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(54) Title: IMMUNOGENIC GLYCOPROTEINS OF HUMAN CYTOMEGALOVIRUS gCII

(57) Abstract

A substantially pure, immunogenic glycoprotein complex of human cytomegalovirus having a molecular weight greater than about 200 kD is disclosed. The complex comprises at least two distinct groups of glycoproteins, which are recognized by two different groups of monoclonal antibodies. The complex is reactive with adult seropositive sera, but is not substantially reactive with sera of congenitally infected infants.

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IMMUNOGENIC GLYCOPROTEINS OF HUMAN CYTOMEGALOVIRUS gCII

Statement Regarding Federally Sponsored Research

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Field of the Invention

The present invention is directed to substantially pure, immunogenic glycoprotein complexes of HCMV which comprise at least two distinct groups of glycoproteins that are recognized by two different groups of monoclonal antibodies, to substantially pure component glycoproteins of these complexes, and to vaccines comprising the glycoproteins.

Background of the Invention

The number of individual glycoproteins reported to be present in extracellular human cytomegalovirus (HCMV) has varied. Furthermore, little detailed information has been reported on the interactions and composition of HCMV glycoproteins. HCMV glycoproteins appear to be involved in human immune recognition of the virus. This has been demonstrated by the reactivity of HCMV glycoproteins with human convalescent sera (G.H. Farrar et al., J. Gen. Virol., 65, 1991 (1984); B. Nowak et al., Virology, 132, 325 (1984)) and peripheral blood mononuclear cells (Y.C. Liu et al., J. Virol., 62, 1066-1070 (March 1988)).

Biochemical approaches have been used to determine the composition of some of the HCMV glycoproteins. Farrar et al., supra, were able to detect five polypeptides in the membranes of HCMV strain AD169 which were labeled by carbohydrate-specific methods. These glycoproteins had molecular weights ranging from 57,000 to 250,000. One method they employed labeled galactose (Gal) and/or N-acetylgalactosamine (GalNAc)

residues. With this method, two glycoproteins with molecular weights of 67,000 and 130,000 showed relatively high incorporation of label, suggesting that these glycoproteins have a distinct phenotype marked by relatively high concentrations of Gal and/or GalNAc and possibly high amounts of O-linked oligosaccharides.

Immunological approaches have also been used to identify some of the glycoproteins in HCMV. Several laboratories have generated monoclonal antibodies (MoAbs) which recognize one or more disulfide-linked glycoprotein complexes which contain three individual glycoproteins. Several laboratories have reported molecular weights of 130,000, 92,000-95,000, and 52,000-55,000 daltons for these glycoproteins (G.H. Farrar et al., J. Gen. Virol., 67, 1469 (1986); B.E. Kari et al., J. Virol., 60, 345-352 (1986); K.M. Law et al., J. Med. Virol., 17, 255 (1985); L. Rassmussen et al., J. Virol., 55, 275 (1985)). Kari et al. have disclosed two MoAbs designated 41C2 and 9B7 which recognize these glycoproteins. Hereinafter, the parent glycoprotein complex comprising the 55 kD, 93 kD, and 130 kD glycoproteins will be referred to as gCI.

Another complex, referred to hereinafter as gCIII, has been identified which contains a glycoprotein with homology to glycoprotein H from HSV (Cranage et al., J. Virol., 62, 1416-1422 (1988)). This glycoprotein was initially described by Rasmussen et al. P.N.A.S., 81, 876-880 (1984).

B.E. Kari et al., J. Virol., 60, 345-352 (1986) have described a MoAb designated 9E10 which reacted with glycoprotein complexes having molecular weights of 93,000 and 450,000 daltons, which eluted from an anion exchange column at 0.3 and 0.9 molar NaCl, respectively. When the 93 kD complex was immunoprecipitated under reducing conditions with MoAb 9E10, the most abundant glycoproteins had molecular weights of 50,000-52,000

daltons. A very small amount of a glycoprotein with a molecular weight of 90,000 daltons was also detected.

D.R. Gretch et al., J. Virol., 62, 875-881 (March, 1988) reported that, when solubilized HCMV envelope glycoproteins were subjected to immunoprecipitation with MoAb 9E10, the antibody reacted with a heterogeneous species designated gp47-52 and with an unidentified species of greater than 200 kD. Gretch et al. designated the HCMV glycoprotein complex comprising these glycoproteins as gCII. Subsequently it was shown that the gCII glycoproteins recognized by 9E10 had a high content of O-linked oligosaccharides (Kari and Gehrz, Arch. Virol., 98, 171-188 (before May, 1988)), and were encoded for in the HXLF gene family of HCMV (Gretch et al., J. Virol., 62, 1956-1962 (June, 1988)).

HCMV infection can be a serious problem for a developing fetus, and therefore humoral immune responses to HCMV proteins in pregnant women and their infants have been studied (Ahlfors et al., Scand. J. Infect. Dis., 16, 129-137 (1984); Alford et al., J. Infect. Dis., 158, 917-924 (1988)). Infant and adult cell-mediated immune responses to HCMV proteins have also been studied. Y.C. Liu et al., J. Virol., 62, 1066-1070 (March, 1988) studied the T-lymphocyte proliferation responses of adult and infant subjects to whole HCMV and to HCMV glycoprotein complexes gCI and gCII. Although all adult sera responded to whole HCMV antigen, a broad spectrum of lymphocyte proliferative responses to the complexes gCI and gCII was observed. Peripheral blood mononuclear cells (PBMC) isolated from the three infants did not respond to either complex. In an enzyme-linked immunosorbent assay, however, sera from all seropositive adults recognized both gCI and gCII, while serum from a seronegative adult donor did not react to either complex. Sera from the infants reacted with both gCI and gCII. These results demonstrated the lack of any qualitative

difference in the ability of adult or infant sera to immunoprecipitate gCI and gCII, as well as the apparent lack of a correlation between antibody and lymphocyte proliferative responses to the gCI and gCII glycoproteins.

Accordingly, there exists a need for further study of the human immune response to HCMV glycoproteins, particularly those of the gCII family.

Brief Description of the Invention

The present invention is directed to compositions of matter which are substantially pure glycoprotein complexes or glycoproteins which are derivable from the HCMV gCII family of glycoprotein complexes. These compounds are found on the membrane envelope of the intact HCMV virion, where the glycoproteins are associated via disulfide linkages into the glycoprotein complexes. The glycoprotein complexes and their component glycoproteins are physically accessible to the immune system and to antibodies, and are capable of stimulating both humoral and cellular immunity in humans and other mammals.

It has unexpectedly been found that the gCII complex comprises at least two distinct groups of glycoproteins, which are recognized by two different groups of monoclonal antibodies. Therefore, a preferred embodiment of the present invention is a substantially pure, immunogenic glycoprotein complex, which is on the membrane envelope of HCMV, and which contains

(a) an about 50-52 kD glycoprotein which reacts with a first monoclonal antibody designated herein as a Group 1 monoclonal antibody; and

(b) two glycoproteins having respective molecular weights of about 90 kD and greater than about 200 kD, both of which react with a second

monoclonal antibody designated herein as a Group 2 monoclonal antibody.

The complex, which includes three glycoprotein components, has a molecular weight of greater than about 200 kD, and is thus designated hereinafter as gCII-200. The Group 1 monoclonal antibody may be selected from the group consisting of monoclonal antibodies designated herein as 9E10, 8B4, and 26E2. The Group 2 monoclonal antibody may be selected from the group consisting of monoclonal antibodies designated herein as 15F9, 12G9, 15G5, 23B10, 25C8, 27B4, and 40B7.

The gCII-200 complex was further analyzed in terms of human humoral immune response to the complex and its component glycoproteins. It was unexpectedly found that under reducing condition the component glycoproteins of gCII-200 are reactive with adult seropositive sera, but are not reactive with congenitally-infected infant sera. However, both the adult and the infant sera are reactive with the 52 kD and 158 kD glycoproteins of the gCI complex. Therefore, the present invention is further directed to the gCII-200 complex wherein the complex is reactive with HCMV-seropositive adult human sera, but is not substantially reactive with HCMV-seropositive, congenitally infected infant human sera. Preferably, the adult human sera and the infant human sera are both reactive with gCI glycoproteins having a molecular weight of about 52 kD or 158 kD.

It has also been determined that the Group 1 and Group 2 MoAbs which react with different glycoproteins of gCII-200 may be further distinguished by their reactive site within HCMV-infected cells. Preferably, the Group 1 monoclonal antibodies are reactive with the plasma membrane of HCMV-infected living human cells and with the cytoplasm of HCMV-infected fixed human cells. Additionally, the Group 1 MoAb 9E10 is preferably reactive with the nucleus of HCMV-infected

fixed human cells. The Group 2 MoAbs are preferably reactive with the cytoplasm of HCMV-infected fixed human cells, but are not reactive with the plasma membranes of HCMV-infected living human cells.

