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**Human Cytomegalovirus Interactions with
Human Fibroblast Cells:**

Characterization of a Neutralization Epitope

and

Identification of Cell Signalling Events

**A thesis submitted to the School of Graduate Studies at the University of
Ottawa in partial fulfillment of the requirements for the degree of Master of
Science, Department of Microbiology and Immunology, Faculty of Medicine.**

By Mathias Haun

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ABSTRACT

Human cytomegalovirus (HCMV) is a widely prevalent herpesvirus and infects approximately half of North American adults. Immunocompromised individuals such as transplant recipients and AIDS patients, as well as congenitally-infected newborns are at greatest risk from the effects of HCMV infection. HCMV has also been implicated in atherosclerotic disease.

HCMV hyperimmune globulins or intravenous immunoglobulin (IVIG) have been widely used as a means of passive immunoprophylaxis. The efficacy of these blood products has not been clearly demonstrated in terms of the correlation between anti-HCMV titres assayed by enzyme-linked immunoassay (ELISA) and viral neutralizing activity measured in vitro by plaque reduction assay.

Recent advances in the characterization of HCMV surface glycoproteins have led to the identification of the immunodominant regions of these molecules. The mouse monoclonal anti-HCMV antibody CMVB1 was utilized to study the properties of a neutralization epitope. Chemical cross-linking experiments with a photoactivable, heterobifunctional and cleavable reagent identified a viral antigen with an apparent molecular weight of approximately 70 kDa. Microtitre plate binding assays verified that the epitope was unique to HCMV and not homologous to herpesvirus-1 or -2 (HSV-1,-2) antigenic sites. The lectin concanavalin A was found to partially block

CMVB1 from binding to the viral epitope, suggesting that carbohydrate moieties may be part of the antigen. Lectin specific sugars such as D-glucose, D-mannose and N-acetylglucosamine did not block CMVB1 binding to HCMV.

Cell signalling events induced by HCMV particle binding to human foreskin fibroblast cells were also examined. Central molecules involved in three major signal transduction pathways were studied. Tyrosine phosphorylation was investigated with immunoprecipitation and immunoblotting techniques. Intracellular cyclic adenosine monophosphate (cAMP) concentrations were measured by immunoassay and protein kinase C (PKC) activation was established by a peptide radiolabelling assay. With the methodologies utilized, no evidence of HCMV-induced tyrosine phosphorylation or elevated cAMP concentrations was detected. PKC activity was found to increase to levels typically observed in activated cells.

Others had reported that HCMV particles binding to lung fibroblasts stimulated a dramatic increase in mRNA levels for oncogene *c-fos* protein (Fos). Immunoprecipitation of radiolabelled Fos from HFF cells stimulated with HCMV particles was unable to detect virus-induced changes in the phosphorylation state of the protein.

Dedication

This undertaking would never have been possible without the tremendous encouragement and support from my wife Roselee. For making sacrifices for three years which many would not have been willing to make, I dedicate this thesis to her.

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I wish to acknowledge the constant moral support and outstanding scientific guidance of my graduate supervisor, Dr. Safia Wasi. I thank Dr. Peter Gill for providing the fiscal and administrative means to support my graduate studies. I also thank Debbie Adamson for excellent handwriting interpretation and typing skills. Finally, I thank my co-workers for helpful discussions and support.

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INTRODUCTION

Human cytomegalovirus (HCMV) is a widely prevalent herpesvirus which infects 50% of North American adults. In the vast majority of individuals, HCMV is present in a latent form and the infection is asymptomatic (1). However, it continues to be a major cause of morbidity and mortality in newborns as well as in immunocompromised individuals such as transplant recipients or Acquired Immune Deficiency Syndrome (AIDS) patients.

