetyl glucosamine is O-glycosidically linked to the protein (Benko *et al.*, 1988). This form of glycosylation also occurs in nuclear proteins associated with the nuclear pore (Hart *et al.*, 1988).

The fifth phosphorylated matrix-tegument protein, pp28, is hydrophilic and highly immunogenic. Its function is not known (Meyer *et al.*, 1988).

INTERACTION OF HCMV WITH β_2m

While attempting to develop an enzyme-linked-immunosorbant assay (ELISA) system for the detection of HCMV, McKeating *et al.* (1985) observed that, although virus harvested from cell culture fluids was readily detectable by the test, virus from fresh urine failed to bind to the monoclonal antibodies attached to the ELISA plate. When the urine samples were re-tested after a period of storage, many of the samples which had originally been assayed as negative, became positive. They concluded that fresh urine contained a factor which bound to the HCMV virion and masked antigenic determinants, but which was released, possibly by proteolysis, on storage. The same workers later showed that virus isolated from both urine and cell culture fluids was able to bind to the host protein beta-2 microglobulin (β_2m) (McKeating *et al.*, 1986) and

furthermore, that β_2m inhibited the attachment of specific monoclonal antibodies to whole HCMV virions (McKeating *et al.*, 1987).

β_2m is a 12 kDa protein which is non-covalently associated with the 45 kDa heavy chain of the class 1 HLA molecule. This protein complex is abundantly expressed on the surface of all nucleated cells and its major function is to bind peptides derived from the degradation of proteins synthesized within the cell, and to present them to T cells (Roitt, 1988). β_2m is shed from the surface of the cell and may be detected in small amounts in both serum and urine (Evrin and Wibell, 1972).

Studies by Grundy *et al.*(1987a; 1987b), indicated that viruses in cell culture did not associate with β_2m intracellularly, but bound β_2m only after the release of virions from cells. In addition they reported that attachment of β_2m to HCMV, resulted in enhanced infectivity, and that β_2m and HCMV virions compete for similar binding sites on human fibroblasts. Because binding of HCMV to Raji cells (a lymphocyte line that expresses class I HLA antigens) was greater than binding to Daudi cells (which do not express class I HLA molecules), they concluded that HCMV uses the class I HLA antigen as a receptor for entry into cells. This conclusion has been challenged by Taylor and Cooper (1990) who claim that the major receptor for HCMV on human fibroblasts is a 30 kDa membrane glycoprotein. These workers were unable to demonstrate binding of virions to either β_2m or the heavy chain of the HLA class I molecule.

By means of immunoprecipitation studies, Grundy *et al.*(1987a) showed that the β_2m-binding property of HCMV was associated with two virion proteins with molecular weights of 36 and 65 kDa. Because these proteins could be solubilized by treatment of virions with Triton X-100, it was assumed that the β_2m-binding components were viral envelope glycoproteins. However, Stannard (1989) demonstrated, by means of immunogold electron microscopy, that β_2m bound, not to the envelope, but to the tegument of the virion.

In view of the fact that β_2m normally occurs as a complex with the heavy chain of the class I HLA molecule, it was of great interest when Beck and Barrell (1988) discovered an open reading-frame in the HCMV genome which codes for a protein with homology to the HLA class I heavy chain. The sequence of

the open reading frame (termed UL18) suggests that the gene product, if expressed, would be a membrane protein with a predicted unglycosylated size of 45 kDa.

STRUCTURAL COMPONENTS OF IgG

IgG is a "Y"-shaped molecule (fig.1). It consists of four polypeptide chains; a pair of heavy (H) chains and a pair of light (L) chains. Each heavy chain consists of four homologous stretches of amino-acids which fold to form repeating globular units, called domains. There is one variable (V) domain, designated VH, and three constant (C) domains designated CH1, CH2 and CH3. Each light chain consists of one variable domain (VL) and one constant domain (CL). The third and fourth globular domains (CH2 and CH3) of the two heavy chains associate with each other to form the stem part of the "Y" (or Fc portion). The first two domains of each heavy chain, VH and CH1, associate with a light chain to form the two arms of the "Y" (or Fab portion of IgG). The distal end of each Fab arm is the part of the IgG molecule that interacts with a specific antigen. The two antigen-combining sites are each composed of portions of the variable domains of one heavy and one light chain. Between the Fc and Fab components is a hinge region of variable length. It is at either end of the hinge region that the IgG molecule is sensitive to proteolytic attack (Metzger, 1990) and can be cleaved to form Fc and Fab fragments.

Digestion with the proteolytic enzyme, pepsin, cleaves the IgG molecule at the Fc aspect of the hinge region to yield an abbreviated Fc portion, termed Fc', and an $F(ab')_2$ fragment in which the two Fab arms are still linked by means of the hinge. Digestion with papain, cleaves the IgG molecule at the Fab end of the hinge, to yield two separate Fab fragments and one Fc fragment. Because of their homogeneity, purified Fc fragments have the propensity to crystallize. This property, first noticed by Porter (1959), resulted in the term "Fraction crystallizable", or Fc.

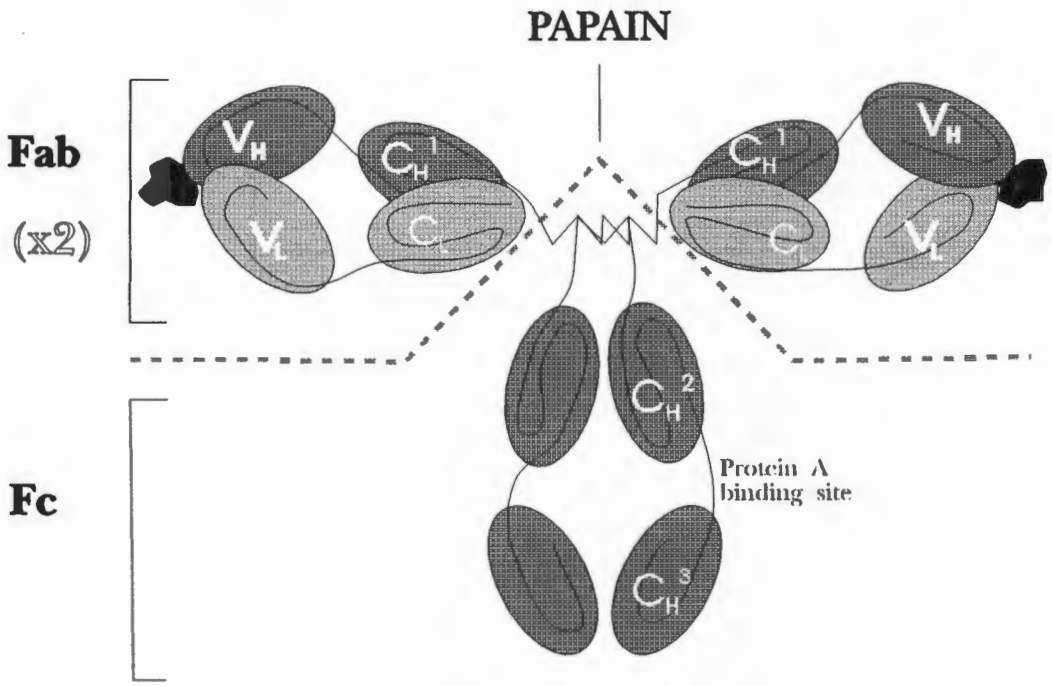


Fig. 1

Model of an IgG molecule; ovals represent immunoglobulin domains. Those labelled CH1, CH2, CH3 and CL are the constant domains of the heavy and light chains respectively. Those labelled VH and VL are the variable domains of the heavy and light chains. The site at which papain cleaves IgG is indicated.

Fc RECEPTORS

Antibodies have two major functional regions, they bind to specific antigens via the two Fab antigen-binding sites, and they stimulate other essential functions of the immune system via the Fc portion of the molecule. The interaction of the Fc with specialized receptors on the surface of cells of the immune system, triggers various responses. When an Fc receptor engages its ligand, a signal is transmitted across the membrane of the cell. The nature of the cellular response, is determined by the cytoplasmic portion of the Fc receptor. An effector cell may, for example, be triggered to release inflammatory mediators (Dessaint *et al.*, 1990); or to phagocytose an immune complex and deliver it for degradation to a lysosome (Ravtetch and Anderson, 1990); or it may be triggered to lyse an antibody coated cell (Segal, 1990). In addition, Fc Receptors (FcR's) on the surface of T and B lymphocytes appear to play

important regulatory functions in the development of the specific immune response (Lynch and Sandor, 1990).

The Fc receptors on mammalian cells have been best characterized in man and the mouse, and have been identified on macrophages, monocytes, T and B lymphocytes, natural killer cells, granulocytes and platelets (Kinet, 1989; Gergely and Sarmay, 1990; Ravetch and Anderson, 1990). All FcR's (with one exception) are integral membrane glycoproteins which have an extracellular portion, a transmembrane segment and a cytoplasmic tail. The extra-cellular portions consist of two or three globular domains which share significant homology with immunoglobulin domains, and on this basis are included in the immunoglobulin superfamily of molecules (Williams and Barklay, 1988) (fig.13, page 59). Three groups of FcR have been described:-

Group I contains the FcγRI of man and the mouse. This molecule has a molecular weight of 72 kDa. It binds monomeric IgG with high affinity and is found only on the surface of macrophages and monocytes.

Group II includes the FcγRII of humans and the FcγIIb of the mouse. This receptor is widely expressed on monocytes, macrophages, T and B lymphocytes, neutrophils and platelets. It has a low affinity for monomeric IgG. Effective binding only occurs when many of these receptors are bound simultaneously to complexed IgG. Different forms of this receptor are expressed on different cells. They vary mainly with respect to their cytoplasmic domains which are the regions involved in signal transduction. The different forms therefore probably mediate different effector functions, appropriate to the particular cell type expressing the receptor. In humans, the Fcγ RII is coded for by at least three genes. Further variation in the structure arises from alternative splicing of mRNA transcripts.

Group III includes FcγRIII in the human and FcγRIIa in the mouse. This molecule is expressed on granulocytes, macrophages and natural killer cells. Two forms of this protein, encoded by two separate genes, exist in man. One form (FcγRIIIa), which is expressed on monocytes, macrophages and natural killer cells, is a conventional integral membrane protein with two extra-cellular immunoglobulin domains, a transmembrane region and a cytoplasmic tail. The second form (FcγRIIIb), which is expressed exclusively on neutrophils, has an

identical extracellular portion, but no trans-membrane or cytoplasmic region. It is, instead, attached to a phosphatidyl-inositol lipid in the outer layer of the cell membrane by a glycan moeity

Receptors for the other classes of immunoglobulin, (IgA, IgE and IgM) are also abundantly expressed on the surface of cells (Kinet, 1989; Underdown, 1990).

Soluble immunoglobulin-binding factors for all classes of immunoglobulin have been detected in vitro, in the growth medium of cultured, activated lymphocytes. Some of these soluble FcR's may arise by cleavage of the membrane-bound Fc receptors by the action of cellular proteases (Fridman and Sautes, 1990).

Fc receptors have also been identified on non-haematopoetic cells. Receptors which play a role in the transport of IgG across epithelial surfaces have been identified on the surface of neonatal intestinal cells, various cell types in the placenta, and breast tissue in lactating mammals (Simister, 1990). Maternal IgG is transmitted to foetal or neonatal animals and provides humoral immunity during the first few weeks or months of life. Suckling calves, rats and mice receive maternal IgG from colostrum. In these animals, the receptor for IgG is expressed on the apical surface of enterocytes in the proximal part of the small intestine (Simister and Mostov, 1989). It is a hetero-dimer, consisting of a 50 kDa sub-unit non-covalently associated with a protein of 12 kDa. The 12 kDa component has recently been identified as β_2m. The 50 kDa sub-unit is a member of the immunoglobulin superfamily consisting of three extra-cellular immunoglobulin domains, a trans-membrane region and a cytoplasmic tail. It has strong homology with the heavy chain of the MHC Class I molecule, another protein known to associate with β_2m.

The binding of IgG to this complex is pH dependent. Maximal binding occurs at a pH of 6.5 (the normal pH of the intestinal lumen). The IgG-receptor complex is endocytosed, transported across the enterocyte to the baso-lateral surface of the cell, and the IgG is released from the complex at a pH of 7.4 (the pH of the fluid in the interstitial space).

In humans maternal IgG is passed across the placenta to the foetus. FcR's have been identified on three cell types: cells of the syncytiotrophoblast, placental

macrophages and foetal endothelial cells. In late pregnancy, the syncytiotrophoblast forms a single cell layer separating the maternal and foetal circulations, and receptors on these cells are likely to be important in the transport of IgG from mother to foetus (Simister, 1990). Three Fc-binding proteins, with molecular weights of 64-67, 52-56 and 30 kDa, have been immunoprecipitated from detergent extracts of placental tissue. In addition, two clones have been isolated from a cDNA library constructed from human placental mRNA, which, when transfected into cells, code for proteins that have the capacity to bind Fc. Both gene products are integral membrane proteins with extracellular domains homologous to those of the immunoglobulin superfamily.

Little is known about FcR's expressed on other cell types. Although they have been reported to be present on the surface of endothelial cells, myocytes and fibroblasts, they have not been characterized and their function is not known. Receptors on endothelial cells may play a role in the transport of IgG to the extravascular compartment (Simister, 1990), while those expressed on fibroblasts and myocytes may be important for the catabolism of IgG (Mariani and Strober, 1990).

OTHER Fc-BINDING PROTEINS

Several proteins which have the property of binding to the Fc portion of immunoglobulin, have been identified in micro-organisms which commonly infect man. Best characterised of these are the bacterial cell-wall proteins, protein A and protein G (Langone, 1982). Protein A, present in the cell wall of staphylococci, binds to human IgG of subclasses 1, 2 and 4. The binding site, on the Fc portion of IgG, appears to be between the second and third constant domains (CH2 and CH3). Other classes of human immunoglobulin (IgA, IgM and IgE), have also been shown to interact with protein A, but in these cases, binding appears to be via Fab structures (Inganas, 1981). Protein G of streptococci binds to the Fc portion of all subclasses of human IgG. Another IgG-binding protein has been identified on the surface of the human parasite Shistosoma mansoni (Torpier *et al.*, 1979). This protein has an affinity for both IgG and β_2m.