The present invention is also directed to a substantially pure, immunogenic glycoprotein having a molecular weight of about 50-52 kD, wherein the glycoprotein is derivable from the membrane envelope of HCMV and can be associated with other envelope glycoproteins by means of disulfide bonds, and wherein the about 50-52 kD glycoprotein reacts with a monoclonal antibody selected from the group consisting of 9E10 (IVI-10118), 8B4 and 26E2. Under reducing conditions, it has been found that the glycoprotein reacts with Group 1 MoAb 9E10 and does not substantially react with Group 2 MoAb 12G9. Under non-reducing conditions, however, it has been found that the 50-52 kD glycoprotein reacts with both Group 1 and Group 2 MoAbs, including the monoclonal antibodies 9E10, 12G9, 26E2, 15F9, and 40B7.

As used herein, "non-reducing conditions" means that glycoprotein complexes were isolated by detergent extraction from HCMV virion envelopes, and immunaffinity purified as described hereinbelow with gCII-specific monoclonal antibodies to obtain purified, unreduced gCII complexes, and "reducing conditions" means that purified, unreduced gCII complexes were additionally solubilized, with subsequent alkylation, as described hereinbelow. Following reduction, the individual glycoproteins could then be separated by SDS-PAGE and analyzed in Western blot, as described hereinbelow.

The present invention is also directed to a substantially pure immunogenic glycoprotein having a molecular weight of about 39-48 kD, designated gp39-48(II), which can be detected in whole Towne HCMV in Western Blot using a Group 2 MoAb.

The present invention also includes two substantially pure, immunogenic glycoproteins having molecular weights of about 90 kD or greater than about 200 kD, designated gp90(II) and gp200(II), respectively. These glycoproteins are derivable from the membrane envelope of HCMV, can be associated with other envelope glycoproteins by means of disulfide bonds, and are reactive with the Group 2 MoAbs defined above. The gp90(II) and gp200(II) glycoproteins can be obtained by reducing the gCII-200 complex described above. It has also been determined that these glycoproteins are synthesized late in infection. Furthermore, it has been found that these glycoproteins are detectable by HCMV-seropositive adult human sera in Western blot, but are not substantially detectable by HCMV-seropositive, congenitally infected infant human sera. Both adult and infant sera are detectable by gCI proteins of about 52 kD or about 158 kD molecular weight, however. The gp90(II) and gp200(II) glycoproteins were also not substantially detectable by sera from HCMV sero-positive mothers of the congenitally infected infants, when the maternal sera was analyzed within 0-63 months post partum.

Due to the immunogenic properties of the present glycoprotein complexes and their component glycoproteins, the present invention also provides a vaccine comprising an amount of one or more of the gp90(II) or gp200(II) glycoproteins which is effective to produce an immune response against HCMV in a mammal, when administered thereto, in combination with a pharmaceutically-effective vehicle such as a liquid carrier. For a description of a vaccine employing the gCI complex of HCMV, see L. Pereira (U.S. Patent No. 4,689,225), the disclosure of which is incorporated by reference herein.

Apart from their use in vaccines, the present glycoprotein complexes and their component glycoproteins

may be useful in the production of monoclonal antibodies, such as the gCII-specific monoclonal antibodies described above, which can in turn be used to diagnose HCMV, or to treat HCMV infections by blocking HCMV infectivity and/or toxicity. The present complexes and glycoproteins are also useful to produce clonal populations of antigen-specific T-helper lymphocytes or antigen-specific T-cytotoxic lymphocytes, which in turn can be used for HCMV therapy. Therefore, the present invention also includes a method for inducing T helper cell (T_h) and T cytotoxic cell (T_c) response against HCMV, which includes the step of administering to a patient a vaccine against HCMV including an immunologically effective amount of gp90(II) or gp200(II) in combination with a pharmaceutically acceptable carrier.

Detailed Description of the Invention

The present invention includes HCMV glycoprotein complexes such as gCII-200 and their corresponding reduced glycoproteins such as gp(90)II which immunoreact with gCII-specific monoclonal antibodies. A summary of the various HCMV glycoprotein complexes, their constituent glycoproteins, the monoclonal antibodies which recognize them, and the abbreviations used herein to designate these entities appears in Table 1, below.

TABLE 1.
HCMV ENVELOPE GLYCOPROTEIN COMPLEXES AND GLYCOPROTEINS

Glycoprotein Complex (M.W. in kD)	Glycoprotein Component(s) (M.W. in kD)	Recognized by Monoclonal Antibodies
gA or gCI(130-190)	GLP-A[1] (55), (93), (130)	41C2 (IVI-10119),[4] 9B7 (IVI-10117)[4]
gB or gCII (>200)[3]	Group 1 glycoproteins: GLP-B[1] (50-52)	Group 1 MoAbs: 9E10 (IVI-10118)[4], 8B4, 26E2
	Group 2 glycoproteins: (39-48), (90), (>200)[2]	Group 2 MoAbs: 15F9 (IVI-10182)[4] 12G9, 15G5, 23B10, 25C8, 27B4, 40B7

1 Designation in commonly-assigned U.S. patent application Serial No. 06/933,789, the entire disclosure of which is incorporated by reference herein; the 93 kD glycoprotein of gCI is designated gp93(I) herein.
2 These glycoproteins are designated gp39-48(II), gp90(II), and gp200(II), respectively, herein.
3 This complex is designated gCII-200 herein.
4 In Vitro International (Linthicum, MD, U.S.A.) access code.

Materials and Methods

1. Production and Characterization of gCII MoAbs

Production and characterization of monoclonal antibodies used in these studies was performed following the method of B.E. Kari et al., J. Virol., 60, 345-352 (1986), the disclosure of which is incorporated by reference herein.

Briefly, adult BALB/c mice were immunized with 100 ug of whole Towne strain HCMV emulsified in complete Freund adjuvant and given a booster immunization with 100 ug whole Towne HCMV at 3 weeks. After several weeks a

final boost was given using 20 ug of purified gCII which had been purified by anion exchange high performance liquid chromatography as previously reported by B.E. Kari et al., supra. Three days following the final boost with gCII, the mice were sacrificed and the spleen cells were fused with Sp2-O-Ag14 myeloma cells. Cloning was done as previously described by B.E. Kari et al., supra.

To identify positive hybridomas, culture medium was assayed for antibody against gCII by enzyme linked immunosorbant assay (ELISA). For this assay, purified gCII was fixed in 96 well dishes. Hybridomas were sub-cloned twice by limiting dilution. Clonality was further assayed by agarose iso-electric focusing of purified MoAbs.

2. Radioactive labeling and Immunoaffinity Purification of HCMV Proteins

Glycoproteins were radiolabeled with [^{14}C]GlcN or [^{3}H]Arg (New England Nuclear) as previously described by B.E. Kari et al., supra. Labeled proteins were extracted from either extracellular virus or from infected whole cells using a Tris buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2mM phenylmethylsulfonyl fluoride (PMSF)) containing 1.0% NP-40. Insoluble material was removed by centrifugation.

Monoclonal antibodies, biotinylated as previously described by Gretch et al., Anal. Biochem., 163, 270-277 (1987), the disclosure of which is incorporated by reference herein, were added to these extracts along with streptavidin agarose beads (BRL). These solutions were mixed for 1 hour at room temperature and centrifuged to pellet the agarose beads. After several washes with PBS containing 0.1% NP-40, bound proteins were solubilized with SDS-PAGE sample solubilization buffer by heating at 100°C for 3 min.

After SDS-PAGE, radioactive proteins were detected by fluorography using ^{3}H Enhance (New England Nuclear).

For Example 7 below, whole HCMV antigen was prepared by differential centrifugation of virions obtained from supernatants of roller bottle fibroblast cultures infected with Towne HCMV at a multiplicity of infection (MOI) of 1. Glycoprotein complexes were obtained by detergent extraction from virion envelopes, and individual gCI and gCII complexes were immunoaffinity purified using gCI-specific and gCII-specific monoclonal antibodies designated as 41C2 and 15F9, respectively. After reduction, individual glycoproteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose paper, as described below.