There are 5,000-7,000 cases annually in the United States alone, of congenitally-acquired HCMV infection resulting in varying degrees of mental retardation and hearing loss (1). Post-transplantation HCMV infections occur in two-thirds of all organ transplant recipients (2) and half of all bone marrow transplants (3). Clinical manifestations of HCMV infection in these patients may include fever, pneumonia, hepatitis, myocarditis, gastrointestinal haemorrhage, and chorioretinitis.

Ninety percent of patients with AIDS develop HCMV infections and 25% of these experience significant disease states, including chorioretinitis, pneumonia, colitis, and Hepatitis A (4). HCMV has also been suggested as a possible co-factor in the accelerated development of AIDS in HIV-infected individuals (5). It has also been suggested that HCMV plays a role in coronary restenosis and atherosclerotic disease (6,7).

Drug therapy to combat HCMV infections consists of treatment with ganciclovir, a thymidine analogue which inhibits viral DNA polymerase. Although effective, the drug has significant side effects, primarily neutropenia, which precludes continued treatment in one-third of patients (4). A ganciclovir-resistant clinical isolate of HCMV has been reported (8), suggesting that drug-resistant strains of the virus may reduce the efficiency of this treatment strategy in the future.

Infusion of HCMV hyperimmune globulins or intravenous immunoglobulin (IVIG) as a means of passive immunoprophylaxis has been widely applied to transplant recipients (9-12). The results of a large number of clinical studies have varied widely in terms of demonstrating a significant benefit to the patient. A lack of standardized approach to such key factors as patient characteristics, graft-versus-host-disease (GVHD), prophylaxis, blood product utilization and supportive care, has rendered direct comparison of data difficult. Furthermore, evaluation of hyper immune plasma or IVIG has been conducted with a range of assays (12) whose results are difficult to compare. Those studies which employed a standardized set of assays to evaluate a panel of commercially-available IVIG's or HCMV hyper immune globulins found no correlation between enzyme-linked immunosorbent assay (ELISA) titres and *in vitro* functional activity as measured by neutralization assay titres (13). It was noteworthy that HCMV hyper immune globulin prepared from donors selected for their high titre of anti-HCMV antibodies by ELISA or complement fixation assay, exhibited no more *in vitro* anti-viral activity in a

neutralization assay than IVIG prepared from non-immune or low-titre donors (13).