Fc-RECEPTORS OF HERPESVIRUSES

A number of the members of the herpesvirus family induce Fc receptors in the cells that they infect. This phenomenon has been demonstrated in the case of HSV (Westmoreland and Watkins, 1974), HCMV (Keller *et al.*, 1976) and VZV (Ogata and Shigeta, 1979). Best studied is the Fc receptor induced by HSV. Johnson and Feenstra (1987) showed that the receptor, which binds monomeric IgG, is a complex of two virus-encoded glycoproteins, gE and gI. Neither gE, nor gI, alone is able to bind monomeric IgG, indicating that co-expression of the two glycoproteins is necessary (Johnson *et al.*, 1988). It has subsequently been shown that gE alone has some capacity to bind aggregated or polymeric IgG (Dubin *et al.*, 1990). Unlike the major HSV glycoproteins gB, gC and gD, which are present in the virion envelope, (Stannard *et al.*, 1987), the HSV glycoprotein gE has not been found on the virion itself, only on cell membranes (L.M.Stannard, personal communication).

Little is known about the Fc receptors induced by HCMV. A search of the HCMV genome has revealed that there is no homologue for the HSV genes that encode gE and gI (the two proteins that make up the Fc receptor of HSV). The induction of an Fc receptor by HCMV was first described in 1976 by Keller *et al.* who demonstrated, by means of immunofluorescence, that cells infected with HCMV aquire the ability to bind non-immune IgG, but not IgA or IgM. Frey and Einsfelder (1984) indentified an Fc receptor, apparently present on the surface of fibroblasts infected by HCMV, which bound aggregated IgG preferentially. Maximal expression of the receptor occurred late in infection. Xu *et al.* (1989) examined detergent extracts of HCMV-infected cells for the presence of Fc binding proteins. IgG was shown to bind to electro-transfers of six proteins of molecular weights 200, 130, 85, 65, 50 and 38 kDa.

Much about the Fc receptor(s) of HCMV remains to be elucidated. By analogy with the FcR's expressed on the surface of mammalian cells, it has always been assumed that the receptor is a membrane-bound protein, but whether it is a product of a viral gene or a cellular one, is not known. Nor is it known whether the protein is present in the virion itself. Recent studies in this laboratory (Stannard and Hardie, 1991), have demonstrated that HCMV virions do contain a protein which interacts with the Fc portion of human IgG, but surprisingly, this Fc-binding protein was found to be present in the tegument of

the virion, and thus not membrane-associated. The present dissertation describes the isolation and characterization of the relevant tegument protein, and discusses possible roles for the presence of an Fc-binding protein in such an unusual location.

MATERIALS AND METHODS

VIRUS

All experiments were conducted using the laboratory-adapted strain AD169 of HCMV. Stock suspensions were kept frozen at -70^0 C. Virus samples were thawed and passaged several times in human embryonic fibroblasts (HEF) before use in experiments.

CELL CULTURE

Human embryonic fibroblasts (HEF) were grown to semi-confluence in plastic tissue culture flasks (Falcon), in Eagle's minimum essential medium (MEM), containing 10% foetal calf serum. Growth medium was removed and cell mono-layers were inoculated with AD169 at a high multiplicity of infection (m.o.i.). The inoculum was allowed to adsorb for 2 h, after which fresh medium, containing 4% foetal calf serum, was added. Cells were monitored daily for the development of cytopathic effect (c.p.e.), and culture fluid was harvested when more than 80% of the cells showed signs of infection. The harvest fluid was centrifuged at low speed to remove cell debris, and stored at 4^0 C.

PREPARATION OF IgG FROM HUMAN SERUM

Human serum, which was assayed as negative for antibodies to HCMV by ELISA, was obtained from Western Province Blood Transfusion Service. The serum was warmed to 25^0 C, and IgG was precipitated by the addition of 18% (w/v) sodium sulphate and incubation at 25^0 C for 30 min. Precipitated proteins were concentrated by centrifugation at 1500 g for 30 min. The supernatant fluid was discarded and the pellet redissolved in the original volume of distilled water. The precipitation step was repeated and the second precipitate was dissolved in approximately 20 ml of 0.02 M sodium phosphate buffer, pH 8.0, and dialysed against the same buffer. The sample was then applied to an anion exchange column of diethyl-amino-ethyl cellulose (DEAE). IgG, which eluted with the starting buffer (0.02 M Phosphate buffer), was concentrated using an Amicon filter.

DIGESTION OF HUMAN IgG WITH PAPAIN

IgG purified on DEAE cellulose, was concentrated to 18 mg/ml and dialysed against 0.1 M sodium phosphate buffer, pH 7.0, containing 0.01% sodium azide.

Digestion required 1 mg of papain (Sigma) per 100mg of IgG. The papain was "activated" by dissolving it in 100 μl of 0.1 M sodium phosphate buffer pH 7.0, containing 0.01 M cysteine and 0.002 M ethylene-diamine-tetra-acetic acid (EDTA) (di-sodium salt).

Papain was added to 4 ml aliquots of IgG, and the mixture was incubated at 37^{0}C for 17 h. Digestion was terminated by dialysis against 0.02 M phosphate buffer, pH 7.6 (which removed the cysteine).

SEPARATION OF Fab AND Fc FRACTIONS

Separation of Fab and Fc fragments was performed according to the method of Franklin and Prelli (1960).

After papain digestion, the IgG was dialysed against 0.02 M phosphate buffer pH 7.6, containing 0.01% sodium azide, and applied to a column of carboxy-methyl Sephadex C50 (Pharmacia).

Fc fragments were eluted with the starting buffer (0.02 M phosphate buffer), and Fab fragments eluted after the application of a step-wise sodium chloride gradient (0.05-0.4M). Both samples were contaminated with undigested IgG and required further purification.

Fc purification:

Purification of Fc fragments was performed either by crystallization (Sanderson and Lanning, 1970) or by gel filtration.

Crystallization of Fc fragments was achieved by repeated cycles of concentration (using Amicon filters) and dialysis against 0.002 M phosphate buffer, pH 7.6. The combination of low molarity buffer and high protein concentration caused crystals of Fc to precipitate out of solution. These crystals

were removed by centrifugation, resuspended and washed four times in 0,002 M phosphate buffer. Fc protein was stored in crystalline form at 4^0 C until use.

For purification of Fc by gel filtration, the sample was first concentrated and dialysed against 0.05 M phosphate buffer containing 0.15 M sodium chloride (PBS), and then applied to a Sephacryl S-200 (Pharmacia) column. The Fc fraction eluted ahead of the undigested IgG. 0,05 M PBS was used as eluant.

Fab purification:

Fab was separated from undigested IgG by gel filtration chromatography. Protein was concentrated to 20 mg/ml, dialysed against 0.05 M PBS, and applied to a Sephacryl S-200 (Pharmacia) column. IgG eluted immediatly ahead of the Fab, and therefore rechromatography of the Fab fraction was performed to ensure complete separation.

DETERMINATION OF PROTEIN CONCENTRATION

The concentration of protein in a solution was calculated by first measuring its absorbance of light at wavelengths of 280 and 260 nm and then using these values in one of the following formulae:

i)Where the protein was thought to be pure:-

$$\text{concentration of protein (mg/ml)} = \frac{\text{absorbance at 280 nm x 10}}{\text{extinction coefficient at 280 nm}}$$

Extinction coefficients (i.e. the absorbance of a 10 mg/ml solution at 280 nm):

IgG = 13.6

Fab = 15.0

Fc = 12.0

ii)Where the protein was not pure,

Protein concentration (mg/ml)= (1.55 x absorbance at 280nm) - (0.76 x absorbance at 260nm)

DETERMINATION OF PURITY OF IgG SUB-FRAGMENTS

i)Immunoelectrophoresis

A glass slide (100 mm x 60 mm) was covered with 9 ml of 1% agarose gel in tris-glycine barbital buffer pH 8.8. Samples of Fc and Fab fractions were loaded into 3 wells, 2 mm in diameter, which were cut in the gel halfway along its length. The slide was placed in an electrophoresis tank and strips of cotton lint were used to establish electrical connection between the ends of the gel and the electrode chambers which were filled with the same Tris-glycine barbital buffer, pH 8.8 (see appendix I). A current of 25 mA was applied across the gel for 60 min. Thereafter, two troughs, 80 mm in length, were cut lengthwise in the agar, equi-distant from the wells on either side. The troughs were filled with a 1:15 dilution of goat antiserum (Sigma) to human Fc or Fab. Gels were incubated at room temperature in a humidified chamber for 24 to 48 h and then examined for the presence of immunoprecipitation lines. Unprecipitated protein was washed out of the gel with several changes of distilled water, and the precipitin lines were stained with a solution containing 0.025% Coomassie blue (see appendix II).

ii) Sodium-dodecyl-sulphate polyacrylamide Gel Electrophoresis (SDS-PAGE)

A modified version of the Laemmli (1970) discontinuous buffer system for SDS-PAGE was used. Samples of proteins were diluted approximately 1:2 in sample buffer and boiled for 5 min. The samples were then loaded on to 10% polyacrylamide slab gels with a 3% stacking gel and electrophoresed at 15 mA until the dye front (bromophenol blue) reached the bottom of the gel. Proteins were stained with a solution containing 0.025% Coomassie blue. (see appendix II for recipes of SDS-PAGE reagents)

PREPARATION OF COLLOIDAL GOLD

Colloidal gold particles of less than 10 nm in diameter were prepared according to the method of Slot and Geuze (1985). This involved reduction of chloro-auric acid with a mixture of sodium citrate and tannic acid. Different sizes of gold particles (between 3 nm and 10 nm) could be obtained varying the amount of tannic acid present in the reduction mixture. For preparation of colloidal gold particles of 20 nm in diameter, tannic acid was omitted in the reduction step (see appendix IV).

COUPLING OF Fc, Fab AND WHOLE IgG TO COLLOIDAL GOLD

The coupling of proteins to colloidal gold particles is an electrostatic process (as yet little understood). It is impaired in the presence of exess ions, which cause the gold to flocculate. For this reason, the proteins, whole IgG and Fab fragments, were dialysed against 0.0002 M Borax-HCl buffer, pH 9.0, prior to coupling. Because Fc fragments were highly insoluble at low molarity, this protein was, instead, rapidly diluted in the same buffer just prior to coupling. The pH of the colloidal gold suspension was adjusted with 0.2 M K_2CO_3 to a pH close to the isoelectric point of the protein to be coupled. For IgG and Fab' fragments, the pH of the gold sol was adjusted to approximately pH 8.0, and for Fc fragments, gold sols were adjusted to pH 7.0.

To determine the minimum concentration of protein required to stabilize the gold, doubling dilutions of the protein were made in the same buffer (borax-HCl, pH 9.0), using 10 μl volumes. A 10-fold excess (100 μl) of colloidal gold (at the appropriate pH) was added. Gold-protein mixtures were mixed well and allowed to stand for two minutes at room temperature. Thereafter, 11 μl of 10% NaCl was added to each tube. The presence of salt causes flocculation of unstabilized gold, and this is detectable by an alteration in the colour of the solution from pink to blue. Titration established the minimal concentration of protein required to stabilize the gold and prevent its flocculation in the presence of 1% NaCl.

One volume of protein, appropriately diluted in 0.0002 M Borax-HCl buffer, and 10 volumes of colloidal gold were mixed rapidly and allowed to stand for

10 min. Stabilization of the gold was confirmed by examining a small aliquot of the prepared probe for its resistance to flocculation in the presence of 1% NaCl. Bovine serum albumin was added to the protein-gold mixture to a final concentration of 1%. Excess ligand was removed by three cycles of ultracentrifugation and resuspension of the gold probe in 0.02 M tris buffer pH 8.2, containing 0.15 M NaCl and 0.5% BSA (TBSA). Probes were finally suspended in TBSA containing 0.01% sodium azide. They were stored in the dark at 4^0 C until use.

ELECTRON MICROSCOPY

HCMV AD169 virions were concentrated from cell culture fluids of infected HEF by centrifugation at 38 000 g for 90 minutes and placed on formvar coated grids. The grids were allowed to dry, floated on drops of colloidal gold probes coupled to either Fc or Fab fragments of IgG and incubated at 37^0 C for 2 h. The grids were then washed with distilled water, negatively stained with 1% phosphotungstic acid (PTA) and examined in an Hitachi 600 electron microscope.

After treatment of HCMV virions with Triton X-100, insoluble components were separated from the Triton phase by centrifugation in a microfuge. The insoluble components were placed on a formvar-coated grid, exposed to Fc-gold and examined by electron microscopy in the same manner as whole virions. A sample of the Triton phase was mixed with Fc-gold probes and incubated at room temperature for 18 h. Thereafter, it was diluted in 5 ml PB and centrifuged at 38 000 g for 45 min. The pellet was suspended in a small drop of distilled water, placed on formvar coated grids, negatively stained with 1% PTA and examined in the electron microscope.

PREPARATION OF VIRAL PROTEINS

Viral particles were concentrated from the culture fluid of infected HEF by ultracentrifugation at 38 000 g for 90 min. Virus pellets were washed once by resuspension in PB and repeat centrifugation. The final pellets were pooled and resuspended in 0.01 M Tris buffer, pH 7.3, containing 0.15 M sodium chloride; 0.01 M calcium chloride; 0.002 M phenylmethylsulphonyl fluoride (PMSF); 1% ethanol and 1% Triton X-100. After thorough agitation, the sample was

centrifuged for 10 min in a microfuge. The supernatant (Triton phase) was removed and stored at 4°C for use in subsequent experiments. The viral proteins contained in this fraction were termed "Triton-soluble"

IMMUNOPRECIPITATION STUDIES

Precipitation of specific proteins from Triton extracts of HCMV virions, was facilitated by the use of large (10-20 nm) colloidal gold particles attached to specific ligands. Probes to be used as immunosorbants, were coupled to one of the following proteins:

Monoclonal antibody F5, specific for the HCMV envelope glycoprotein gB (gp 52)

Human IgG (whole molecule)

Human Fc fragments of IgG

Human β_2m (Sigma)

All the human immunoglobulin used in these experiments was extracted from serum assayed as negative for antibody to HCMV.

Aliquots of Triton-soluble viral proteins were mixed with specific colloidal gold probes and incubated at 37°C for 18 h. After incubation, gold conjugates were concentrated by ultracentrifugation at 38 000 g for 60 min and washed by three cycles of centrifugation in phosphate buffer. The pellets were suspended in electrophoresis sample buffer containing 5% beta-mercapto ethanol (BME) (see appendix II), boiled for five minutes, and subjected to SDS-PAGE on 10% polyacrylamide gels.

DIGESTION WITH N-GLYCOSIDASE F

(Elder and Alexander, 1982)

Enzymatic digestion with the endoglycosidase, N-glycosidase F (Boeringer-Mannheim), was performed according to the method recommended by the manufacturers.