3. SDS-PAGE and Western blot

SDS-PAGE was performed with either 7% or 10% polyacrylamide gels following the method of U.K. Laemmli, Nature, 227, 680-684 (1970), the disclosure of which is incorporated by reference herein. Proteins in the gels were then electroblotted onto nitrocellulose paper, and the paper was blocked with 3% gelatin in Tris-phosphate buffered saline (TBS, 20 mM Tris, 500 mM NaCl, phenylmethylsulfonyl fluoride (PMSF) 2 mM, pH 7.5). Strips of the blocked paper were incubated overnight with MoAbs in ascites diluted 1 to 500 or with human sera diluted 1 to 30 in TBS containing 1% gelatin. Strips were washed with TBS containing 0.05% Tween 20 and then with TBS before incubation with phosphatase labeled goat anti-mouse IgG (H + L) or goat anti-human IgG (H + L)(Kirkegaard and Perry, Gaithersburg, Maryland) diluted 1 to 1000 with TBS containing 1% gelatin. After a 1 hour incubation, the paper was washed and the substrate 5-bromo-4-chloro-3-indoyl phosphate in 0.1 M Tris buffer (Kirkegaard and Perry) was added. After visualization,

the reaction was stopped by immersing the strips in water.

4. Immunofluorescence

Cells infected with Towne strain HCMV were examined as living and fixed cells. Cells in 6 well culture dishes were infected with Towne strain HCMV at a MOI of 3×10^{-4} or 3. Cultures of the cells infected at an MOI of $3X10^{-4}$ were harvested at 12 days post infection, while cultures of the cells infected at an MOI of 3 were harvested at 4 and 7 days post infection. Living cultures were then stained by indirect immunofluorescence using gCII MoAbs (Group 1 MoAbs 9E10, 26E2, and 8B4 and Group 2 MoAb 15F9) to detect gCII glycoproteins in the plasma membrane. Duplicate cultures which had been fixed with acetone methanol were stained at the same time to detect internal gCII glycoproteins.

Cells to be stained were incubated with the gCII specific MoAbs in phosphate buffered saline (PBS) pH 7.5 at 37°C for 1 hour. Cultures were washed and incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin (Cooper Biomedical Inc., West Chester, Pennsylvania). Following a final wash, cultures were examined with a Zeiss phase fluorescence microscope.

5. HCMV Antisera

For Example 6 below, convalescent human anti-HCMV sera and non-immune human sera were obtained from healthy adults. Sera were also obtained from congenitally infected infants at 1 to 17 months of age. All infants were symptomatic for HCMV infection at birth. Rabbits were given subcutaneous injections of Towne strain HCMV or uninfected human skin fibroblasts in complete Freund's adjuvant, in order to obtain rabbit anti-HCMV serum or anti-skin fibroblast serum, respectively.

For Example 7 below, sera were obtained from peripheral blood from infants with symptomatic congenital HCMV infection, mothers of HCMV-infected infants, and normal adult donors, and frozen at -70°C prior to analysis for HCMV-specific antibody.

6. Analysis of Human Antisera by Immunofluorescence

For Example 7 below, serial dilutions of human sera were tested for antibody reactivity with viral proteins expressed on HCMV-infected fibroblasts in an indirect immunofluorescence assay, using polyvalent goat-anti-human immunoglobulin conjugated to fluorescein isothiocyanate (FITC). Antibody reactivity with individual HCMV glycoproteins was determined by Western blot analysis as described above.

7. Reducing and Non-Reducing Conditions

Purified, unreduced glycoprotein complexes were obtained under non-reducing conditions by isolating glycoprotein complexes by detergent extraction from HCMV virion envelopes, and immunoaffinity purification of the complexes with gCII-specific monoclonal antibodies.

Individual, reduced glycoproteins were obtained under reducing conditions as follows: unreduced gCII complexes purified as above were solubilized in the presence of reducing agents (2-mercaptoethanol [2-BME]) alone or in combination with dithiotheitriol at a final concentration of 10 mM in TN buffer adjusted to pH8 containing 8 M urea. Alkylation was then done by adding 5-10 milligrams iodoacetamide and allowing the reaction to proceed at room temperature for an additional 2 hours. Following reduction, the individual glycoproteins were then separated by SDS-PAGE and transferred to nitrocellulose as described above.

Example 1.
Production and Characterization of gCII Monoclonal Antibodies

B.E. Kari et al., J. Virol., 60, 345-352 (1986) described a MoAb (9E10) which reacted with HCMV glycoprotein complexes having molecular weights of 93,000 and 450,000, which eluted from an anion exchange column at 0.3 and 0.9 molar NaCl, respectively. The major glycoproteins of the 93,000 complex recognized by 9E10 in electrophoresis after reduction had molecular weights ranging from 50-52 kD. A very small amount of a 90 kD glycoprotein was also detected by Kari et al. B.E. Kari and R.C. Gehrz, Arch. Virol., 98, 171-188 (before May, 1988) reported that complex gCII-93, immunoprecipitated by MoAb 9E10, contained a 90 kD glycoprotein, as well as a 50-52 kD glycoprotein (gp52(II-93)), and that the complex they designated gCII-200, also immunoprecipitated by MoAb 9E10, contained a material ranging in molecular weight from 90-200 kD (gp90(II-200)) and a glycoprotein with molecular weight greater than 200 kD (gp200(II-200)), as well as a 50-52 kD glycoprotein (gp52(II-200)). Based on several observations, Kari and Gehrz reported that gp52(II-93) and gp52(II-200) appeared to be the same glycoprotein, which they referred to collectively as gp52(II).

Therefore, in order to better understand the composition of the gCII family of complexes, the present studies involved the production of additional gCII-specific MoAbs which were made using partially purified gCII as the immunizing antigen in mice. Several MoAbs were obtained and characterized. Based on the parameters described below, these MoAbs could be placed into two groups. The MoAbs characterized as Group 1 MoAbs included three MoAbs having different subtypes, 8B4 (IgM), 9E10 (IgG3), and 26E2 (IgG2a). The MoAbs characterized as Group 2 MoAbs included five MoAbs (15F9, 12G9, 23B10, 27B4, and 40B7) which had an IgG2a subtype,

MoAb 15G5 which had an IgG1 subtype, and MoAb 25C8 which had an IgA subtype.

Example 2.

Western blot Analysis of gCII Monoclonal Antibodies

The monoclonal antibodies made to gCII as described above were next analyzed to determine what molecular weight proteins they recognized. Western blot analysis was performed using whole Towne HCMV as the antigen. A mouse ascites (SP2), which did not contain HCMV antibody, was used as a negative control. The mouse ascites did not react in Western blot.

Following Western blotting and probing with Group 1 MoAbs 8B4, 9E10, and 26E2, bands were detected corresponding to 47,000 to 63,000 dalton molecular weight proteins. Bands corresponding to higher molecular weight proteins were weakly detected, but not studied further. Thus, the MoAbs characterized as Group 1 MoAbs recognized gCII glycoproteins with molecular weights ranging from 47,000 to 63,000. These 47-63 kD glycoproteins are believed to include the 50-52 kD glycoprotein designated as gp52(II) by Kari and Gehrz, supra.

Following Western blotting and probing with the Group 2 MoAbs 15F9, 12G9, 23B10, 27B4, 40B7, 15G5, and 25C8, bands were detected which corresponded to proteins having molecular weights between about 39,000 to about 48,000. Other bands were detected which corresponded to proteins having molecular weights of about 90,000. In addition, other bands were detected which corresponded to proteins having a molecular weight of greater than about 200,000. Thus, the MoAbs characterized as Group 2 MoAbs recognized proteins with a molecular weight ranging from 39,000 to 48,000, other proteins with a molecular weight of about 90,000, and other proteins with a molecular weight of greater than about 200,000. Therefore, the Group 2 MoAbs recognized proteins with a wider range of molecular weights than the Group 1 MoAbs. The 90

kD and the greater than 200 kD proteins recognized by the Group 2 MoAbs were purified and repeatedly exposed to urea, SDS, and reducing reagents, without any further change in their molecular weights. These results indicate that the high molecular weights of these proteins were not the result of incomplete reduction.

Example 3.
Radioimmunoprecipitation of gCII Complexes and Purification of gCII Glycoproteins

Representative Group 1 and Group 2 gCII MoAbs were examined for their ability to immunoprecipitate gCII complexes from non-ionic detergent extracts of radioactively labeled Towne strain HCMV infected cells. Extracts containing [^{14}C]GlcN labeled HCMV glycoproteins were first immunoprecipitated with either Group 1 MoAb 9E10 or Group 2 MoAb 12G9.