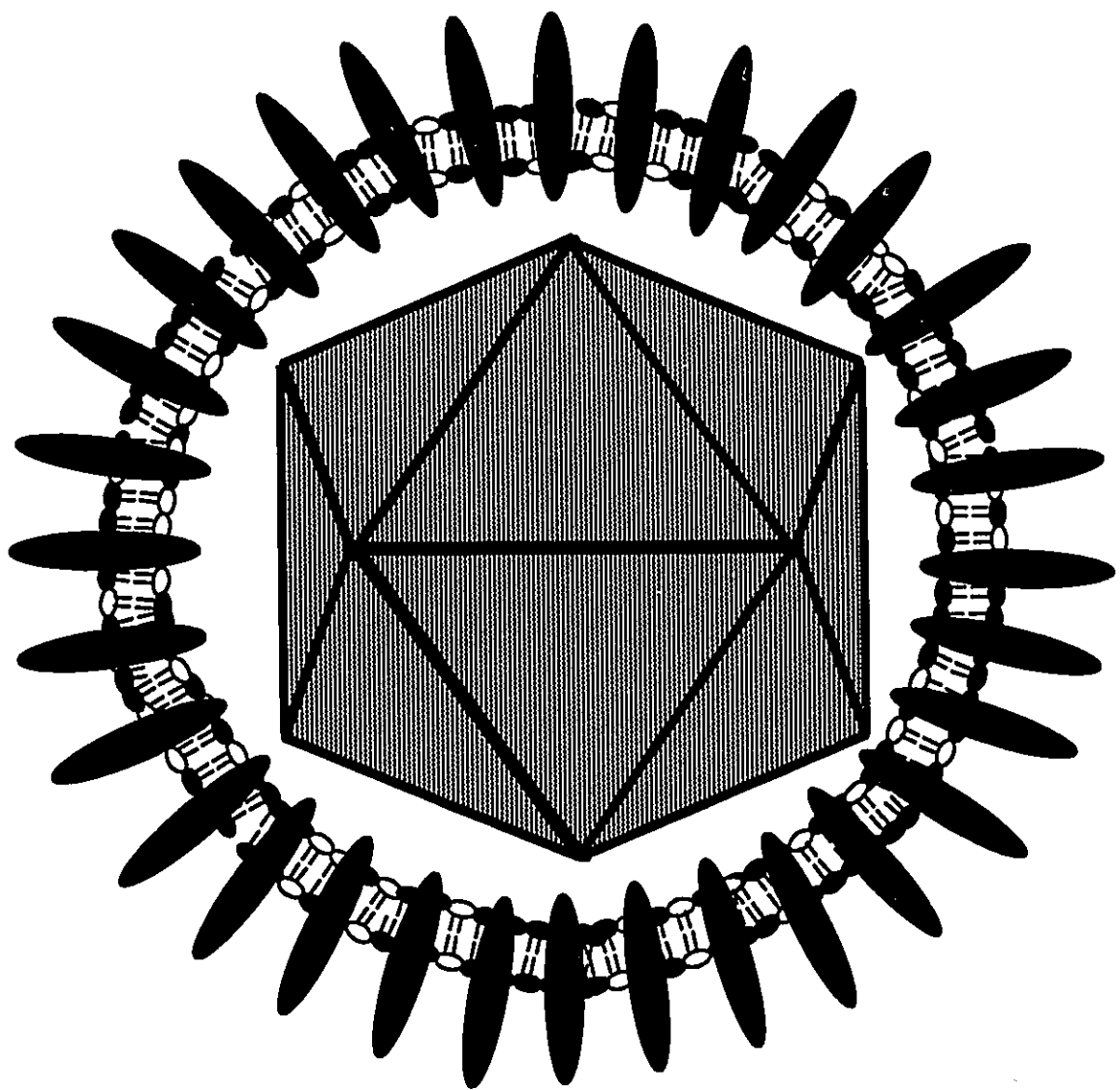
Significant research efforts have been invested towards the development of an HCMV vaccine able to provide protection for women before pregnancy and for patients receiving organ transplants. Initial studies in this area utilized the Towne strain of HCMV (14), previously isolated from the urine of a congenitally-infected infant and passaged 129 times. Inoculation of seronegative individuals with this attenuated live virus elicited both humoral and cellular immune responses (15). HCMV-neutralizing antibodies and lymphoproliferative responses (LPR) were detectable up to ten years post-inoculation in the majority of vaccines. When challenged with infectious doses of a different HCMV strain (Toledo), vaccinated individuals required a dose at least ten times greater than seronegative individuals before exhibiting any symptoms of HCMV disease. More striking, however, was the observation that vaccinated renal transplant recipients receiving seropositive (HCMV-infected) organs had a six-fold lower incidence of severe HCMV disease compared to seronegative patients receiving seropositive organs. Furthermore, the proportion of renal transplant recipients (from cadaver donors) surviving after ten years was twice as high in the vaccinated group compared to the initially seronegative, non-vaccinated group (16).

Concern over the oncogenic potential of HCMV (17,18) has driven vaccine research in the direction of a subunit vaccine, focusing on the immunoreactive

components of the HCMV viral envelope. Monoclonal antibody technology has provided a powerful tool for the characterization of HCMV surface glycoproteins and their immunodominant epitopes (19-23). Two glycoprotein complexes have been identified as the primary targets for neutralizing antibodies. Glycoprotein B, also reported as gC1, (24,25) is present as a 160, 190, or 300 kDa complex in its non-reduced form. Upon treatment with a reducing agent, the complex dissociates into two proteins of 58 kDa (gp58) and 90-130 kDa (gp116). Using HCMV-positive human sera, it has been shown that 40-70% of the total neutralizing capacity of the sera is directed against the gB glycoprotein (26). A second, less-well characterized HCMV glycoprotein, known as gH or gp86, is an 86 kDa viral surface molecule and is targeted by some neutralizing antibodies as well (27).