Triton-soluble viral proteins were mixed with gold particles coupled to: a) Mab F5 (anti-gB of HCMV), or b) Fc of human IgG, and incubated at 37°C for 18 h. Gold-protein conjugates were concentrated by ultracentrifugation and washed twice with phosphate buffer. The pellets were resuspended in a buffer containing: 0.1 M sodium phosphate pH 7.6; 0.5 % SDS; 0.1% BME; 0.01 M EDTA (the total volume of sample was approximately 30 μl) and boiled for 10 min. The samples were then diluted to a volume of 150 μl in a 0.1 M sodium phosphate buffer containing 2 % Triton; 0.1 % BME; 10 mmol EDTA and 2 mmol PMSF and cooled to 4°C.

Each sample was divided into two aliquots, one to be subjected to enzyme treatment, and one to remain untreated as a control sample. 0.6 units (3 μl) of N-glycosidase F was added to each test sample, and an equal volume of distilled water was added to the two control samples. The specimens were incubated for 18 h at 37°C, then diluted in sample buffer to yield a final concentration of 2.5% SDS and 5% BME. Mixtures were boiled and subjected to SDS-PAGE, on a 10% polyacrylamide gel. Protein bands were visualized by staining with Coomassie blue.

WESTERN BLOTS

Proteins separated by PAGE were electro-transferred to nitrocellulose membranes either in a tank filled with transfer buffer or by the dry blot technique. The gel and nitrocellulose were placed face to face, and, in the case of the dry blot procedure, sandwhiched between sponges soaked in transfer buffer.

In the tank procedure, the gel and membrane were clamped in a cassette between Scotch-Brite pads and submerged in a tank filled with transfer buffer. Application of a current of 200 mA between the electrodes for 2 h was sufficient to transfer most of the protein in the gel to the nitrocellulose membrane.

After transfer, membranes were soaked over-night in a 1% solution of bovine serum albumin. The membranes were cut into strips and covered with colloidal gold particles coupled to various proteins, including, human IgG (whole molecule), Fc fragments, β_2m, Mab F5 and a number murine Mab's of sub-

classes IgG1, 2a, 2b and 3, specific for antigens unrelated to HCMV. After an incubation period of 7 to 18 h at room temperature, strips were washed in distilled water, and adsorbed probes were visualized by enhancement of the gold with silver ions (Intense M, Janssen).

PREPARATION OF Fc AFFINITY COLUMN

Cyanogen bromide ($CNBr^-$), covalently attached to sepharose beads, can be used to immobilize proteins of choice, thus forming effective substrates for affinity chromatography. $CNBr^-$ captures protein by covalent linkage to free NH_2 groups.

A sample containing 8 mg of purified Fc fragments was prepared for coupling by dialysis for 18 h against a 0.01 M $NaHCO_3$ buffer, pH 8.3, containing 0.5 M NaCl. One g of $CNBr^-$-activated sepharose 4B (Pharmacia) was suspended in, and extensively washed with, 0.001 M HCl, under suction in a buchner funnel. Wet gel was added quickly to the protein solution and rotated end-over-end for 4 h at room temperature. The gel was separated from the supernate by low speed centrifugation, and the fluid was tested spectrophotometrically for the presence of unattached protein.

To saturate residual binding sites, the gel was resuspended in a solution containing 1 M ethanolamine (pH 9.0) and rotated endover-end at 4^0 C for 18 h. Thereafter, the gel was washed alternately with three changes of Tris-HCl, pH 8.0, in 0.5 M NaCl; and 0.1 M acetate buffer, pH 4.0, in 0.5 M NaCl. Finally the gel was resuspended in 0.05 M Tris-HCl, pH 7.5 containing 0.01% sodium-azide, and poured into a column. This was stored at 4^0 C until use.

EXTRACTION OF Fc BINDING PROTEINS BY AFFINITY CHROMATOGRAPHY

It is recommended that affinity columns be washed before use with the dissociating agent which will subsequently be used for elution. This will remove protein that is loosely attached to the stationary phase which would otherwise elute with the specific ligand. As the nature of the interaction between the viral Fc-binding proteins and the Fc portion of IgG was not known, the choice of a suitable dissociating agent was difficult. For this reason, the Fc

affinity column was pre-treated with a number of dissociating agents, including, 10% Dioxane, 1 M acetic acid and 3 M potassium thiocyanate (KSCN). After each treatment, the column was washed with 20 column volumes of 0.05 M Tris-HCl containing 0.15 M sodium chloride (TBS).

To ensure that proteins did not adsorb non-specifically to the sepharose, Triton-soluble HCMV proteins were first passed through a 5 ml sepharose 4B column and then through the Fc affinity column. By connecting the outlet of the sepharose-4B column to the inlet of the Fc-affinity column, viral proteins were cycled three times through both columns at a flow rate of approximately 0.1 ml/min. Thereafter, the Fc-column was washed with 20 column volumes of TBS to remove any unbound protein. Elution of specifically bound proteins was attempted by washing the affinity column with successively: 1 M acetic acid, 3 M KSCN, 10% dioxane and 1% SDS. The absorbance at 280 nm of the eluates was monitored with a uvicord. After each attempt at elution, the eluate was collected, concentrated and subjected to SDS PAGE to examine for the presence of protein.

DOT BLOTS

Approximately 6 μl aliquots of the protein solution which had been eluted from the Fc-affinity column, were spotted onto nitrocellulose membranes which had been presoaked in transfer buffer. Samples were applied to the membranes under suction in a Buchner funnel. The membranes were then soaked for 18 h in 1% BSA. Separate blots were allowed to react for 24 h with suspensions of colloidal gold coupled to: Fc, β_2m or anti-gB (Mab F5). Membranes were washed, and the presence of adsorbed gold particles was determined by enhancement with silver ions.

IMMUNOFLUORESCENCE

Confluent mono-layers of human fibroblasts were treated with a solution containing 0.025% Trypsin, 0.02% EDTA and 0.02% KCl in PBS. The cells were suspended at a seeding rate of 3 x 10^5 cells per ml in MEM containing 10% foetal calf serum and 5% sodium bicarbonate, and drops of the cell suspension were applied to wells, 8mm in diameter, on Teflon coated glass slides (Flow Laboratories). The slides were incubated in a humidified chamber

at 37^0 C for 24 h in the presence of 5% carbon dioxide (CO_2). Thereafter, the growth medium was removed and medium containing AD169 at a high m.o.i. was added to the cell mono-layers. The cells were again incubated at 37^0 C in the presence of 5% CO_2. The infection cycle was terminated at 16, 23, 40 and 48 h, by fixing the infected cell sheets for 10 min in cold acetone. The slides were dried and stored at 4^0 C until use.

Acetone-fixed mono-layers of infected fibroblasts were flooded with 1% TBSA and incubated at 37^0 C for 10 min. The TBSA was removed and wells were covered with purified Fc fragments of human IgG, diluted in PBS, or with PBS alone. After incubation for 1.5 h, unbound antibody was removed by extensive washing of the cell sheets with PBS. The presence of bound human Fc was assessed by immunofluorescence using a poly-clonal antiserum to human IgG, which was labelled with fluorescein-isothiocyanate (FITC). This antibody was also applied to wells which had not been exposed to Fc in order to identify any non-specific adsorption of the secondary antibody. After incubation for 1 h at 37^0 C, the unbound FITC conjugate was removed by extensive washing with PBS, as before. The mono-layers were covered with glycerol under glass cover-slips and examined microscopically using incident light with a wavelength of 390 nm. The results were photographed on Ilford FP5, 400 ASA film, using exposures of between 1 and 6 min.

HOMOLOGY COMPARISONS OF THE PRIMARY AMINO ACID SEQUENCES OF AD169 PROTEINS AND THOSE OF HUMAN FcR'S

The following gene sequences were imported from the Genembl data bank

:Hs5MV301	HCMV H301 gene for the glycoprotein homologous to MHC class 1 antigens
Hs5p28p	HCMV 28 kDa structural phosphoprotein
Hs5ppbc	HCMV phosphorylated matrix protein (pp65) and related pp71
Hs5p67k	HCMV 67 kDa phosphorylated protein
HUMFCRHB	Human high affinity IgG receptor (Fcγ R-I)
HUMIGGFCRA	Human low affinity IgG receptor (Fcγ R-II)
HUMIGGRLAA	Human low affinity IgG receptor CD16 (Fcγ R-III)

Using the computer programme Genepro, the nucleotide sequences corresponding to open reading frames were translated into proteins and the primary amino-acid sequences of the viral proteins were aligned with those of the human FcR's. A percentage homology score was recorded for the "best fit" alignment.

RESULTS

SEPARATION OF IgG FRAGMENTS AFTER DIGESTION WITH PAPAIN

Separation of IgG fragments on a CM sepharose column yielded two main components (fig.2):

Fraction I (eluted with the starting buffer) contained mainly Fc fragments, but was also contaminated with undigested IgG. Initially the Fc fraction was separated from the undigested IgG by repeated crystallization in low molarity phosphate buffer, but this resulted in a low yield of Fc because much of the crystalline material did not redissolve after storage. Subsequently, therefore, separation was performed by gel filtration on Sephacryl S-200, which yielded Fc preparations of high purity (fig. 3a).

Fraction II (eluted with 0.4 M NaCl) contained a mixture of Fab fragments and undigested IgG. Separation of the two components was achieved by gel filtration on Sephacryl S-200. Initially, the IgG eluted immediately ahead of the Fab (fig.3b), resulting in incomplete separation of the two fractions. The Fab fraction was therefore reapplied to the column. Pure Fab, visible as a single symmetrical peak (fig.3c), was collected.

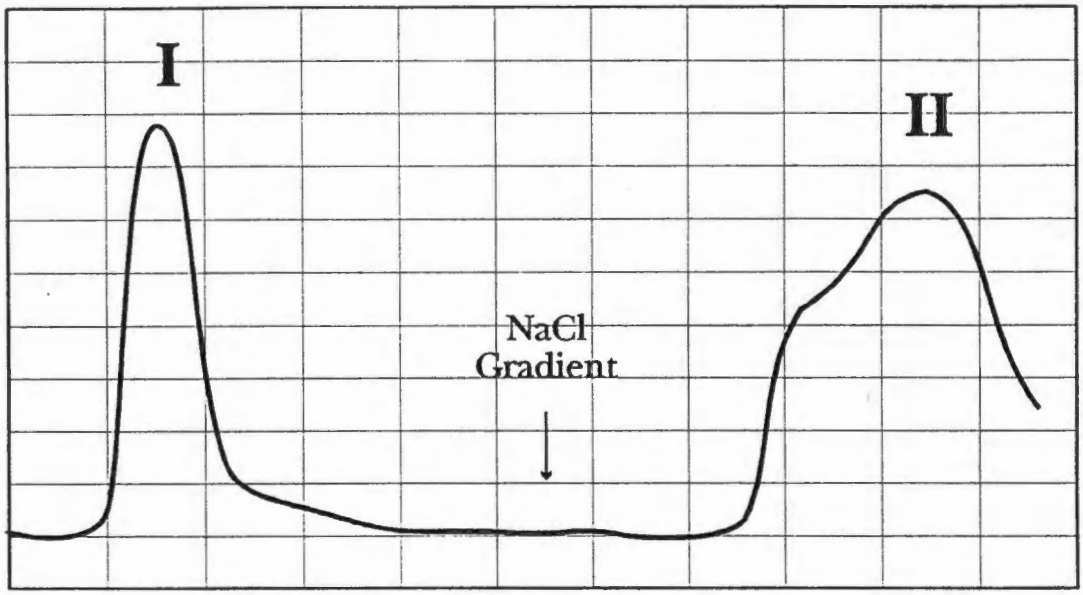


Fig. 2

Elution pattern of IgG fragments after digestion with papain and separation by CM Sephadex C50 chromatography. Peak I contains mainly Fc fragments, contaminated with undigested IgG. Peak II contains mainly Fab fragments, contaminated with undigested IgG.

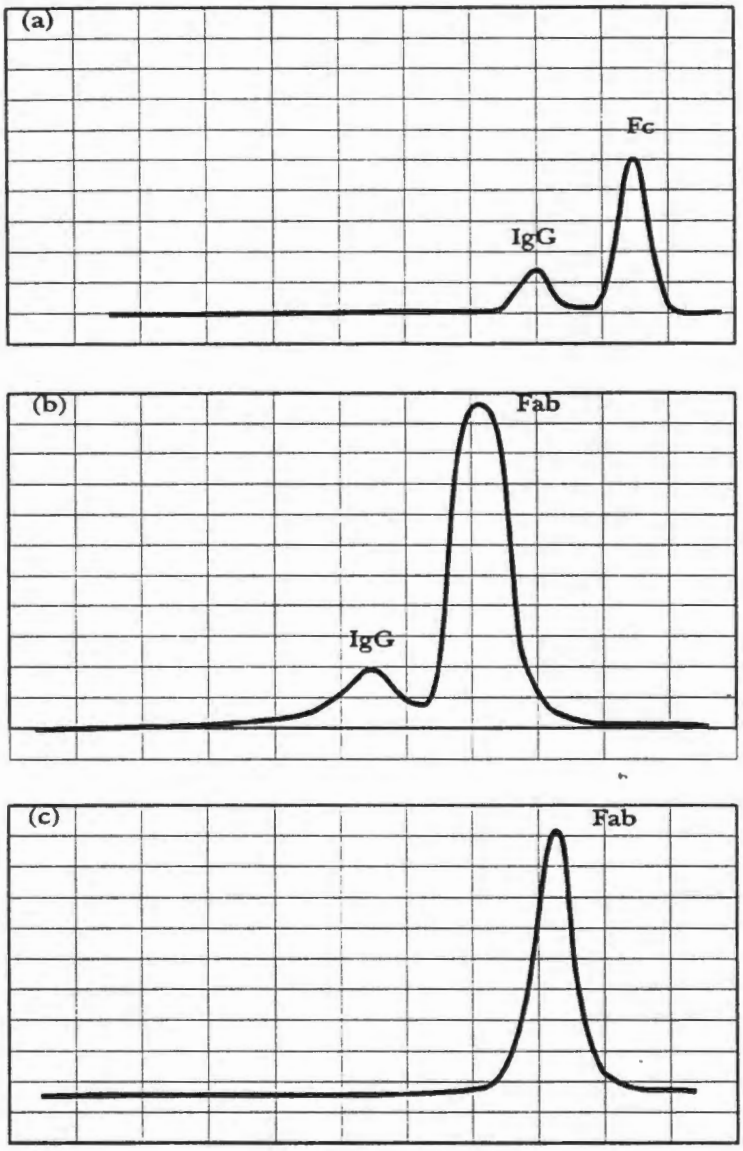


Fig. 3

a) Elution pattern of Fc and undigested IgG (obtained from peak I, fig.2) separated on Sephacryl S-200. The first fraction contains whole IgG and the second, pure Fc fragments.

b) Elution pattern of Fab and undigested IgG (obtained from peak II, fig.2) separated on Sephacryl S-200. The first fraction contains whole IgG. It is incompletely separated from the second fraction which contains Fab fragments.

c) Elution pattern after the second major fraction (fig.3b) was reapplied to the Sephacryl S-200 column. A single peak containing pure Fab is seen.