Immunoprecipitated glycoproteins were separated by SDS-PAGE under reducing conditions using a 7% polyacrylamide gel. Radioactive glycoproteins were detected by fluorography. When immunoprecipitates from the [^{14}C]GlcN labeled extracts were examined under these reducing conditions, bands were detected in the 7% gel for Group 1 MoAb 9E10 which corresponded to glycoproteins with molecular weights of 47-52,000. These bands were not detected with Group 2 MoAb 12G9, however. Bands were also detected in the gels for both Group 1 MoAb 9E10 and for Group 2 MoAb 12G9 which corresponded to glycoprotein complexes having molecular weights of about 93 kD and greater than about 200 kD. The molecular weight range of the 47-52,000 glycoproteins recognized by MoAb 9E10 was similar to that of the 47-63,000 molecular weight glycoproteins recognized by MoAb 9E10 in Western blot, described above. Kari and Gehrz, Arch. Virol., 98, 171-188 (before May, 1988) have previously shown that MoAb 9E10 can immunoprecipitate 50,000-52,000 molecular weight glycoproteins from gCII complexes independently

of other gCII glycoproteins. Therefore, based on their molecular weights, the 47,000-52,000 molecular weight glycoproteins detected in the present 9E10 immunoprecipitates are uncomplexed glycoproteins, and include the 50-52 kD glycoprotein (gp52(II)) described by Kari and Gehrz, supra. Thus, under the reducing conditions described above, the present 50-52 kD glycoprotein reacts with Group 1 MoAb 9E10 but not with Group 2 MoAb 12G9.

Immunoprecipitated glycoproteins were also separated by SDS-PAGE under non-reducing conditions using a 10% polyacrylamide gel. After separation, no substantial difference was detected when comparing glycoproteins immunoprecipitated with either Group 1 MoAb 9E10 or Group 2 MoAb 12G9. Bands were detected in the 10% polyacrylamide gels for either MoAb which corresponded to proteins having molecular weights of about 47-63 kD, about 90 kD, and greater than about 200 kD. Furthermore, immunoprecipitates obtained with either MoAb contained the same relative abundance of the 47-63,000 molecular weight glycoproteins. Therefore, these 47-63,000 molecular weight glycoproteins, including gp50-52(II), were disulfide-linked to the 90 kD and greater than 200 kD glycoproteins.

Finally, immunoprecipitations of unreduced gCII were performed under non-reducing conditions with Group 1 MoAb 26E2 and Group 2 MoAbs 15F9 and 40B7. Bands were detected in the 10% polyacrylamide gels for all three MoAbs which corresponded to proteins having molecular weights of about 47-63 kD, about 90 kD, and greater than about 200 kD. Thus, under non-reducing MoAbs 26E2, 15F9, and 40B7 also immunoprecipitated gCII complexes containing the same molecular weight glycoproteins as detected in complexes immunoprecipitated by 9E10 and 12G9.

To investigate the differences in glycoproteins immunoprecipitated and detected under reducing versus non-reducing conditions, serial immunoprecipitations of [^{14}C]GlcN labeled HCMV glycoproteins were performed with one MoAb from each group. Extracts were immunoprecipitated two to three times with either a Group 1 MoAb or a Group 2 MoAb until no further radioactivity could be precipitated, and were then immunoprecipitated with a MoAb from the opposite group. Following serial immunoprecipitation under non-reducing conditions with first Group 2 MoAb 12G9, and then Group 1 MoAb 9E10, immunoprecipitates were obtained which corresponded to glycoproteins with molecular weights from 47,000 to 52,000. Disulfide-linked complexes ranging in molecular weight from 93,000 to greater than 200,000 were also detected. When these complexes were reduced, a major broad band was detected with molecular weights ranging from 47,000 to 63,000. A darker band within the 47-63 kD band could be detected which corresponds to the 50-52 kD glycoprotein (gp52(II)) described above.

Following serial immunoprecipitation under non-reducing conditions with first Group 1 MoAb 9E10, and then Group 2 MoAb 12G9, two disulfide-linked complexes were obtained which had molecular weights of about 130,000 and of greater than about 200,000. After reduction, the most abundant glycoproteins had molecular weights of about 90,000 and greater than about 200,000.

These data indicate that disulfide-linked glycoprotein complexes and their constituent glycoproteins were present in the extracts which are recognized by Group 1 MoAbs and not by Group 2 MoAbs, and other complexes and glycoproteins which are only recognized by Group 2 MoAbs and not by Group 1 MoAbs. The greater than 200 kD complex was recognized by both the Group 2 MoAb 12G9 and the Group 1 MoAb 9E10, however. In any case, regardless of whether a Group 1 MoAb or a

Group 2 MoAb was initially used in the serial immunoprecipitations, only 14% of the total radioactivity was immunoprecipitated by the other (second) MoAb, indicating that most gCII complexes contain glycoproteins recognized by both groups of MoAbs. However, some complexes contain glycoproteins recognized only by Group 1 MoAbs, as well as glycoproteins recognized only by Group 2 MoAbs.

Example 4.
Indirect Immunofluorescence of Infected Cells Using gCII Monoclonal Antibodies

Van der Voort et al., J. Virol., 63, 1485-1488 (1989) reported that immunoprecipitation of ^{125}I surface labeled antigens of HCMV infected fibroblasts showed that polypeptides belonging to glycoprotein complexes designated gCI and gCIII are recognized by human sera positive for HCMV. However, proteins comparable in molecular weights to the gCII proteins described herein were not detected in the immunoprecipitations performed by Van der Voort et al., indicating that gCII glycoproteins probably are not on the surface of infected cells.

Therefore, experiments were performed to determine if gCII complexes or glycoproteins are on the surface of HCMV infected cells. Indirect immunofluorescence of living and acetone methanol fixed cells was performed with both Group 1 MoAbs (9E10, 8B4, and 26E2) and Group 2 MoAbs (15F9, 12G9, 15G5, 23B10, 25C8, 27B4, and 40B7). Human skin fibroblasts were infected with Towne strain HCMV at a multiplicity of infection (MOI) of $3X10^{-4}$. Cultures were harvested 12 days post infection, then stained by indirect immunofluorescence using gCII MoAbs as unfixed cultures. When living cells were infected at an MOI of $3X10^{-4}$ and stained with Group 1 MoAbs at 12 days post infection, staining of the plasma membranes was detected. When

cells infected with an MOI of $3X10^{-4}$ were stained with Group 2 MoAbs, however, only background staining was detected. The same result was obtained with an MOI of 3 at 4 or 7 days post infection.

To detect internal antigens, cells were fixed with acetone methanol prior to staining. Skin fibroblasts were infected at an MOI of 3 and harvested at 4 days post infection. These cultures were fixed and examined by indirect immunofluorescence using gCII MoAbs. Only diffuse background staining was detected with fixed cells when no MoAb was used. With fixed infected cells, all Group 1 and 2 MoAbs showed cytoplasmic staining at either MOI. However, Group 1 MoAb 26E2 showed variable cytoplasmic staining, with some cells showing intense staining, and others very weak staining. This result was obtained with either MOI.

Staining of the nucleus could also be detected with Group 1 MoAb 9E10. The nuclear staining appeared to be of two types. In some cells there was a diffuse staining of the whole nucleus, while in other cells intensely stained dots as well as diffuse staining were detected. The intense dots were also occasionally observed in the cytoplasm. No other Group 1 or 2 MoAb showed nuclear staining.

The only other Group 1 MoAb, 8B4, showed strong cytoplasmic staining in all cells. All Group 2 MoAbs showed strong cytoplasmic staining. Immunofluorescence was also performed with uninfected fibroblasts and no gCII MoAb stained these cells.

Example 5.
Synthesis of the High Molecular Weight gCII Glycoproteins in Infected Cells

Gretch et al., J. Virol., 62, 1956-1962 (June, 1988) have previously shown that the glycoproteins recognized by Group 1 gCII MoAbs are first synthesized

between 48 to 72 hours post infection, and have molecular weights ranging from 47,000 to 52,000. Thus, it was of interest to determine when the higher molecular weight glycoproteins of gCII which are recognized by Group 2 MoAbs were synthesized.

Accordingly, human skin fibroblasts were infected with Towne strain HCMV at an MOI of 1. Cells were scraped from culture dishes at 24, 48, 72, and 96 hours post infection. Culture extracts from these cells were immunoprecipitated using Group 2 MoAb 15F9. The purified gCII was reduced, subjected to SDS-PAGE, and examined by Western blot using MoAb 15F9. An SP2 ascites, used for negative control, did not react. Proteins with molecular weights of about 90,000 and greater than about 200,000 were clearly detected at 72 hours post infection. This data indicated that proteins recognized by Group 2 MoAbs such as 15F9 are late proteins.