DETERMINATION OF PURITY OF Fab AND Fc FRACTIONS

The purity of both Fc and Fab preparations was assessed by SDS PAGE and immunoelectrophoresis. Both techniques indicated that the final preparations were homogeneous.

i) SDS PAGE

After SDS PAGE, under non-reducing conditions, protein bands of characteristic size for each fragment were observed. Whereas whole IgG (fig.4, lane 2) migrated as a diffuse band at about the 150 kDa position, no equivalent bands were detected in either the Fc or Fab fractions. Fab fractions (fig.4, lanes 3 and 4), migrated to approximately 46 kDa. A minor band, present at approximately 100 kDa, may represent $F(ab')_2$ fragments which resulted from incomplete digestion of the IgG. One of the Fab fractions contained some degraded material, visible as a smear of protein less than 20 kDa (fig.4, lane 4). This Fab fraction was therefore discarded. The Fc fractions (fig.4, lanes 5 and 6) migrated to a position corresponding to approximately 55 kDa. Faint bands at the 110 and 160 kDa positions probably represent dimers and trimers which may have formed during the concentration process.

ii) Immunoelectrophoresis

Immunoprecipitin arcs were observed where antigen-antibody interactions occurred. Both Fc and Fab preparations formed immunoprecipitin arcs with their specific antisera only. No reaction was observed between Fc and anti-Fab, or Fab and anti-Fc (fig.5). Fc and Fab fragments were seen to have different electrophoretic mobilities; the Fc fragment (negatively charged at pH 8.8) migrated towards the positive electrode, and the Fab fraction (uncharged) was carried towards the negative electrode by endosmosis.

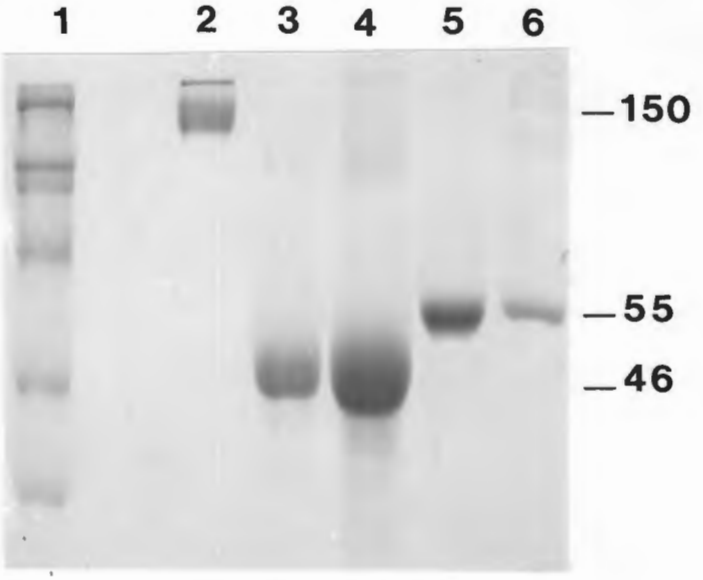


Fig. 4

Purity of the Fc and Fab fractions of human IgG was determined by SDS PAGE (under non-reducing conditions) on a 10% gel; proteins were stained with Coomassie blue. Lane 1, molecular weight markers; lane 2, whole IgG, seen as a diffuse band at approximately 150 kDa; lanes 3 and 4, purified Fab fragments are approximately 46 kDa in size, a faint band at the 100 kDa position may represent $(Fab')_2$ fragments; lanes 5 and 6, purified Fc fragments are approximately 55 kDa in size, faint bands visible at the 110 and 160 kDa positions may represent dimers and trimers.

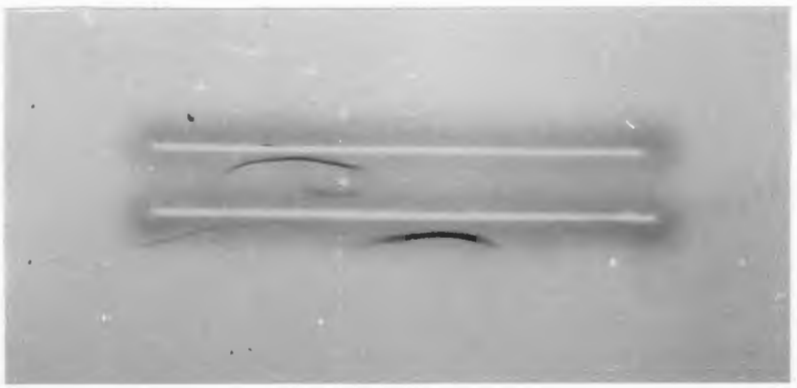


Fig. 5

Purity of Fc and Fab fractions determined by immunoelectrophoresis. Purified Fab placed in the top and bottom wells and Fc in the centre well. Troughs were filled with goat anti-Fc (upper trough) and anti-Fab (lower trough). Single immunoprecipitation arcs indicate the homogeneity of each fraction.

E.M. OBSERVATIONS OF INTERACTIONS WITH Fc- AND Fab-GOLD PROBES

Negative staining electron microscopy allowed the visualization of precise sites of interaction between Fc- or Fab-gold probes and components of extracellular HCMV virions. The Fc-gold probes bound solely to the tegument and not to the envelopes of the virions (fig.6a). The binding of Fab-gold probes to the tegument was markedly reduced relative to the binding of Fc (fig.6b). Nevertheless, a low level of interaction between Fab and tegument was consistently seen. Neither envelopes nor capsids of the virions were labelled with Fab-gold.

Treatment of virions with Triton X-100 removed the envelopes and some of the tegument proteins. Naked capsids, which were observed in the Triton insoluble fraction, did not interact with the Fc-gold probes (fig.7).

After incubation of the Fc probes with the Triton soluble fraction, small aggregates of gold particles were seen by electron microscopy. The gold particles appeared to be cross-linked by protein.

IMMUNOPRECIPITATION STUDIES

Treatment of the virions with Triton X-100 resulted in the solubilization or dispersion of envelope proteins, and certain tegument proteins, into the Triton phase. Selected proteins could be extracted from the Triton phase by precipitation with specific ligands, coupled to colloidal gold. Proteins recovered in this manner were subjected to SDS-PAGE to determine their molecular weights.

Polypeptides of approximately 70 and 33 kDa were precipitated with Fc-gold (fig.8, lane 3). These polypeptides were identical in size to those which were precipitated with β_2m-gold (fig.8, lane 2). Only one protein of approximately 55 kDa was precipitated using gold particles coupled to a Mab specific for gp52 (gB) of HCMV (fig.8, lane 1).

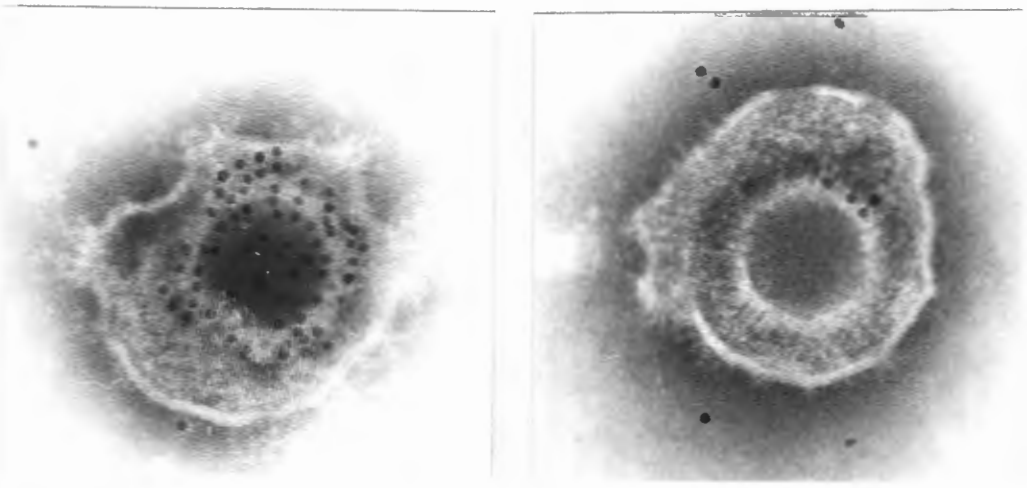


Fig. 6

HCMV virions after reaction with colloidal gold probes; a) Fc-gold probes bind strongly to the tegument of the virion; b) Fab-gold probes show markedly reduced binding to the virion. (magnification x 200 000)

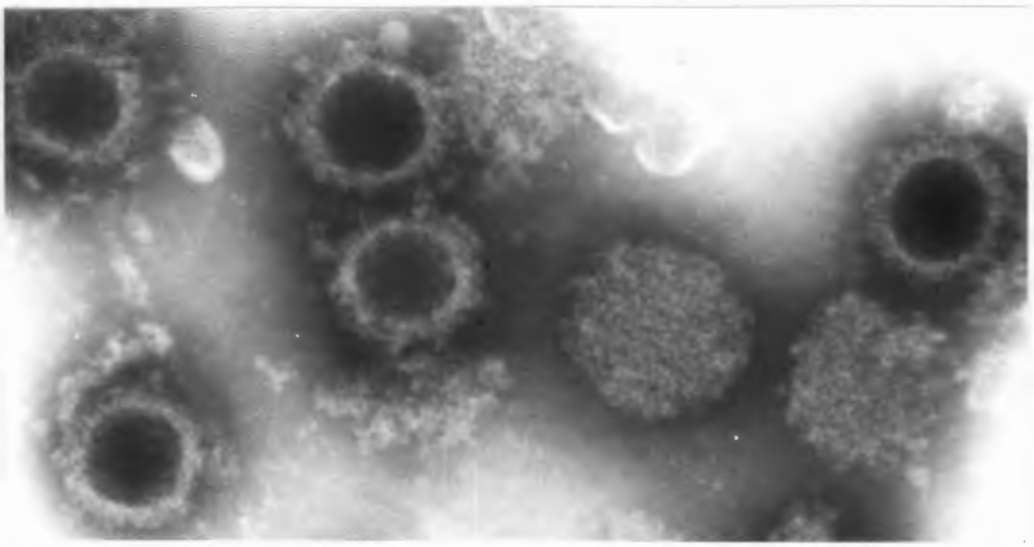


Fig. 7

Treatment with Triton X-100 removes the envelopes from HCMV virions. Fc-gold probes do not bind to naked capsids. (magnification x 150 000)

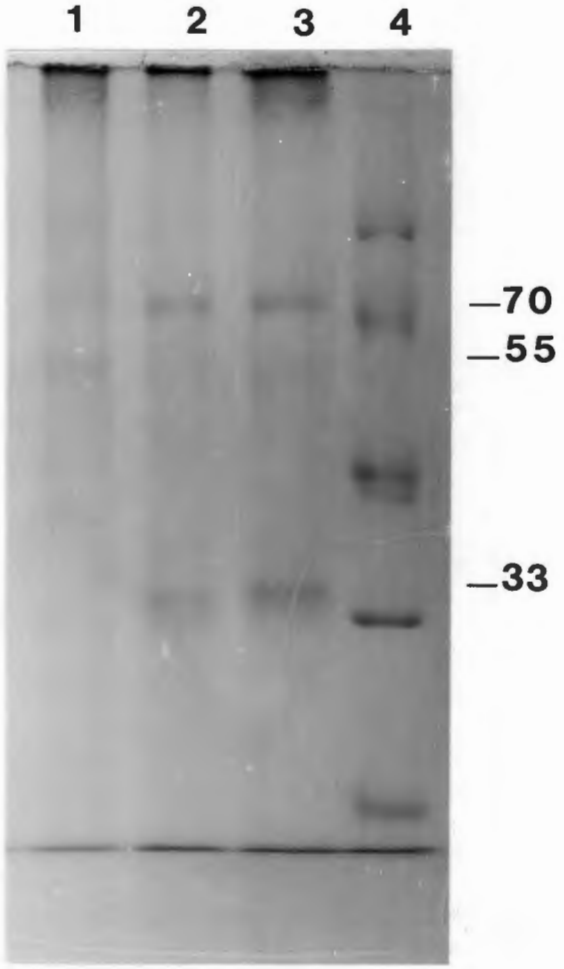


Fig. 8

After treatment of HCMV particles with Triton X-100, specific viral proteins were immunoprecipitated from the Triton phase and subjected to SDS PAGE on a 10% gel. Protein bands were stained with Coomassie blue. A single protein of 55 kDa was immunoprecipitated by Mab F5, (lane 1); two proteins of 70 and 33 kDa were immunoprecipitated by both β_2m (lane 2) and Fc (lane 3). Lane 4 contains molecular weight markers.

ENDOGLYCOSIDASE TREATMENT

Fc-binding proteins, which had been selectively recovered by immunoprecipitation from Triton extracts of HCMV virions, were subjected to digestion with N-glycosidase F to determine the presence or absence of N-linked glycosylation. Treated and untreated proteins were subject to SDS-PAGE, where an alteration in electrophoretic mobility would indicate loss of N-linked sugars. After N-Glycosidase treatment, the electrophoretic mobility of both the 70 and the 33 kDa proteins remained unaltered (fig. 9, lanes 2 and 3), indicating the absence of N-linked glycosylation.

To confirm the efficacy of the enzyme, parallel tests were performed on samples of the envelope glycoprotein gB, which had been immunoprecipitated in a manner similar to that used for the Fc-binding proteins. Treatment of gB with the enzyme resulted in a reduction in the molecular weight of the gB protein by approximately 9 kDa (fig. 9, 4 and 5).

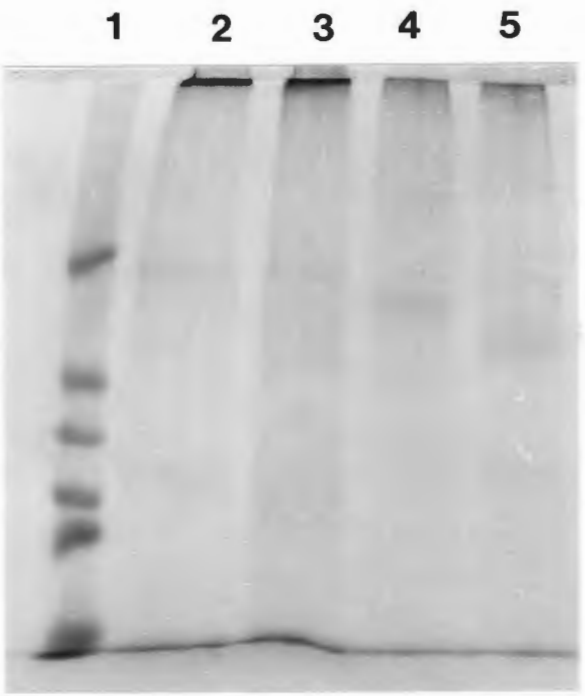


Fig. 9

Effect of N-glycosidase F treatment on the electrophoretic mobility (size) of the Fc-binding proteins of HCMV. Specific viral proteins were obtained by immunoprecipitation from Triton extracts of HCMV virions, treated with N-glycosidase F and subjected to SDS PAGE on a 10% gel. The molecular weights of the proteins precipitated by Fc were the same before treatment (lane 2) as after treatment (lane 3) with enzyme. The HCMV envelope glycoprotein gB, used as control, had a molecular weight of 55 kDa before digestion with N-glycosidase F (lane 4) and a molecular weight of 46 kDa after treatment (lane 5).

WESTERN BLOTS OF TRITON SOLUBLE VIRAL PROTEINS

It was of interest to determine the spectrum of specific ligands with which the Fc-binding proteins were capable of interacting, and also whether the ability of these proteins to bind Fc (or other ligands) was impaired after reduction. Triton-soluble proteins were therefore subjected to SDS-PAGE (under both reducing and non-reducing conditions) and electrotransferred to nitrocellulose, after which they were allowed to react with a variety of ligands attached to colloidal gold particles. Specific interactions on the Western blot could be ascertained by direct visualisation of bound gold probes after enhancement with silver ions. These reactions were visible as discrete bands of brown colouration.

It was found that a 33 kDa protein was able to bind, not only the Fc portion of human IgG (fig.10, lane 2), but also human β_2m (fig.10, lane 3). Occasionally, weak reactions with both ligands were also observed at the 70 kDa position. Reduction did not alter the capacity of the proteins to bind these ligands.

A variety of murine monoclonal antibodies with specificities for antigens unrelated to HCMV, were also used as probes in the blotting experiments. Three of these Mab's, namely antibodies specific for alkaline-phosphatase, a class II HLA antigen (expressed on NM melanoma cells), or the HSV envelope glycoprotein gC, were all bound by the 33 kDa protein (fig.10, lanes 4, 5 and 6). The only known property which all three Mab's had in common was that they were all sub-class I of murine IgG. Other Mab's of sub-classes 2a, 2b or 3 did not bind to the transferred proteins (table 1). Likewise, the anti-gB Mab F5 (subclass 2a) did not bind to either the 33 or 70 kDa proteins, but did react with the gB-protein complex (130 kDa), under non-reducing conditions (fig.10, lane 1).

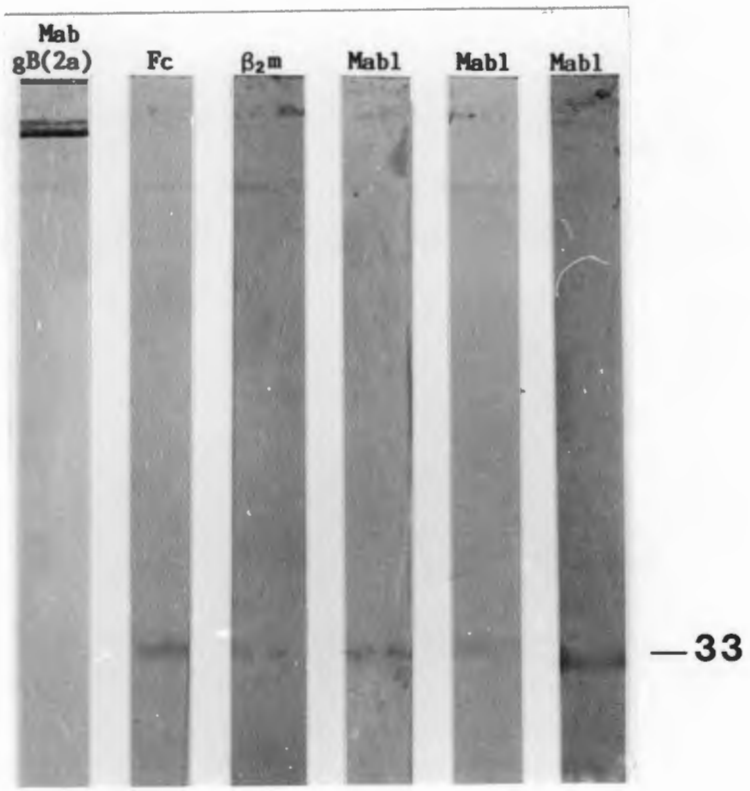


Fig. 10

Triton soluble HCMV proteins were separated by SDS PAGE under non-reducing conditions and transferred to nitrocellulose. Electro-transfers were reacted with colloidal gold particles coupled to various proteins and the presence of adsorbed probes was visualized by enhancement with silver ions; lane 1, Mab F5 binds to a high molecular weight complex which does not enter the gel under non-reducing conditions. Both Fc (lane 2) and β_2m (lane 3) bind to proteins of 33 kDa; various murine Mab's of IgG subclass 1 (lanes 4-6) also bind to a protein of 33 kDa; lane 4, anti-alkaline phosphatase; lane 5, anti-HSV gC; lane 6, anti-melanoma cells.

TABLE 1

MURINE MAB'S TESTED FOR REACTIVITY WITH HCMV PROTEINS ON WESTERN BLOTS:

Mab	Class	Specifity	Reactivity with HCMV proteins on Western blots
	IgG1	anti-NM (melanoma cells)	33 kDa protein
	IgG1	anti-alkaline phosphatase	33 kDa protein
	IgG1	anti-pp150 (of HCMV)	33 kDa protein
	IgG1	anti-gC (of HSV)	33 kDa protein
	IgG1	anti-gH (of HSV)	33 kDa protein
	IgG2a	anti-gH (of HSV)	no binding
F5	IgG2a	anti-gB (of HCMV)	high molecular weight complex (non reduced)
III-114	IgG2b	anti-gD (of HSV)	not tested *
II-512-3	IgG3	anti-gC (of HSV)	not tested *
I-59-2	IgG3	anti-gB (of HSV)	not tested *

.* No binding to tegument by E.M.

CONFIRMATION THAT THE PROTEINS THAT BIND Fc AND β_2m ARE IDENTICAL

In order to determine whether the 70 and 33 kDa proteins which bound human Fc on Western blots, were the same proteins as those which bound β_2m, further experiments were performed. In these studies, the relevant proteins were first precipitated from Triton solutions by either Fc or β_2m using specific gold probes, and then subjected to SDS PAGE and electrotransferred to nitrocellulose. Duplicate transfers were reacted with either Fc-gold or β_2m-gold. In both cases, positive reactions were observed with both the homologous and the heterologous probes, indicating that the proteins which bound Fc, were indeed the same as those which bound β_2m.

PURIFICATION OF Fc-BINDING PROTEINS BY AFFINITY CHROMATOGRAPHY

Affinity chromatography was employed for the purification of Fc-binding proteins from a mixture of Triton-soluble HCMV proteins. After extensive exposure of the column to the viral proteins, elution was attempted with a number of dissociating agents. The presence of protein in the eluate was determined spectrophotometrically. Treatment with, successively, 1 M acetic acid, 3M KSCN and 10% dioxane, failed to dissociate proteins from the stationary phase. Treatment with 1% SDS, however, resulted in elution of sufficient protein to produce a visible deflection on the Uvicord tracing. The protein from this fraction was concentrated and subjected to SDS PAGE on a 10% gel, and stained with Coomassie blue. A single band at approximately 33 kDa was detected (fig.11).

Dot blots of the material which was eluted from the Fc affinity column, confirmed the presence of a protein which could bind both Fc-gold and β_2m-gold, but which did not bind Mab F5 (Mab against gB of HCMV).

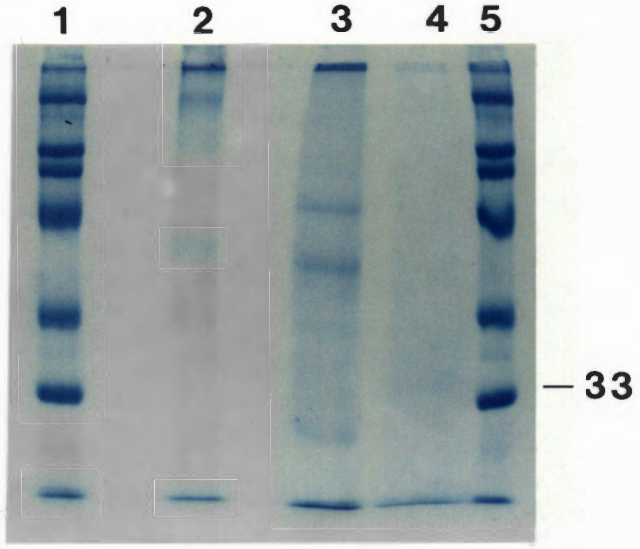


Fig. 11

Fc-binding HCMV proteins, purified by Fc-affinity chromatography, were concentrated and subjected to SDS PAGE on a 10% gel. Proteins were stained with Coomassie blue. Mixture of Triton-soluble HCMV proteins before application to the column, non-reduced (lane 2) or reduced (lane 3). A single protein of 33 kDa was eluted off the column (lane 4); molecular weight markers in lanes 1 and 5.

IMMUNOFLUORESCENCE

Using immunofluorescence, it was possible to demonstrate that infection of HEF with HCMV (AD169) induces intracellular Fc receptors which become increasingly prominent with time after infection.

No specific reactivity with the Fc portion of human IgG could be demonstrated in cells harvested 16 h post infection, except for possible faint outlining of some nuclei (fig.12a). By 23 h post infection, prominent foci of fluorescence were observed which were concentrated in large aggregates in a juxta-nuclear position. Discrete pockets of fluorescence were also observed towards the periphery of the cell. Fluorescence was not prominent in the nuclei, although tiny fluorescent foci could be detected in most nuclei. Multinucleate giant cells were present. In these syncytia, the fluorescent material was concentrated in the centre and the nuclei were found at the periphery (fig.12b and c). No obvious cell membrane fluorescence could be seen. This pattern of fluorescence was unchanged at 40 h post infection.

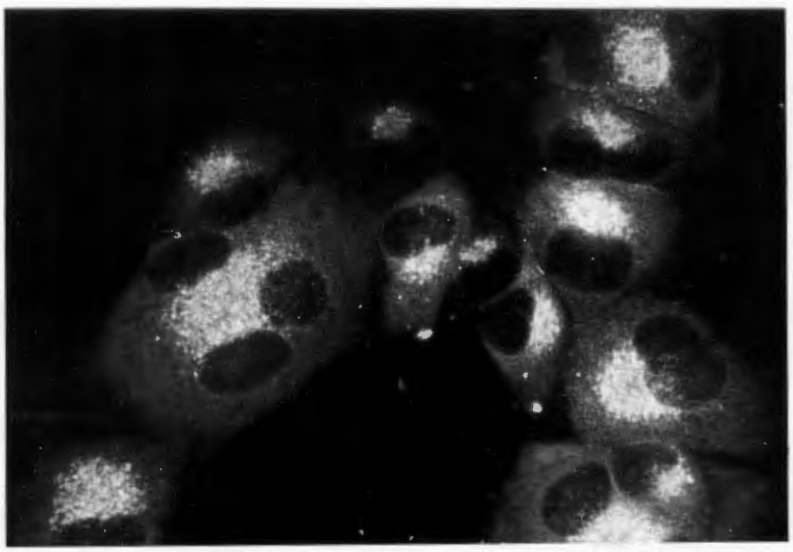
No fluorescence was observed when uninfected cells were reacted with human Fc, indicating that the Fc-binding protein is not present in uninfected cells, but is induced by infection with AD169. Fluorescence was also not observed in infected cells which were reacted only with the FITC-anti-human IgG conjugate, indicating that the fluorescence was not caused by either non-specific adsorption, or specific Fc-binding by the secondary antibody.

Fig. 12

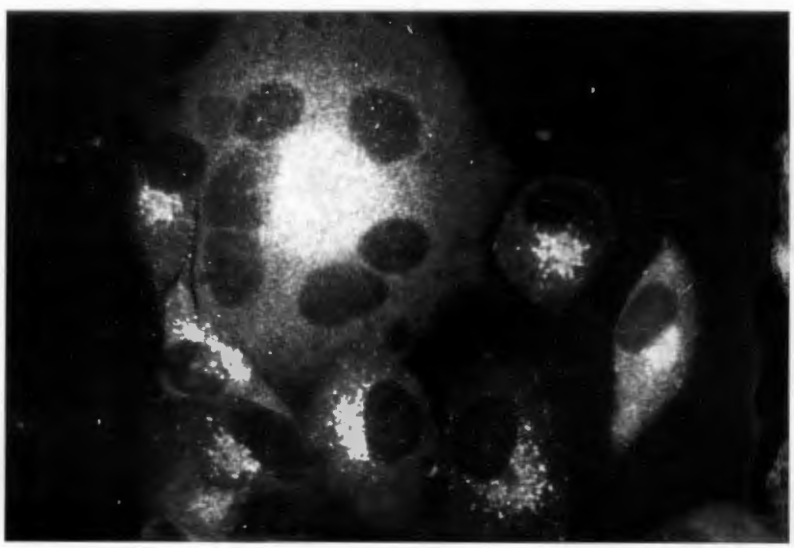
Indirect immunofluorescence demonstrated the presence of Fc receptors in mono-layers of HCMV-infected HEF. Acetone-fixed cells were reacted first with purified Fc fragments of human IgG and then with FITC goat anti-human IgG. a) At 16 h post infection, only faint outlining of nuclei can be seen (film exposed for 6 min). By 23 h post infection (b and c), prominent paranuclear fluorescence can be seen.

a

b



c



HOMOLOGY COMPARISONS OF THE PRIMARY AMINO ACID SEQUENCES OF AD169 PROTEINS AND THOSE OF HUMAN FcR'S

In order to determine whether any significant sequence homology existed between the known tegument proteins of AD169 and human FcR's, the primary amino acid sequences of the proteins were compared and scored according to their percentage homology. Homology scores varied from 8 to 20 % (table 2). Greater homology was seen when short amino acid sequences were compared with the three FcR sequences. The degree of homology observed was not considered significant.