Example 6.
Human Immune Response to gCII Proteins

The human humoral immune response to gCII proteins was examined by Western blot using gCII which had been immunoaffinity purified with Group 2 MoAb 15F9. Purified gCII was reduced, the resulting proteins separated by SDS-PAGE, and electroblotted onto nitrocellulose paper. Strips of nitrocellulose paper were probed with rabbit sera, adult and infant human sera, and monoclonal antibodies as described below.

In one set of experiments, approximately 100-150 ug of gCII protein purified with Group 2 MoAb 15F9 was used for analysis with seropositive rabbit and adult convalescent HCMV sera. In these experiments, each of the following was used as a probe of the purified gCII: rabbit anti-skin fibroblast serum (RSF); rabbit preimmune serum (R-); rabbit anti-Towne HCMV serum (R+); mouse

ascites negative control (SP2); MoAb 35F10, which is specific for a 28,000 molecular weight HCMV protein; Group 1 MoAb 9E10; Group 2 MoAb 12G9; Group 2 MoAb 15F9; six adult positive sera (A^+); and two adult HCMV negative sera (A-).

When Group 1 MoAb 9E10 was used as a probe, bands were detected which corresponded to glycoproteins having a molecular weight of about 47-63 kD. These glycoproteins included the glycoprotein gp50-52(II). When Group 2 MoAbs 12G9 and 15F9 were used as probes, however, bands were detected which corresponded to glycoproteins having molecular weights of about 39-48 kD and other glycoproteins having molecular weights of about 90 kD and of greater than about 200 kD.

Examination of the gCII proteins with HCMV positive rabbit serum (R+) produced a pattern substantially similar to that obtained with Group 2 MoAbs 12G9 and 15F9. Thus, the gCII proteins are believed to be immunogenic in rabbits as well as in humans. However, no reactivity was detected with rabbit anti-skin fibroblast serum (RSF), and only one peptide with a molecular weight of 100,000 gave non-specific reaction with rabbit preimmune serum (R-). Thus, the immunogenicity of gCII proteins may be specific to rabbit serum obtained from rabbits inoculated with Towne strain HCMV.

Western blot analysis was next performed with purified gCII proteins using the six HCMV-seropositive adult human convalescent sera. The titers for these HCMV positive sera were as indicated in Table 2, below.

TABLE 2.
HCMV IMMUNOFLUORESCENCE TITERS FOR HUMAN SERA AND THEIR REACTIVITY WITH HCMV PROTEINS IN WESTERN BLOT

	Serum Sample	IF Titer[1]	Age (Months)	gCI[2]	gCII[3]	28K[4]
ADULT CONVALESCENT SERA	A1+	160		+	+	+
	A2+	40		+	+	+
	A3+	160		+	+	+
	A4+	40		+	+	+
	A5+	40		+	+	+
	A6+	80		+	-	+
	A9+	320		+	+	+
	A7-	<10		-	-	-
	A8-	<10		-	-	-
INFANT SERA	I1+	5120	10	+	-	+
	I2+	ND	17	+	-	+
	I3+	19,240	1	+	-	+
	I4+	640	5	+	-	+
	I5+	160	10	+	-	+
	I6+	320	1	+	-	+
	I7+	320	5	+	-	+
	I8+	20,480	4	+	-	+
	I9+	20,480	1	+	-	+
	I10+[5]	80	1	+	+	+
	I11+[5]	80	1	+	+	+

[1] Immunofluorescence titers (IF) were determined in the clinical virology laboratory at the University of Minnesota and are expressed as the reciprocal of serum dilution. Indirect immunofluorescence titers of <10 were considered negative.

[2] Sera positive with at least two gCI glycoproteins in Western blot.

[3] Sera reactive with gCII proteins of 90 kD and greater than 200 kD which were recognized by Group 2 MoAbs 15F9 and 12G9 in Western blot, and with gCII proteins of 47-63 kD which were recognized by Group 1 MoAb 9E10 in Western blot.

[4] Sera reactive with the 28,000 molecular weight matrix protein in Western blot.

[5] I10 presented with anemia, hepatomegaly, and petecchiae due to abruptio placenta. CMV was isolated from the urine in the first week, but was not necessarily related to the patient's clinical symptom's. I11 did not have CMV infection in early infancy, but acquired CMV at six-months-of-age.

Five of the adult positive sera showed strong reactivity with gCII proteins in Western Blot, again giving a pattern similar to that obtained with Group 2 MoAbs 12G9 and 15F9. Specifically, bands were detected which corresponded to glycoproteins having molecular weights of about 39-48 kD and other glycoproteins having molecular weights of about 90 kD to greater than 200 kD. One HCMV positive adult serum reacted with only a single protein having an apparent molecular weight of 100,000, which may have been non-specific since faint reactivity was detected with the negative sera.

All seven positive adult human sera were also reactive in Western Blot with a 28,000 molecular weight protein not recognized by any of the gCII MoAbs. However, the 28,000 molecular weight protein was recognized by another HCMV MoAb, 35F10. The 28,000 molecular weight protein was not associated with gCII by disulfide bonds, and is believed to be a contaminating HCMV protein.

Finally, two human sera which were negative for HCMV by immunofluorescence failed to react with gCII proteins, but did show weak non-specific reactivity with 100,000 and 70,000 molecular weight proteins.

Purified gCII proteins were also examined with 11 sera obtained from congenitally infected infants. With one exception, the infant sera were obtained during the first year after birth, and thus a major portion of the antibody detected is likely to represent maternal antibody. The exception was infant I2+, who was 17 months old. HCMV antibody titers for the infant sera are also summarized in Table 2, above. Approximately 200 ug of gCII protein purified by Group 2 MoAb 15F9 was used in Western blot analysis with infant sera. The following were used as probes in Western Blot: HCMV MoAb 35F1; mouse ascites negative control (SP2); eleven infant sera

positive for HCMV; two adult negative serum (A-); and seven adult positive serum (A+).

While most adult sera reacted with gCII proteins, 9 of 11 infant sera showed little or no reactivity with reduced gCII proteins. The other 2 of 11 infant sera which contained gCII-specific antibodies were fraternal twins. The first, I10, presented with anemia, hepatomegaly, and petecchiae due to abruptio placenta. CMV was isolated from the urine during the first week but may not have been related to the patient's clinical symptoms. A second twin, I11, did not have congenital CMV infection, but acquired CMV at six-months-of-age. Neither infant suffered any permanent injury to CMV. The lack of gCII antibodies in the first nine infants with severe, irreversible injury from congenital CMV infection, and the presence of gCII antibodies in the fraternal twins who did not experience serious illness from CMV provides further evidence as to the protective role of gCII antibodies.

Reactivity with the 28,000 molecular weight protein was clearly detected with all infant sera, however. Table 2 indicates that the two infants who did react with gCII proteins had very low HCMV IF titers, as compared to the nine infants who did not react with gCII proteins.

Since the lack of reactivity by the infant sera could have been due to an inability to react with reduced HCMV glycoproteins, the reactivity of the infant sera with reduced glycoproteins from the HCMV glycoprotein complex designated gCI was also examined. Gretch et al., J. Gen. Virol., 69, 1205-1215 (1988) reported that immunoaffinity purified gCI contains three glycoproteins with molecular weights of 158,000, 93,000, and 52,000 daltons. The proteins with molecular weights of 158,000 and 52,000 are recognized by several of the gCI-specific

MoAbs described by Lussenhop et al., Virology, 164, 362-372 (1988).

In the present studies, approximately 200 ug of immunoaffinity purified gCI was used in Western blot analysis with infant sera. The following were used as probes: HCMV MoAb 35F10, which is specific for the 28,000 molecular weight HCMV protein; mouse negative ascites control (SP2); gCI-specific MoAb 39E11; eleven HCMV positive infant sera; two negative adult sera; and seven adult HCMV positive sera.

In Western blot, all the infant sera tested in these studies reacted with the 158 kD and 52 kD gCI glycoproteins. Two infant sera also reacted with the 93 kD gCI protein. All the adult sera tested also reacted with the 52 kD and 158 kD gCI glycoproteins. The 28,000 molecular weight HCMV protein was not detected in the gCI preparation, however.

Thus, a major difference between adult and infant sera was the lack of infant sera reactivity with gCII proteins. These results are summarized in Table 2, above.

Example 7.
Humoral Immune Response of Infants with Symptomatic Congenital HCMV infection and Their Mothers

Human cytomegalovirus (HCMV) is the most common congenital infection in the United States, affecting approximately 1% of all newborn infants. The consequences of primary maternal HCMV infection to the fetus range from asymptomatic viruria to severe cytomegalic inclusion disease (CID) with microcephaly and psychomotor retardation. These infants develop HCMV-specific antibodies but have a defect in HCMV-specific lymphocyte proliferation which may predispose them to the persistent viral infection and possibly to clinical disease. In contrast, fetal infections resulting from reactivation of latent HCMV in seropositive pregnant

women are not generally associated with clinical illness or neurological sequelae. Passive transfer of maternal HCMV antibody prior to fetal exposure therefore appears to protect the infant.