Table 2

AD169 Protein	Human Fcγ R 1	Human FcγR II	Human FcγR III
pp65 first exon (93 amino acids)	14	17	20
pp65 second exon (456 amino acids)	8	8	10
pp71 (574 amino acids)	7	7	8
pp28 (191 amino acids)	10	8	12
pp67 (564 amino acids)	9	8	15
MHC class 1 homologue (369 amino acids)	14	14	10

DISCUSSION

Although it is well recognized that infection with herpesviruses (including HCMV) can induce the formation of Fc-receptors in the cells that they infect, the Fc-binding proteins which have been isolated and characterized in this study are novel in two respects. Firstly, they are found in the HCMV virus particles themselves, and secondly, they are not present in the membranous envelope, but are found instead, in the proteinaceous layer between envelope and capsid, termed the tegument. The location within the virion of the Fc-binding protein was clearly demonstrated by electron microscopy using specific gold probes.

The non-involvement of the virion envelope in Fc-virion interactions was of special interest since most Fc-receptors (FcR's) thus far identified in mammalian cells, are membrane-associated proteins (Kinet, 1989; Ravetch and Anderson, 1990); either integral membrane proteins, or else attached to the lipid bilayer by a phosphatidyl-inositol glycan tail (Simmons and Seed, 1988). Although non-membrane-bound immunoglobulin binding proteins have been identified, they are usually derived from cleavage of conventional membrane-associated proteins (Fridman and Sautes, 1990).

The results pertaining to this HCMV tegument FcR will be discussed in three sections: the findings related, respectively, to the characterization of the protein, a comparison of the properties of the FcR with those of known structural proteins of HCMV and, finally, a discussion of its significance and the possible role that it may play in the pathogenesis of infection by the virus.

CHARACTERIZATION

The first step in the characterization of this unusual FcR was to extract the relevant tegument protein from the virions. Emulsification of concentrated virions in the non-ionic detergent Triton X-100, resulted in "solubilization" of the virion envelopes and release of the Fc-binding proteins into the Triton-phase. Viral capsids remained intact after Triton treatment and immunogold electron microscopy demonstrated that the capsids did not participate in Fc-binding.

Isolation of the Fc-binding proteins from Triton solutions was primarily accomplished by specific precipitation with purified Fc fragments. Because the Fc fragments were first coupled to large colloidal gold particles, separation of the precipitates was easily accomplished by differential centrifugation. After adequate washing, exposure of the precipitates to SDS, released the viral proteins captured by the Fc, and allowed their characterization by PAGE. Two proteins with molecular weights of 70 and 33 kDa were precipitated using this technique.

By means of Western blots, it was shown that Fc probes reacted most strongly with the 33 kDa protein, and that treatment of this protein with BME did not affect the interaction. Binding of Fc probes at the 70 kDa position was only occasionally observed. These findings suggest that the Fc-binding proteins may exist as a non-covalently-associated dimer, in which the Fc-binding site resides predominantly in the 33 kDa subunit.

In addition to binding the Fc portion of human IgG, the HCMV Fc-binding proteins were also shown, on Western blots, to react non-specifically with a number of murine Mab's of IgG subclass I. The possibility of specific (Fab) binding, was excluded as the IgG1 Mab's used were all directed against antigens unrelated to HCMV proteins. As in the case of human Fc, strongest reactions were observed at the 33 kDa position and weaker reactions at the 70 kDa positions. The observation that class I murine Mab's can interact non-specifically with HCMV proteins is of practical importance as Mab's are commonly used in the diagnostic laboratory to detect the presence of specific viral proteins in patient samples. The non-specific reactivity of murine IgG1 should be borne in mind when interpreting the results of such investigations.

Treatment of the purified Fc-binding proteins with the enzyme N-glycosidase F (an endoglycosidase that selectively cleaves N-linked oligo-saccharides at their point of attachment to asparagine residues in the polypeptide) did not result in an alteration in the molecular weight of either the 70 or the 33 kDa proteins. This indicates that these proteins are not N-glycosylated. It is known that N-linked oligo-saccharides are attached to proteins in the endoplasmic reticulum (Stryer, 1988). HCMV virions are assembled in the nucleus of the cell and acquire their envelopes by budding through the inner nuclear membrane. Enveloped particles are transported to the surface of the cell in vesicles,

probably derived from elements of the outer nuclear membrane and the golgi apparatus. Although envelope proteins could be glycosylated during this process, it is unlikely that virion proteins, not present in the envelope, would be exposed to glycosyl-transferases present in cytoplasmic organelles, and thus the presence of N-linked oligo-saccharides on tegument proteins would be unexpected. It has, however, recently been reported that one of the tegument proteins of HCMV, the 149 kDa basic phospho-protein, contains residues of N-acetyl-glucosamine attached by an unusual O-linkage (Benko *et al.*, 1988). This modification is found on a number of nuclear and cytoplasmic proteins (Hart *et al.*, 1988), and the presence of similar O-linked modifications on the Fc-binding tegument proteins cannot be excluded.

An important finding in this study, was that the HCMV tegument proteins which interact with the Fc portion of IgG, are identical to those which combine with β_2m. Initial evidence came from the fact that proteins, precipitated from Triton-extracts of HCMV virions by either Fc or β_2m, were of the same size, namely 70 and 33 kDa. The sizes of these proteins were similar to those obtained by Grundy *et al.* (1987a), who immunoprecipitated two proteins of 36 and 65 kDa using a polyclonal rabbit anti-serum to β_2m. Even stronger evidence that the proteins which bind Fc are the same as those which bind β_2m, came from an experiment in which proteins, immunoprecipitated with Fc, were found to bind β_2m on Western blots, and that proteins precipitated with β_2m, were likewise able to bind Fc. A similar dual affinity was demonstrated when the 33 kDa protein which was purified by Fc affinity chromatography, was shown to react with both β_2m and Fc-gold on dot blots.

Immunofluorescence was used to determine whether the tegument protein of HCMV virions could account for the Fc-binding property displayed by cells infected by HCMV. The presence of these intracellular Fc receptors was first described by Keller *et al.* (1976). Using indirect immunofluorescence they demonstrated that Fc-specific reactivity was first detectable 36 h post infection as diffuse cytoplasmic fluorescence. Fluorescent foci (which they described as granules) later coalesced to form areas of intense fluorescence in a juxta-nuclear position. This became detectable 72 h post infection. The pattern of Fc-specific fluorescence observed in this study was similar to that found by Keller *et al.* except that it was observed at a much earlier stage, namely 23 h post

infection. In addition, faint intranuclear staining, not described by Keller *et al.*, was also observed in this study. Although there is no conclusive evidence that the Fc-binding activity demonstrated by fluorescence, is caused by the virion-associated Fc-binding protein, the intracellular distribution of the Fc receptors is in keeping with the accepted route of egress of mature virions, and suggests that they may well be virion associated. Virions, after budding from the inner nuclear membrane, are believed to accumulate at a para-nuclear location, possibly in the golgi apparatus. From this site, they are transported in vesicles to the plasma membrane. The para-nuclear fluorescence may thus reflect the accumulation of virions in the golgi, and the discrete fluorescent foci which were observed towards the periphery of the cell, might represent virions in vesicles which are being transported to the cell membrane.

That an Fc receptor may be expressed on the surface of infected fibroblasts, was suggested by the work of Frey and Einsfelder (1984). In the present study, however, Fc specific membrane fluorescence could not be detected. This may be accounted for by the fact that the cells used in these studies were fixed with acetone.

COMPARISON WITH OTHER KNOWN STRUCTURAL PROTEINS OF HCMV

It was of interest to know whether the Fc-binding protein in the tegument of the virion was one of the known structural proteins of HCMV. Of the five tegument proteins which have been identified to date, three have molecular weights similar to the 70 kDa protein precipitated by Fc-gold. These include: the "lower matrix" protein (pp65), which has a molecular weight of 65-69 kDa and which is present in both virions and in dense bodies (Rueger *et al.*, 1987); the "upper matrix" protein (pp71), which is present in small amounts in both infected cells and virions, but not in dense bodies (Rueger *et al.*, 1987); and pp64, a 64-67 kDa protein, present in virions and infected cells (Davis and Huang, 1985). On the other hand, the only known tegument protein in the size range of the 33 kDa protein, is the highly immunogenic, pp28 (Meyer *et al.*, 1988). Amino-acid sequence homology is possibly the best indication of protein identity, but until the primary amino-acid sequences of the Fc-binding proteins are known, no direct homology comparisons can be performed. It was thought

that a comparison of the primary amino-acid sequences of the known tegument proteins, with those of mammalian FcR's might be rewarding. However, no significant homology was revealed and on this basis, none of the tegument proteins previously characterized could be implicated as Fc-binding proteins. The nature of the interaction between virion tegument proteins and Fc portions of IgG, may of course be totally dissimilar to those between Fc and conventional mammalian FcR's. The latter are reported to bind predominantly to the C2 domain of IgG, close to the hinge region (Gergely and Sarmay, 1990). Other Fc-binding proteins bind elsewhere; protein A, for example, binds to IgG at a site situated between the C2 and C3 domains (Langone, 1982). It was shown by electron microscopy, that pre-treatment of Fc-gold probes with protein A blocked their attachment to the HCMV virion (Stannard and Hardie, 1991), thus the site on the IgG molecule (to which the HCMV tegument Fc-binding protein attaches) is likely to be close to the binding site for protein A.

It is possible that the 70-33 kDa heterodimer has previously been characterized as a non-tegument structural protein of HCMV. Because of its solubility in the non-ionic detergent, Triton X-100, Grundy *et al.* (1987) mistakenly concluded that the HCMV virion protein which interacts with β_2m, was an envelope glycoprotein. It was subsequently shown, by means of immunogold electron microscopy (Stannard, 1989), that the protein(s) that bound β_2m are present, not in the envelope, but in the tegument of the virion. In other studies of HCMV structural proteins, workers have also assumed that extracts obtained with Triton X-100 contain only envelope proteins (Gretch *et al.*,1988; Farrar and Greenaway, 1986). In the present study, it was found that treatment of virions with Triton resulted in the solubilization of, not only envelope proteins, but also some of the tegument proteins. Data obtained from earlier experiments may need to be re-evaluated in order to establish whether other tegument proteins have been mistakenly labelled as envelope components.

In contemplating the possible identity of the Fc-binding tegument protein, consideration was given to its equally strong interaction with β_2m. It was therefore of interest that Beck and Barrell (1988) identified an open reading frame (now termed UL18) in the genome of HCMV, which has significant homology with the gene for the heavy chain of the class I HLA molecule. It was natural to wonder whether either the 70 or 33 kDa tegument proteins,

which could be precipitated by β_2m, did in fact represent the protein encoded by the UL18 gene. The DNA sequence suggests that the UL18 gene product, if expressed, would have a molecular weight of 45 kDa. Also, that it is likely to be membrane associated. Neither its predicted size, nor its potential membrane association makes the putative UL18 gene product a likely candidate for the Fc- and β_2m-binding protein found in the tegument. Browne *et al.* (1990) constructed vaccinia virus recombinants containing either the UL18 gene or the gene for β_2m. Co-infection of cells with these two recombinants, resulted in increased expression of β_2m on the cell surface. In addition, β_2m and a 67 kDa protein, presumed to be the UL18 gene product, could be co-precipitated with a monoclonal antibody to β_2m, which led them to conclude that β_2m was indeed bound by the UL18 gene product. They suggest that glycosylation could account for the increased molecular weight from the predicted 45 kDa to 67 kDa.

The intracellular processing of a protein, which has been encoded by an isolated viral gene inserted into vaccinia virus, is not necessarily the same as that which occurs during natural infections. Thus, while the UL18 gene product may be a membrane associated protein when expressed in cells infected with vaccinia virus recombinants, it may occur in an entirely different form in cells naturally infected with the entire HCMV genome. It is not yet known whether some form of the UL18 gene product is present in the virion; this can only be determined once we have access to either specific Mab's or the UL18 gene product itself.

Few reports analysing the FcR's induced by HCMV have been published. One recent publication by Xu-bin *et al.* (1989) describes PAGE analysis of Fc-binding proteins from cells infected with the Town strain of HCMV and also from purified HCMV virions. These workers detected six Fc-binding proteins, present in infected cells, ranging in size from 200 to 38 kDa and two proteins of 65 and 130 kDa present in purified HCMV virions. In these experiments, the FcR's were detected using radio-iodinated IgG and it is possible that the iodination procedure may have altered the binding properties of the probes. In this study, Fc fragments of IgG coupled to colloidal gold particles were used as probes to identify the presence of Fc-binding proteins. Proteins associate with colloidal gold particles by means of electro-static interactions (Geoghegan and

Ackerman, 1977) and, when attached in this way, retain their native conformation and are therefore more likely to display their natural binding properties. It is possible therefore that the the differences in the results obtained by Xu-bin *et al.* and those of the present study may reflect differences in the binding properties of the probes used in the experiments.

SIGNIFICANCE AND POSTULATED ROLE IN HCMV INFECTIONS

The presence of an Fc-receptor inside the virion particle is intriguing. What role a receptor located in the virion tegument might play in the pathogenesis of HCMV infections is perplexing. A number of hypotheses may be considered. It is possible that its presence may allow unenveloped virions to become coated with normal host proteins and thereby avoid immune recognition by the host immune system. The masking of antigenic epitopes as a result of the acquisition of a coat of host proteins is a strategy commonly employed by a number of human pathogens, including the parasite Shistosoma mansoni (Torpier *et al.*, 1979). The same strategy could help to preserve the infectivity of unenveloped HCMV particles in vivo. However, the manner in which particles coated with Fc-anchored IgG might attach to target cells and initiate infection is not clear.

Viruses gain entry to cells by attaching to specific receptors present on the cell surface. Two groups of workers claim to have identified cell surface receptors for HCMV virions : Keay *et al.* (1989) identified a 92 kDa cell surface protein on human fibroblasts and Taylor and Cooper (1990) have identified a 30 kDa receptor, present on lymphocytes and human fibroblasts. They also observed weak binding of HCMV virions to 28 and 92 kDa cell surface molecules.

Although not frequently taken into account, the envelope of herpesviruses is not essential for infectivity. *In vitro* studies with Allerton bovine herpesvirus have shown that removal of virion envelopes by sonication, does not result in a loss of infectivity (L.M. Stannard, personal communication). The attachment of unenveloped particles to cells presumably involves the interaction of receptors which differ from those for enveloped virions. It is possible that the Fc-binding tegument protein, is involved in this interaction once virion envelopes have been damaged or removed.

The interaction between virus particles and cell surface receptors may occur either by direct attachment, or via a bridging molecule of host origin. That soluble proteins of host cell origin might facilitate attachment of virions to the cell surface, is well described. For example, the attachment of hepatitis B virus to hepatocytes, is said to be enhanced by polymerized serum albumin (Weiss, 1991). It has also been suggested that HSV gains entry into cells by first adsorbing to the host protein, basic fibroblast growth factor (bFGF) and by this means, attaching to receptors for bFGF on the surface of the cell (Baird *et al.*, 1990).

Antibody molecules frequently function as bridging molecules. After specific attachment of antibody to viral epitopes, antibody-virus complexes may be bound by FcR's expressed on the surface of monocytes and macrophages and enter the cell by phagocytosis. In this way, infection with viruses that are capable of replicating in macrophages or monocytes, may be enhanced (Peiris and Porterfield, 1981; Halstead, 1979).

With regard to possible mechanisms for attachment of HCMV, this study has shown that antibody can be bound in reverse orientation to unenveloped virions, i.e. via the Fc portion of the molecule. Under these circumstances, the Fab portions of the molecule would be most available for interaction with a receptor on the cell surface and, unlike specific immune complexes of viruses, IgG-coated HCMV capsids would be unable to utilize Fc receptors to enter the cell. Of relevant interest, is the recent report by Lenert *et al.* (1990), that the CD4 molecule of T lymphocytes can bind the Fab portion of IgG. This raises the question of whether CD4, or indeed any other unknown receptor, either for Fab constant domains or for another part of the IgG molecule, could serve as cell surface receptors for unenveloped HCMV virions.

The relevance of the Fc-binding property of the 33 kDa HCMV tegument protein, must be assessed in the light of its dual affinity for β_2m. It has previously been suggested that β_2m-coated HCMV virions might attach to target cells by interacting with class I HLA molecules expressed on the cell surface (Grundy *et al.*, 1987b). This theory is rather improbable, however, as it implies that β_2m is bi-valent. Since β_2m is mono-valent, it cannot form a bridge between virion and cell.

In humans, β_2m associates with the heavy chain of the class I HLA molecule and also with the the CD1 antigen, a family of MHC class I-like proteins expressed on the surface of immature thymocytes (Martin *et al.*, 1986). In mice and rats, β_2m is known to associate with yet another MHC-like protein which is also an Fc receptor. This receptor is expressed on enterocytes in the upper small intestine of neonatal animals and has provisionally been termed FcRn (neonate). Its function is to transport IgG across the enterocyte from the intestinal lumen (Simister and Mostov, 1989). The FcRn molecule is markedly similar to the heavy chains of both class I HLA molecules and CD1 antigens. Another protein known to have affinity for both the Fc portion of IgG and β_2m, is a protein present on the surface of the parasite Schistosoma mansoni (Torpier *et al.*, 1979). It appears, therefore, that shared affinity for Fc and β_2m (such as we have shown to exist in the 33 kDa tegument protein) is not an uncommon phenomenon in nature.

Both β_2m and the IgG molecule, as well as many other proteins involved in recognition events occurring at the cell membrane, have been shown to contain one or more immunoglobulin units or domains (fig. 13). The immunoglobulin domain is characterized by a conserved tertiary structure consisting of a pair of anti-parallel β pleated sheets, held together by a di-sulphide bond formed between a pair of highly conserved cysteine residues. Proteins containing this structure are said to belong to the immunoglobulin superfamily of molecules. The presence of an immunoglobulin domain in so many different proteins suggests evolution from a single primordial gene. An important feature of proteins of the immunoglobulin superfamily, is that they are complementary to one another and as a result, strong non-covalent inter-domain interactions commonly occur between members of the protein family. Examples of this type of interaction are, the association of the heavy and light chains in the immunoglobulin molecule itself, and the association of β_2m with the MHC class I heavy chain (Williams and Barklay, 1988).

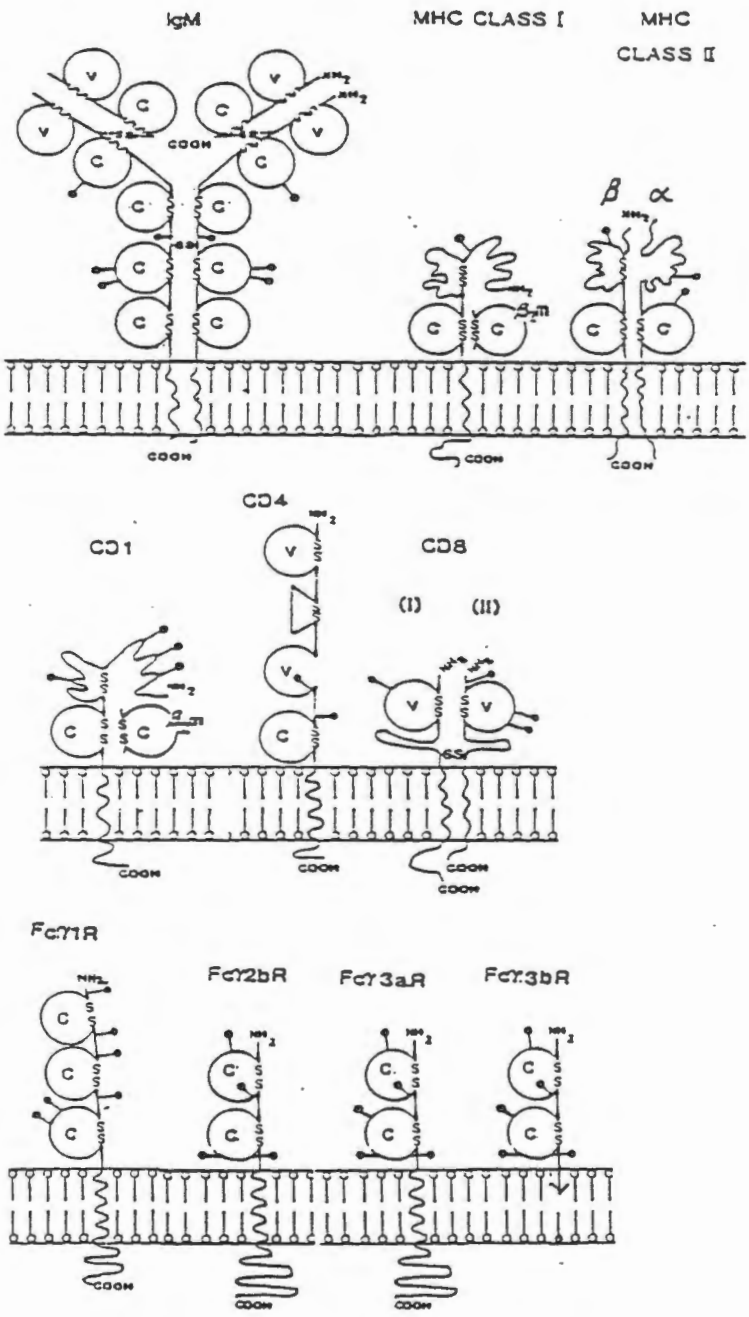


Fig. 13

Models for selected members of the immunoglobulin superfamily. The circles represent sequence segments that fold as an immunoglobulin domain. Segments labelled C correspond to domains similar in structure to the constant domains of immunoglobulins, and those labelled V resemble the variable domains of immunoglobulins. Intra- and inter-chain di-sulphide bonds are shown by SS symbols. Predicted N-linked glycosylation sites are indicated. The presence of a phosphatidyl-inositol glycan anchor is indicated by an arrow for FcγbR. (adapted from Williams and Barklay, 1988)

It is possible that similar properties may be attributed to the 33 kDa tegument protein. If the tegument protein itself, were also a member of the immunoglobulin superfamily, its interaction with either Fc or β_2m (or both) might occur as a result of domain homology. It would also be possible for the 33 kDa protein to have equal (or greater) affinity for yet another ligand, also a member of the immunoglobulin superfamily. It is perhaps relevant, that a low level of interaction between Fab fragments of IgG and the tegument of HCMV virions was also observed by immunogold electron microscopy. This might represent interactions between similar domains in the Fc-binding tegument protein and the Fab portion of IgG. A number of cell surface molecules, belonging to the immunoglobulin superfamily, have been identified as virus receptors (White and Littman, 1989). These include : the CD4 molecule, which acts as the receptor for HIV (Maddon *et al.*, 1986); the inter-cellular adhesion molecule (ICAM-1), the major rhinovirus receptor (Staunton *et al.*, 1989); and, an as yet unidentified protein, the poliovirus receptor (Mendelsohn *et al.*, 1989). It is thus possible that a cell surface molecule of the immunoglobulin superfamily acts as the receptor for unenveloped HCMV virions and that the 33 kDa HCMV tegument protein represents the attachment protein on the virion. If this were so, then the Fc- and β_2m-binding properties of the 33 kDa protein may simply be red herrings.

In conclusion, an Fc receptor present in the tegument of the HCMV virion itself, has been isolated and characterized. These results are novel, as the existence of a protein of this nature has not been revealed by previous work on HCMV. Positive identification of the protein can only be made once its sequence has been determined. Knowlege of the protein's structure will enable the role that it plays in the pathogenesis of infection with HCMV to be defined more readily.

APPENDIX I

0.08 M Barbital-glycine-tris buffer, pH8.6 for immunoclectrophoresis:

solution A:

Sodium Barbital 6.5g

Barbital 1.04g

dissolved in 0.5 litres of distilled water

Solution B:

Glycine 28.1g

Tris base 22.6g

dissolved in 0.5 litres of distilled water.

Mix equal volumes of solutions A and B.

APPENDIX II

REAGENTS FOR PAGE

Sample Buffer:

0.01 M Tris-HCL, pH 8.0

0.001 M EDTA

1% sodium dodecal sulphate (SDS)

10% glycerol

0.1% Bromophenol blue

Reducing Sample buffer:

5% Beta-mercapto-ethanol (BME) added to sample buffer.

Separating Gel Buffer:

0.375 M Tris-HCL, pH 8.8

0.1% SDS

Stacking Gel Buffer:

0.125 M Tris-HCL, pH 6.8

0.1% SDS

Electrophoresis Buffer:

0.05 M Tris-HCL, pH 8.3

0.384 M glycine

0.1% SDS

Acrylamide Stock Solution:

30% w/v acrylamide

0.8% bis-acrylamide

in distilled water

Coomassie Blue Stain:

10 % acetic acid

30 % methanol

0.025 % w/v Coomasie Brilliant Blue R

APPENDIX III

Transfer Buffer:

22.4 g Glycine

4.8 Tris base,

made up to 1600 ml in distilled water. Add 400 ml methanol and adjust pH to 8.3 with concentrated HCl

APPENDIX IV

PREPARATION OF COLLOIDAL GOLD

To make 50 ml of colloid:

Solution A:

39.875 ml deionised, microfiltered water

0.125 ml 4% Chloro-auric acid

Solution B:

2 ml 1% trisodium citrate (prepared freshly)

0-2.5 ml 1% tannic acid

0-2.5 ml 0.015 K_2CO_3 (equivalent volume to tannic acid used)

De-ionised, micro-filtered water up to 10 ml

The size of the gold particles varies inversely with the amount of tannic acid added.

Guide for the amount of tannic acid to use: (approximately)

1.5 ml tannic acid for 3 nm gold

0.1-0.05 ml tannic acid for 6-8 nm gold

25 μl tannic acid for 10 nm gold

Method:

Heat both solutions to 60^0 C. Add solution B to solution A whilst rapidly mixing. Stir at 60^0 C for 20 minutes then bring to boiling point and boil for about 2 minutes.The colour of the gold mixture will change from blue to red indicating the reduction end point.

LIST OF ABBREVIATIONS

β_2m - beta 2-microglobulin

BME - beta-mercapto ethanol

BSA - bovine serum albumin

bFGF - basic fibroblast growth factor

^{0}C - degrees Celsius

CID - cytomegalic inclusion disease

c.p.e. - cytopathic effect

C - constant (domain)

cDNA - complimentary DNA

DNA - deoxyribo-nucleic acid

ds - double stranded

DEAE - diethyl-amino-ethyl

EDTA - ethylene-diamine-tetra-acetic acid

ELISA - enzyme-linked immunosorbant assay

E.M. - electron microscopy

EBV - Epstein-Barr virus

Fab - fraction antigen binding

Fc - fraction crystallizable

FcR - Fc receptor

FcγR - Fc receptor for IgG

FcεR - Fc receptor for IgE

FcRn - Fc receptor-neonate

FITC - fluorosceine isothiocyanate

g - grammes

mg - milligramme

µg - microgramme

g - gravitational force

gp - glycoprotein

gB - glycoprotein B

gC - glycoprotein C

gD - glycoprotein D

gE - glycoprotein E

gI - glycoprotein I

HCMV - human cytomegalovirus

HSV - herpes simplex virus

HEF - human embryonic fibroblasts

h - hour(s)

H - heavy (chain of IgG)

HLA - human leucocyte antigen

IgA - immunoglobulin A

IgE - immunoglobulin E

IgG - immunoglobulin G

IgM - immunoglobulin M

kDa - kilodaltons

l - litres

ml - millilitres

µl - microlitres

L - light (chain of IgG)

M - molar solution

m.o.i. - multiplicity of infection

mA - milliamps

mm - millimetres

min - minute(s)

mRNA - messenger RNA

Mab - monoclonal antibody

MHC - major histocompatibility complex

nm - nanometres

NH_2 - amino

PTA - phosphotungstic acid

PMSF - phenyl-methyl-sulphonyl fluoride

pp - phosphoprotein

PBS - phosphate buffer containing 0.15 M NaCl (saline)

PAGE - polyacrylamide gel electrophoresis

SDS - sodium dodecyl sulphate

TBS - tris buffer containing 0.15 M NaCl (saline)

TBSA - TBS containing bovine serum albumin

VZV - varicella-zoster virus

V - variable (domain)

v - volts

kv - kilo-volts

w/v - weight per volume

BIBLIOGRAPHY

ALFORD CA, BRITT WJ (1990) Cytomegalovirus. In. Virology. Edited by BN Fields, DM Knipe. New York: Raven Press Ltd. 1981-2010.

BAIRD A, FLORKIEWICZ RZ, MAHER PA, KANER RJ, HAJJAR DP (1990) Mediation of virion penetration by association of basic fibroblast growth factor with herpes simplex virus type 1. *Nature (London)*, **348**, 344-346.

BECK S, BARRELL BG (1988) Human cytomegalovirus encodes a glycoprotein homologous to MHC Class-I antigens. *Nature (London)*, **331**, 269-272.

BENKO DM, HALTIWANGER RS, HART GW, GIBSON W (1988) Virion basic phosphoprotein from human cytomegalovirus contains O-linked N-Acetyl glucosamine. *Proc. Natl. Acad. Sci. USA*, **85**, 2573-2577.