The purpose of this experiment was to analyze sera from infants with symptomatic congenital HCMV infection and their mothers for antibodies reactive with glycoproteins contained in HCMV virion envelopes. The experimental results indicate that infants with symptomatic congenital HCMV infection may have a deficit in the HCMV-specific humoral immune response.

A. Human Antibody Responses to gC-I and gC-II Glycoproteins

Monoclonal antibodies were used to confirm the presence of individual gCI and gCII glycoproteins in the antigen preparations to be used for Western blot analysis of human sera. Western blotting indicated that the purified gCI contained three glycoproteins. Specifically, following Western blotting of purified gCI and probing with gCI-specific MoAb 41C2, bands were detected in the gel corresponding to a mature glycoprotein having a molecular weight of about 52,000, designated gp52; and to a fully glycosylated precursor glycoprotein having a molecular weight of about 158,000, designated gp158. Following Western blotting of purified gCI and probing with gCI-specific MoAb 3B10, bands were detected in the gel corresponding to a mature glycoprotein having a molecular weight of about 93,000, designated gp93; and the fully glycosylated precursor glycoprotein gp158 which was also recognized by MoAb 41C2. A mouse ascites negative control (SP2) did not react with gCI.

Western blotting with purified gCII indicated that gCII contained two antigenically distinct groups of glycoproteins, designated Group 1 and Group 2.

Specifically, following Western blotting of purified gCII and blotting with gCII/Group 1-specific MoAb 9E10, bands were detected in the gel corresponding to glycoproteins having a molecular weight ranging from about 45,000-63,000, designated gp45-63. Following Western blotting of purified gCII and blotting with gCII/Group 2-specific MoAb 15F9, bands were detected in the gel corresponding to three glycoproteins: one having molecular weight ranging from about 39,000-48,000, designated gp39-48; one having a molecular weight of about 90,000, designated gp90; and one having a molecular weight of greater than about 200,000, designated gp200. A mouse ascites negative control (SP2) did not react with gCII.

An unrelated non-glycosylated protein designated pp28 consistently co-precipitated with either gCI or gCII, and was recognized by MoAb 35F10. This monoclonal antibody recognizes a matrix protein of the virion.

Human antibodies reactive with all three gcI glycoproteins (gp52, gp93, and gp158) were detected in HCMV-seropositive convalescent sera obtained from healthy adult donors, as well as in sera from congenitally infected infants. Sera from 7 of 8 HCMV-positive adult donors contained antibodies reactive with all Group 1 and Group 2 gCII glycoproteins, as well as antibody reactive with the matrix protein pp28. However, one apparently healthy seropositive adult lacked gCII-specific antibodies. None of the sera obtained from infants with symptomatic congenital HCMV infection ranging in age from 2 weeks to 5 years contained any gCII-specific antibodies, but this sera did react with pp28. No gCI-, gCII-, or pp28-specific antibodies were detected in adult seronegative donors.

B. Longitudinal Analysis of HCMV Antibody Responses in Congenital HCMV Infants and Their Mothers

Longitudinal serum specimens were obtained from five infants with symptomatic congenital HCMV infection ranging in age from 1 to 63 months. All patients were initially excreting high titers of HCMV in the urine as determined by 50% tissue culture infections dose ($TCID_{50}$), and their sera contained high titers of HCMV antibody as determined by indirect immunofluorescence. There was a gradual diminution in the quantity of virus excreted, associated with a decrease in HCMV antibody titer in all subjects, as shown by the data of Table 3, below.

TABLE 3
HUMAN ANTIBODY RESPONSE TO HCMV GLYCOPROTEINS IN CONGENITAL HCMV PATIENTS

				Reactivity with HCMV (Glyco)proteins(WB)[d]				
				gcI		gcII		
Patient[a]	Age (months)	HCMV Aby[b] (IF)	HCMV[c] Cultures $TCID_{50}$	gp52	gp93	gp45-63	gp39-48/gp90/gp200	pp28
#1	1	640	10^{-4}	+	+	-	-	+
	3	640	10^{-6}	+	+	-	-	+
	4	2560	10^{-3}	+	+	-	-	+
	9	1280	10^{-3}	+	+	-	-	+
	13	5120	10^{-3}	+	+	-	-	+
	24	80	10^{-2}	+	+	-	-	+
	32	160	10^{-1}	+	+	-	-	+
Mother of #1	32 Post-partum			+	+	-	-	+
#2	1	2560	10^{-5}	+	ND	-	-	-
	1.5	320	10^{-3}	+	ND	-	-	-
	2	320	10^{-3}	+	ND	-	-	-
	3.5	160	10^{-2}	+	ND	-	-	-
	5.5	160	10^{-4}	+	ND	-	-	-
	6	160	10^{-4}	+	ND	-	-	-
	11	40	10^{-3}	+	ND	-	-	-
	27	20	ND	+	ND	-	-	±
	36	80	ND	+	ND	-	-	+
	63	ND	ND	+	ND	±	±	+

TABLE 3 CONT'D
HUMAN ANTIBODY RESPONSE TO HCMV GLYCOPROTEINS IN CONGENITAL HCMV PATIENTS

				Reactivity with HCMV (Glyco)proteins(WB)[d]				
				gcI		gcII		
Patient[a]	Age (months)	HCMV Aby[b] (IF)	HCMV[c] Cultures $TCID_{50}$	gp52	gp93	gp45-63	gp39-48/gp90/gp200	pp28
Mother of #2	63 Post-partum			+	ND	±	±	+
#3	3	ND	10^{-6}	+	ND	±	-	ND
	7	320	10^{-5}	+	ND	-	-	ND
	11	ND	-	+	ND	-	-	ND
	15	80	ND	+	ND	-	-	ND
	25	ND	10^{-3}	+	ND	±	-	ND
	36	ND	-	+	ND	±	-	ND
#4	4	20,480	10^{-6}	+	+	-	-	+
	7	5,120	10^{-4}	+	+	-	-	+
	11	640	10^{-3}	+	+	-	-	+
	12	320	10^{-6}	+	+	-	-	+
#5	1	20,480	10^{-3}	+	ND	-	-	+
	2	20,480	10^{-4}	+	ND	-	-	+
	5	5,120	10^{-3}	+	ND	-	-	+
	8	320	10^{-3}	+	ND	-	-	+
	20	20	10^{-1}	+	ND	-	-	+

a. Sera obtained from infants with symptomatic congenital HCMV infection and their mothers.

b. Antibody reactive with HCMV-infected fibroblasts as determined by indirect immunofluorescence, expressed as reciprocal of serum dilution.

c. Qualitative isolation of HCMV from urine and/or saliva in fibroblast cell culture (+/-) or quantitative excretion of HCMV in urine expressed as reciprocal dilution exhibiting $TCID_{50}$.

d. Individual gcI and gcII glycoproteins were separated from reduced, immunoaffinity purified complexes by SDS-PAGE and transferred to nitrocellulose for Western blot analysis. pp28 co-precipitated with gcII complexes.

e. The "±" symbol indicates equivocal reactivity with the lowest molecular weight glycoprotein in each group; i.e. gp45-63, gp39-48 or pp 28.

As indicated by the data of Table 3, all infant sera contained antibodies reactive with gp52 and gp93. In contrast, none of the infant sera contained antibodies reactive with Group 1 or Group 2 gCII glycoproteins. In particular, infant sera obtained from 0-63 months of life were non-reactive with gCII, indicating a possible deficit in the maternal antibody response to this glycoprotein. Furthermore, sera from mothers of patients 1 and 2 obtained 32 and 63 months postpartum, respectively, did not contain gCII-specific antibodies, but were reactive with gCI glycoproteins, as shown by the data of Table 3.

The patient designated as Patient 1 of Table 3 presented with symptoms of cytomegalic inclusion disease in the newborn period, including intrauterine growth retardation, microcephaly, hepatosplenomegaly, jaundice, and petechiae. After initial clinical improvement, this patient developed HCMV-associated interstitial pneumonia requiring prolonged hospitalization and supplemental oxygen at 10 weeks of age, associated with a quantitative increase in viral excretion and a rise in HCMV antibody titer. Western blot of purified gCI and probing with sera from Patient 1 over a 32-month period indicated that this patient developed a quantitative increase in antibody reactive with gCI glycoprotein gp52 following the episode of HCMV pneumonia. This increase was confirmed by densitometric scanning. Western blot was also performed with purified gCII and probing with sera from Patient 1 over the same 32-month period. Despite repeated antigenic stimulation and clinical relapse, this patient did not develop gCII-specific antibodies over the 32-month period.