BROWNE H, SMITH G, BECK S, MINSON T (1990) A complex between the MHC class 1 homologue encoded by human cytomegalovirus and β_2-microglobulin. *Nature (London)*, **347**, 770-772.

COLE R, KUTTNER AG (1926) A filterable virus present in the submaxillary glands of guinea pigs. *J. Exp. Med.* , **44**, 855-873.

CRANAGE MP, KOUZARIDES T, BANKIER AT, SATCHWELL S, WESTON K, TOMLINSON P, et al (1991) Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. *EMBO J.*, **5**, 3057-3063.

CRANAGE MP, SMITH GL, BELL SE (1988) Identification and expression of HCMV glycoprotein H: neutralization of HCMV infectivity with anti-gH monoclonal antibody. *J. Virol.*, **62**, 1416-1422.

DAVIS MG, HUANG E-S (1985) Nucleotide sequence of a human cytomegalovirus DNA fragment encoding a 67 kilodalton phosphorylated viral protein. *J. Virol.*, **56**, 7-11.

DESSAINT JP, CAPRON M, CAPRON A (1990) Immunoglobulin E-stimulated release of Mediators from mononuclear phagocytes, eosinophils and platelets. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 260-287.

DUBIN G, FRANK I, FRIEDMAN HM (1990) Herpes simplex virus type 1 encodes two Fc receptors which have different binding characteristics for monomeric immunoglobulin G (IgG) and IgG complexes. *J. Virol.*, **64**, 2725-2731.

ELDER JH, ALEXANDER S (1982) endo-B-N-Acetylglucosaminidase F: Endoglycosidase from Flavobacterium meningosepticum that cleaves both high-mannose and complex glycoproteins. *Proc. Natl. Acad. Sci. USA*, **79**, 4540-4544.

EVRIN PE, WIBELL L (1972) Serum levels and urinary excretion of β_2-microglobulin in apparently healthy subjects. *Scand. J. Clin. Lab. Invest.* **29**, 69-74.

FARRAR GH, GREENAWAY PJ (1986) Characterization of glycoprotein complexes present in human cytomegalovirus envelopes. *J. Gen. Virol.*, **67**, 1469-1473.

FRANKLIN EC, PRELLI F (1960) Structural units of human 7S gamma globulin. *J. Clin. Invest.*, **39**, 1933-1941.

FREY J, EINSFELDER B (1984) Induction of surface IgG receptors in cytomegalovirus infected human fibroblasts. *Eur. J. Biochem.*, **138**, 213-216.

FRIDMAN WH, SAUTES C (1990) Immunoglobulin Binding factors. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 335-355.

GEOGHEGAN WD, ACKERMAN GA (1977) Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscopic level: a new method, theory and application. *J. Histochem. Cytochem.*, **25**, 1187-1200.

GERGELY J, SARMAY G (1990) The two binding site models of human IgG binding Fc receptors. *FASEB J.*, **4**, 3275-3283.

GRETCH DR, KARI B, GEHRZ RC, STINSKI MF (1988) A multigene family encodes the human cytomegalovirus glycoprotein complex gcII (gp47-52 complex). *J. Virol.*, **62**, 1956-1962.

GRETCH DR, KARI B, RASMUSSEN L, GEHRZ RC, STINSKI M (1988) Identification and characterization of three distinct families of glycoprotein complexes in the envelopes of human cytomegalovirus. *J. Virol.*, **62**, 875-881.

GRIFFITHS PD (1990) Cytomegalovirus. In: Principles and practice of Clinical Virology. Edited by AJ Zuckerman, JE Banatvala, JR Pattison. : John Wiley and Sons Ltd. 69-102.

GRUNDY JE, AYLES HM, MCKEATING JA, BUTCHER RG, GRIFFITHS PD, POULTER LW (1988) Enhancement of class 1 HLA antigen expression by cytomegalovirus: role in amplification of virus infection. *J. Med. Virol.*, **25**, 483-495.

GRUNDY JE, LUI SF, SUPER M, BERRY NJ, SWENY P, FERNANDO ON, et al (1988) Symptomatic cytomegalovirus infection in seropositive kidney recipients: reinfection with donor virus rather than reactivation of recipient virus. *Lancet*, **ii**, 132-135.

GRUNDY JE, MCKEATING JA, GRIFFITHS PD (1987a) Cytomegalovirus strain AD169 binds β_2-microglobulin in vitro, after release from cells. *J. Gen. Virol.*, **68**, 777-784.

GRUNDY JE, MCKEATING JA, WARD PJ, SANDERSON AR, GRIFFITHS PD (1987b) β_2-microglobulin enhances the infectivity of cytomegalovirus and when bound to the virus enables class I HLA molecules to be used as a virus receptor. *J. Gen. Virol*, **68**, 793-803.

HALSTEAD SB (1979) In vivo enhancement of Dengue virus infection in Rhesus monkeys by passively transferred antibody. *J. Infect. Dis.*, **140**, 527-531.

HART GW, HOLT GD, HALTIWANGER RS (1988) Nuclear and cytoplasmic glycosylation: novel saccharide linkages in unexpected places. *TIBS*, **13**, 380-384.

INGANAS M (1981) Comparison of mechanisms of interaction between protein A from Staphylococcus aureus and human monoclonal IgG, IgA and IgM in relation to the classical Fc and the alterative F(ab')$_2$E protein A reactions. *Scand. J. Immunol.*, **13**, 343-352.

JOHNSON DC, FEENSTRA V (1987) Identification of a novel herpes simplex virus type I-induced glycoprotein which complexes with gE and binds immunoglobulin. *J. Virol.*, **61**, 2208-2216.

JOHNSON DC, FRAME MC, LIGAS MW, CROSS AM, STOW ND (1988) Herpes simplex virus immunoglobulin G Fc receptor activity depends on a complex of two viral glycoproteins, gE and gI. *J. Virol.*, **62**, 1347-1354.

KEAY S, MERIGAN TC, RASMUSSEN L (1989) Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA*, **86**, 10100-10103.

KELLER R, PEITCHEL R, GOLDMAN JN, GOLDMAN M (1976) An IgG-Fc receptor induced in cytomegalovirus-infected human fibroblasts. *J. Immunol.*, **116**, 772-777.

KINET J-P (1989) Antibody-cell interactions: Fc receptors. *Cell*, **57**, 351-354.

LAEMMLI UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680-685.

LANGONE JJ (1982) Protein A of Staphylococcus aureus and related immunoglobulin receptors produced by streptococci and pneumonococci. *Adv. Immunol.*, **32**, 157-252.

LAW KM, WILTON-SMITH P, FARRAR GH (1985) A murine monoclonal antibody recognizing a single glycoprotein within a human cytomegalovirus virion envelope glycoprotein complex. *J. Med. Virol.*, **17**, 255-266.

LENERT P, KROON D, SPIEGELBERG H, GOLUB ES, ZANETTI M (1990) Human CD4 binds immunoglobulins. *Science*, **248**, 1639-1643.

LONNQVIST B, RINGDEN O, WAHREN B, GAHRTON G, LUNDGREN G (1984) Cytomegalovirus infection associated with and preceding chronic graft-versus-host disease. *Transplantation*, **38**, 465-468.

LYNCH RG, SANDOR M (1990) Fc Receptors on T and B lymphocytes. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 305-334.

MADDON PJ, DALGLEISH AG, MCDOUGAL JS, CLAPHAM PR, WEISS RA, AXEL R (1986) The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell*, **47**, 333-348.

MARIANI G, STROBER W (1990) Immunoglobulin Metabolism. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 94-177.

MARTIN LH, CALABI F, MILSTEIN C (1986) Isolation of CD1 genes: a family of major histocompatability complex-related differentiation antigens. *Proc. Natl. Acad. Sci. USA*, **83**, 9154-9158.

MCKEATING JA, GRIFFITHS PD, GRUNDY JE (1987) Cytomegalovirus in urine specimens has host β_2-microglobulin bound to the viral envelope: a mechanism for evading the immune response?. *J. Gen. Virol.*, **68**, 785-792.

MCKEATING JA, GRUNDY JE, VARGHESE Z, GRIFFITHS PD (1986) Detection of cytomegalovirus by ELISA in urine samples is inhibited by β_2-microglobulin. *J. Med. Virol.*, **18**, 341-348.

MCKEATING JA, STAGNO S, STIRK PR, GRIFFITHS PD (1985) Detection of cytomegalovirus in urine samples by enzyme-linked immunosorbant assay. *J. Med. Virol.*, **16**, 367-373.

MENDELSOHN CL, WIMMER E, RACANIELLO VR (1989) Cellular receptor for poliovirus : Molecular cloning, nucleotide sequence and expression of a new member of the immunoglobulin superfamily. *Cell*, **56**, 855-865.

METZGER H (1990) General aspects of antibody structure and function. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 7-11.

MEYER H, BANKIER AT, LANDINI MP, BROWN CM, BARRELL BG, RUEGER B, et al (1988) Identification and prokaryotic expression of the gene coding for the highly immunogenic 28-kilodalton structural phosphoprotein (pp28) of human cytomegalovirus. *J. Virol.*, **62**, 2243-2250.

O'GRADY JG, ALEXANDER GJM, SUTHERLAND S, HARVEY F, PORTMAN B, CALNE RY, et al (1988) Cytomegalovirus infection and Donor/Recipient HLA antigens: interdependent co-factors in pathogenesis of vanishing bile-duct syndrome after liver transplant. *Lancet*, **ii**, 302-305.

OGATA M, SHIGETA S (1979) Appearance of immunoglobulin G Fc receptor in cultured human cells infected with varicella-zoster virus. *Infect. Immunol.*, **26**, 770-774.

PEIRIS JSM, PORTERFIELD JS (1981) Antibody dependent enhancement of plaque formation in cell lines of macrophage origin - a sensitive assay for antiviral antibody. *J. Gen. Virol.*, **57**, 119.

PORTER RR (1959) The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain. *Biochem. J.*, **73**, 119-126.

RAVETCH JV, ANDERSON CL (1990) Fc R Family: proteins, transcripts and genes. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 211-235.

ROITT IT (1988) Essential Immunology. : Blackwell Scientific Publications. 43-48.

ROWE WP, HARTLEY JW, WATERMAN S, TURNER HC, HUEBNER RJ (1956) Cytopathogenic agent resembling human salivary gland virus recovered from tissue cultures of human adenoids. *Proc. Soc. Exp. Biol. and Med.*, **92**, 418-424.

RUEGER B, KLAGES S, WALLA B, ALBRECHT J, FLECKENSTEIN B, TOMLINSON P, et al (1987) Primary structure and transcription of the genes coding for the two virion phosphoproteins pp65 and pp71 of human cytomegalovirus. *J. Virol.*, **61**, 446-453.

SANDERSON AR, LANNING JJ (1970) Crystalline Fc prepared in high yield from normal human IgG. *Nature (London)*, **226**, 356-358.

SEGAL DM (1990) Antibody mediated killing by leukocytes. In: Fc Receptors and the action of antibadies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 291-301.

SIMISTER NE, MOSTOV KE (1989) An Fc receptor structurally related to MHC class I antigens. *Nature (London)*, **337**, 184-187.

SIMISTER NE (1990) Transport of monomeric antibodies across epithelia. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washinton D.C.: American Society for Microbiology. 57-73.

SIMMONS D, SEED B (1988) The Fc receptor of natural killer cells is a phospholipid-linked membrane protein. *Nature (London)*, **333**, 568-570.

SISSONS JGP, BORYSIEWICZ LK, RODGERS B, SCOTT D (1986) Cytomegalovirus - its cellular immunology and biology. *Immunology Today*, **7**, 57-61.

SLOT JW, GEUZE HJ (1985) A new method for preparing gold probes for multiple-labelling cytochemistry. *Eur. J. Cell Biol.*, **38**, 87-93.

STANNARD LM (1989) β_2-microglobulin binds to the tegument of cytomegalovirus: an immunogold study. *J. Gen. Virol.*, **70**, 2179-2184.

STANNARD LM, HARDIE DR (1991) An Fc receptor for human immunoglobulin G is located within the tegument of human cytomegalovirus. *J. Virol.*, **65**, 3411-3415.

STANNARD LM, RIDER JR, FARRAR GH (1989) Morphology and distribution of gp52 on extracellular Human cytomegalovirus (HCMV) supports biochemical evidence that it represents the HCMV glycoprotein B. *J. Gen. Virol.*, **70**, 1553-1560.

STAUNTON DE, MERLUZZI VJ, MARLIN SD, SPRINGER TA (1989) A cell adhsion molecule, ICAM-1, is the major surface receptor for rhinoviruses. *Cell*, **56**, 849-853.

STINSKI MF (1990) Cytomegalovirus and its Replication. In: Virology. Edited by BN Fields, DM Knipe. New York: Raven Press . 1959-1980.

STRYER L (1988) Biochemistry. New York: W.H. Freeman and Co.. 773-774.

TAYLOR HP, COOPER NR (1990) The human cytomegalovirus receptor on human fibroblasts is a 30 kDa membrane protein. *J. Virol.*, **64**, 2484-2490.

TORPIER G, CAPRON A, OUAISSI MA (1979) Receptor for IgG(Fc) and human B2-microglobulin on S. mansoni schistosomula. *Nature (London)*, **278**, 447-449.

TOWBIN H, STAEHELIN T, GORDON J (1979) Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.

UNDERDOWN BJ (1990) Transcytosis by the Receptor for polymeric Immunoglobulin. In: Fc Receptors and the action of antibodies. Edited by H Metzger. Washington D.C.: American Society for Microbiology. 74-93.

WALTZER WL, ARNOLD AN, ANAISE D, HURLEY S, RAISBECK A, EGELANDSDAL B, et al (1987) Impact of cytomegalovirus infection and HLA matching on outcome of renal transplantation. *Transplant. Proc.*, **19**, 4077-4080.

WEISS RA (1991) Games that herpes viruses play. *Curr. Biol.*, **1**, 69-70.

WESTMORELAND D, WATKINS JF (1974) The IgG receptor induced by herpes simplex virus: studies using radioiodinated IgG. *J. Gen. Virol.*, **24**, 167-178.

WHITE JM, LITTMAN DR (1989) Viral receptors of the immunoglobulin superfamily. *Cell*, **56**, 725-728.

WILLIAMS AF, BARKLAY AN (1988) The immunoglobulin superfamily - domains for cell surface recognition. *Ann. Rev. Immunol.*, **6**, 381-405.

XU-BIN, MURAYAMA T, ISHIDA K, FURUKAWA T (1989) Characterization of IgG Fc receptors induced by human cytomegalovirus. *J. Gen. Virol.*, **70**, 893-900.