In order to better understand the composition of the gCII family of complexes, several gCII-specific MoAbs have been made using partially purified gCII as the immunizing antigen in mice. Several MoAbs were obtained

and characterized. The MoAbs showed distinctions between high and low molecular weight glycoproteins found in gCII complexes. Accordingly, the MoAbs were characterized as belonging to either Group 1 or Group 2. The gCII complex contains two distinct groups of proteins which are recognized by the two different groups of monoclonal antibodies.

MoAbs characterized as Group 1 MoAbs immunoprecipitated gCII complexes from non-ionic detergent extracts of HCMV strain Towne, and reacted in Western blot with glycoproteins having molecular weights of 47-63,000. All Group 1 MoAbs stained the plasma membrane of HCMV-infected living cells in an immunofluorescence essay, indicating that the proteins they recognize are expressed on the surface of infected cells.

Further experimentation indicated that the three Group 1 MoAbs obtained (9E10, 8B4, and 26E2) react with different epitopes, and possibly with different products from the HXLF gene family. This may be established by the following observations. First, staining with Group 1 MoAb 26E2 ranged from weak to intense in fixed infected cells using an indirect immunofluorescence assay. In contrast, 95-100% of fixed infected cells always showed strong staining with Group 1 MoAbs 8B4 and 9E10, regardless of the MOI or days post infection. One possibility is that the factors regulating the appearance of the protein recognized by 26E2 are different from those which regulate the appearance of proteins recognized by 8B4 and 9E10. Second, all three of the Group 1 MoAbs stained the cytoplasm in indirect immunofluorescence, but only MoAb 9E10 showed immunofluorescent staining of the nucleus. Stinski et al., J. Gen. Virol., 43, 119-129 (1979) have shown that late HCMV antigens accumulate on the nuclear envelope. The antiserum used in these studies was made

against a non-ionic detergent extract containing the envelope glycoproteins of strain Towne. Some of the antigens they originally detected may have been those recognized by 9E10.

Seven Group 2 MoAbs which immunoprecipitated gCII complexes from non-ionic detergent extracts of HCMV strain Towne also reacted in Western blot with glycoproteins having molecular weights of about 39-48 kD, about 90 kD, and greater than about 200 kD. These proteins were synthesized late in infection. (Proteins recognized by Group 1 MoAbs are also synthesized late in infection, but have much lower molecular weights, as reported by Gretch et al., J. Virol., 62, 1956-1962 (June, 1988)).

The high molecular weight proteins recognized by Group 2 MoAbs could not be reduced in size by repeated exposure to urea, SDS, and reducing agents, indicating that the Group 2 proteins are not the result of incomplete reduction or aggregation of lower molecular weight proteins. However, even though PMSF was used in the initial isolation steps, proteolysis during the isolation could have generated the very low molecular weight Group 2 proteins from higher molecular weight species.

Group 2 MoAbs showed strong immunofluorescence with fixed cells, but not with living infected cells, in contrast with the Group 1 MoAbs which stained the plasma membrane of the living infected cells. These results are further evidence that the proteins recognized by Group 2 MoAbs are different from those recognized by Group 1 MoAbs. The proteins recognized by Group 2 MoAbs may be products of different transcripts encoded by the HXLF gene family, or encoded by a different gene or genes. One possibility is that the gCII complexes recognized on the surface of infected cells are those which were immunoprecipitated by Group 1 MoAb 9E10, but were not

immunoprecipitated by Group 2 MoAb 12G9. Accordingly, the gCII complexes recognized by both groups of MoAbs may only be expressed inside the cell. A less likely possibility is that the epitopes recognized by Group 2 MoAbs may be on the surface of infected cells, but are not exposed enough for antibody binding.

Immunoaffinity purified gCII proteins were also recognized by antibody in adult convalescent sera. The pattern obtained in Western blot of the human sera was very similar to that obtained with Group 2 MoAbs. However, there was overlap in the low molecular weight proteins recognized by both Group 1 MoAbs and Group 2 MoAbs. Some of the low molecular weight proteins recognized by the human sera may include proteins recognized by both groups of MoAbs. While most adult sera reacted with gCII proteins in Western blot, most congenitally infected infants were non-reactive. This non-reactivity with gCII proteins occurred even though the infant sera was reactive with reduced gCI glycoproteins and the 28,000 molecular weight protein in Western blot. These proteins were also recognized by adult sera. Thus, a major difference between adult and infant sera was the lack of reactivity with reduced gCII proteins. The lack of reactivity by the infant sera may have resulted from the fact that the strain of HCMV which they were infected with did not express gCII. However, the presence of antibody to gCII proteins in several adult sera suggested that wild type strains of HCMV do express these proteins.

One clinical isolate was screened with all the gCII MoAbs in indirect immunofluorescence assays. All the gCII MoAbs were reactive, suggesting that this clinical isolate expressed gCII proteins. Another possibility is that antibody to gCII is made after convalescence, or after repeated exposure to HCMV. This

would seem less likely, since at least one adult convalescent serum did not react with gCII proteins.

Passive transfer of maternal antibody is thought to play a major role in reducing the virulence of infection in the fetus. See Stagno, et al., N. Engl. J. Med., 306, 945-949 (1982); Ahlfors et al., Scand. J. Infect. Dis., 16, 129-137 (1984). Alford et al., J. Infect. Dis., 158, 917-924 (1988) have examined the antibody response in primary HCMV infection in pregnant women, and compared antibody responses between women whose offspring either have or have not become infected. They found no difference in the precipitin responses against virus encoded proteins when comparing antibodies from these two groups of women. This result suggested that antibody responses to the HCMV proteins detected were not enough to prevent intrauterine infection. However, they did observe that women who transmitted virus to their fetuses had a higher level and a more intense IgG response then women who did not transmit virus to their fetuses.

In the present work, infants who had gCII antibodies also had very low HCMV antibody titers to whole HCMV as detected by indirect immunofluorescence, as compared to infants who lacked gCII antibodies. Liu et al., J. Virol., 62, 1066-1070 (1988) have previously reported finding no qualitative differences in the ability to immunoprecipitate gCI and gCII when comparing adult convalescent sera and congenitally infected infant sera. Therefore, these infants may have antibody which recognize conformational determinants on gCII complexes, but may lack significant amounts of antibodies which recognize continuous epitopes on gCII glycoproteins.

With respect to Example 7 above, the purpose of the study described therein was to characterize the antibodies to individual envelope glycoproteins of HCMV in sera from symptomatic congenital HCMV infants and

their mothers, and to determine if susceptibility to clinical injury and/or viral persistence might be related to deficits in the antibody responses to these glycoproteins. Previous studies have failed to detect human antibody responses to gp93 of gCI or to any of the group 1 or group 2 gCII glycoproteins. (Pereira et al., Infect. Immunity, 36, 933 (1982); Landihi et al., J. Med. Virol., 17, 303 (1985); Hayes et al., J. Infect. Dis., 156, 615 (1987); Gold et al., J. Infect. Dis., 157, 319 (1988)). This may be due, in part, to differences in the relative abundance of certain glycoproteins in whole viral antigen preparations, differences in immunogenicity among these complexes and their constituent glycoproteins, or similarities in molecular weights among unrelated glycoproteins which make it difficult to discriminate the specificity of individual antibody responses. In the present study, purification of the individual glycoproteins prior to immunological analysis allowed the confirmation of their importance in the human antibody response in normal individuals, and the detection of a deficit in the gCII-specific antibody response in infants with symptomatic congenital HCMV infection and their mothers.

The defect in HCMV-specific T_h proliferation to immunodominant (glyco)proteins previously described by Gehrz et al., Lancet, 2, 844-847 (1977); and Gehrz et al., Clin. Exp. Immunol., 74, 333-338 (1988) may account for the lack of antibody response to gCII glycoproteins in congenitally infected infants, as described in Example 7 above. Furthermore, mothers of congenitally infected infants also appear to lack gCII-specific antibodies, indicating that susceptibility to the pathogenic effects of HCMV may be associated with maternal/fetal non-responsiveness to gCII and implying an essential role for gCII-specific antibodies in host defense. An alternative explanation is that gCII glycoproteins may be present to

varying extents in different strains of HCMV, with virulent strains either not expressing gCII in sufficient quantity to stimulate the immune response, or containing gCII polymorphisms which do not expose or express immunodominant epitopes recognized by neutralizing gCII antibodies. Since these infants do appear to have antibodies recognizing conformation-dependent epitopes on unreduced gCII complexes (Liu et al., J. Virol., 62, 1066-1070 (1988)), it would seem that antigenic variations among constituent glycoproteins is a more likely explanation.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations may be made while remaining within the spirit and scope of the invention.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 9, line ____ of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet [X] [3] 2-29-9B7 (hybridoma)

Name of depositary institution [4]

In Vitro International, Inc.

Address of depositary institution (including postal code and country) [4]

611(P) Hammonds Ferry Road
Linthicum, Maryland 21090

Date of deposit [5]	Accession Number [6]
10 September 1986	IVI-10117

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [✓] This sheet was received with the international application when filed (to be checked by the receiving Office)

Catherine B. Williams
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

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Form PCT/RO/134 (January 1981)

(January 1985)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 9, line ____ of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet [X] [3] 1-48-41C2

Name of depositary institution [4]

In Vitro International, Inc.

Address of depositary institution (including postal code and country) [4]

611(P) Hammonds Ferry Road
Linthicum, Maryland 21090

Date of deposit [5]	Accession Number [6]
10 September 1986	IVI-10119

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [✓] This sheet was received with the international application when filed (to be checked by the receiving Office)

Catherine B. Williams
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

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Form PCT/RO/134 (January 1981)

(January 1985)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 9, line ____ of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet [X] [3] D46-15F9 (hybridoma)

Name of depositary institution [4]

In Vitro International, Inc.

Address of depositary institution (including postal code and country) [4]

611(P) Hammonds Ferry Road
Linthicum, Maryland 21090

Date of deposit [5]	Accession Number [6]
01 August 1988	IVI-10182

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

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Catherine B. Williams
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(January 1985)

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 9, line ____ of the description [1]

A. IDENTIFICATION OF DEPOSIT [5]

Further deposits are identified on an additional sheet ☐ [2] 2-15-9E10

Name of depositary institution [6]

In Vitro International, Inc.

Address of depositary institution (including postal code and country) [6]

611(P) Hammonds Ferry Road
Linthicum, Maryland 21090

Date of deposit [8]	Accession Number [6]
10 September 1986	IVI-10118

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [9] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

Catherine B. Williams
(Authorized Officer)

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was _____

(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

WHAT IS CLAIMED IS:

1. A substantially pure, immunogenic glycoprotein complex which is on the membrane envelope of HCMV, wherein the complex comprises:
   (a) an about 50-52 kD glycoprotein which reacts with a first monoclonal antibody selected from the group consisting of 9E10, 8B4 and 26E2; and
   (b) two glycoproteins with respective molecular weights of about 90 kD and greater than about 200 kD, both of which react with a second monoclonal antibody selected from the group consisting of 15F9, 12G9, 15G5, 23B10, 25C8, 27B4, and 40B7;
   and wherein the complex has a molecular weight of greater than about 200 kD.

2. The glycoprotein complex of claim 1, wherein the complex is reactive with HCMV-seropositive adult human sera but is not substantially reactive with HCMV-seropositive, congenitally infected infant human sera.

3. The glycoprotein complex of claim 2, wherein the adult human sera and the infant human sera are reactive with gCI glycoproteins having molecular weights of about 52 kD or 158 kD.

4. The glycoprotein complex of claim 1, wherein the first monoclonal antibody is reactive with plasma membranes of HCMV-infected living human cells and with cytoplasm of HCMV-infected fixed human cells.

5. The glycoprotein complex of claim 1, wherein the second monoclonal antibody is reactive with cytoplasm of HCMV-infected fixed human cells but which is not reactive with plasma membranes of HCMV-infected living human cells.

6. The glycoprotein complex of claim 1, wherein the monoclonal antibody 9E10 is reactive with the nucleus of HCMV-infected fixed human cells.

7. A substantially pure, immunogenic glycoprotein having a molecular weight of about 50-52 kD, wherein the glycoprotein is derivable from the membrane envelope of HCMV and can be associated with other envelope glycoproteins by means of disulfide bonds, and wherein the about 50-52 kD glycoprotein reacts with a monoclonal antibody 9E10 produced by hybridoma IVI-10118.

8. The glycoprotein of claim 7, wherein under reducing conditions the glycoprotein reacts with the monoclonal antibody 9E10 and does not substantially react with a monoclonal antibody 12G9.

9. The glycoprotein of claim 8, wherein under non-reducing conditions the glycoprotein reacts with the monoclonal antibodies 9E10, 12G9, 26E2, 15F9, and 40B7.

10. A substantially pure glycoprotein of HCMV having a molecular weight of within about 39-48 kD, wherein the glycoprotein is derivable from the membrane envelope of HCMV and reacts with a monoclonal antibody selected from the group consisting of 15F9, 12G9, 15G5, 23B10, 25C8, 27B4, and 40B7.

11. A substantially pure, immunogenic glycoprotein of HCMV having a molecular weight of about 90 kD, wherein the glycoprotein is derivable from the membrane envelope of HCMV and can be associated with other envelope glycoproteins by means of disulfide bonds, and wherein the about 90 kD glycoprotein reacts with a monoclonal antibody selected from the group consisting of 15F9, 12G9, 15G5, 23B10, 25C8, 27B4, and 40B7.

12. A substantially pure, immunogenic glycoprotein of HCMV having a molecular weight of greater than about 200 kD, wherein the glycoprotein is derivable from the membrane envelope of HCMV and can be associated with other envelope glycoproteins by means of disulfide bonds, and wherein the greater than about 200 kD glycoprotein reacts with a monoclonal antibody selected from the group consisting of 15F9, 12G9, 15G5, 23B10, 25C8, 27B4, and 40B7.

13. The glycoprotein of claim 11 or 12, wherein the monoclonal antibody is monoclonal antibody 15F9 produced by a hybridoma IVI-10118, and wherein the glycoprotein is synthesized late in infection.

14. The glycoprotein of claim 11 or 12, wherein under reducing conditions the glycoprotein is detectable by HCMV-seropositive adult human sera but is not substantially detectable by HCMV-seropositive, congenitally infected infant human sera.

15. The glycoprotein of claim 11 or 12, wherein under reducing conditions the glycoprotein is not substantially detectable by HCMV-seropositive, congenitally infected infant sera or by HCMV sero-positive maternal sera within about 0-63 months post partum.

16. The glycoprotein of claim 11 or 12, wherein under reducing conditions the glycoprotein is immunoprecipitable by HCMV-seropositive adult human sera but is not substantially immunoprecipitable by HCMV-seropositive, congenitally infected infant human sera, and wherein the adult human sera and the infant human sera are reactive with gCI glycoproteins having a molecular weight of about 52 kD or about 158 kD.

17. A substantially pure, immunogenic glycoprotein of HCMV having a molecular weight of about 90 kD, wherein the glycoprotein reacts with a monoclonal antibody 15F9 produced by a hybridoma IVI-10118, wherein the glycoprotein is produced by a process comprising reducing the complex of claim 1.

18. A substantially pure immunogenic glycoprotein of HCMV having a molecular weight of greater than about 200 kD, wherein the glycoprotein reacts with a monoclonal antibody 15F9 produced by a hybridoma IVI-10118, wherein the glycoprotein is produced by a process comprising reducing the complex of claim 1.

19. A vaccine against HCMV comprising an immunologically effective amount of the glycoprotein of claim 11 or 12 in combination with a pharmaceutically-acceptable carrier.

20. A method for raising the titer of an antibody against HCMV in the blood of a mammal, comprising the step of administering to the mammal a vaccine against HCMV comprising an immunologically effective amount of the glycoprotein of claim 11 or 12 in combination with a pharmaceutically-acceptable carrier.

21. A method for inducing T helper cell (T_h) and T cytotoxic cell (T_c) response against HCMV, comprising the step of administering to a patient a vaccine against HCMV comprising an immunologically effective amount of the glycoprotein of claim 11 or 12 in combination with a pharmaceutically-acceptable carrier.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04371

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 15/14; A61K 39/00
U.S. CL.: 530/395 424/88

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/395 424/88

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	J. of Virology, Volume 62, No. 3, issued March 1988, Gretch et al., "Identification and Characterization of Three Distinct Families of Glycoprotein Complexes in the Envelopes of Human Cytomegalovirus" pages 875-881, See figure 4.	1,6-13,17-18 1-21
X Y	J. of Virology, volume 62, No. 3 issued March 1988, Liu et al., "Human Immune Responses to Major Human Cytomegalovirus Glycoprotein Complexes", pages 1066-1070, see Table 1.	2-5, 14-16 2-5, 14-16

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
27 September 1990	19 DEC 1990
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	SHELLY J. GUEST

Form PCT/ISA/210 (second sheet) (May 1986)