A strategy of identifying and characterizing the viral components which are essential to the infection process would focus on the molecules being targeted by the immune response. Clearly those viral surface glycoproteins which elicit a strong neutralizing antibody response would be key candidates for subunit vaccines. Furthermore, characterization of those key glycoproteins could potentially lead to an assay system which screens human sera for neutralizing antibody activity (28). The efficacy of hyperimmune globulins selected through such a screening process would theoretically be superior to existing products which are immunoreactive to infected cell lysates (29). Existing assays for detection of HCMV-neutralizing antibody are limited to time-consuming and labour

Figure 1. A schematic representation of the HCMV particle structure. Icosadeltahedral capsid is enclosed by a lipid bilayer membrane. Glycoprotein spikes project outward from the viral surface and are the target of many neutralizing antibodies. (Modified figure courtesy of Dr. E.L.G.Pryzdial.)



-intensive plaque reduction assays (30).

Existing approaches to the characterization of immunodominant regions of viral glycoproteins have focused on measuring antibody reactivity to recombinant fusion proteins (31,32) or synthetic peptides (33,34). Although some antigenic sites may not be affected by the altered conformational structure of a fusion protein or peptide, relative to the native structure, it is conceivable that antibodies specific for conformational epitopes would not easily be studied with these approaches.

OBJECTIVE

The intent of this body of work was to characterize a potent neutralizing anti-HCMV mouse monoclonal antibody and its interaction with purified whole HCMV virus particles. Chemical cross-linking methodologies were utilized to study antibody binding to viral antigens under native conditions, and a series of binding assays which used purified whole virus particles were developed to study the specificity of the antibody and the biochemical properties of the antigen. Identification and isolation of the antigen targeted by neutralizing antibody could permit the development of an assay to screen donor sera for anti-HCMV neutralizing activity.

A second series of experiments were carried out in which the interaction between HCMV viral particles and human fibroblast cells were studied. It has previously been reported that the binding of HCMV particles to the fibroblast cell surface

induced a rapid intracellular signalling process (35-37). Given that many cell signalling processes are initiated by a cell-receptor phosphorylation event (38,39), identification of a rapidly phosphorylated cellular protein could permit characterization of the cell receptor for HCMV particles.

Conventional approaches to the study of intracellular phosphorylation were employed. Incorporation of ^{32}P orthophosphate by activated phosphoproteins such as *c-fos* protein (Fos), phospholipase $\text{C}\gamma$ -1 ($\text{PLC}\gamma$ -1) and tyrosine-phosphorylation of cellular proteins induced by HCMV virion binding to fibroblast cell surfaces were examined. Protein kinase C (PKC) activity and intracellular cyclic adenosine monophosphate (cAMP) concentrations were also measured in response to HCMV virion binding to cells.

OBJECTIVE

Identification of a secondary messenger molecule phosphorylation event or concentration change would provide a detectable indication of cell activation. The ability of an anti-HCMV antibody or hyperimmune globulin product to block this cell activation could provide a useful assay of product efficiency.

Materials and Methods - Part A

Purification of anti-HCMV monoclonal antibody

Frozen ascites fluid (5 ml) was thawed and filtered through a 0.45 μ m cartridge (Millipore, Bedford, MA). Mouse IgG was loaded onto Protein G Sepharose (Pharmacia, Montreal, PQ) and unbound material was discarded. Bound protein was eluted with glycine-HCl buffer (250 mM, pH 2.7) which was immediately adjusted to pH 7.4 with 10%(w/v) Tris (Tris(hydroxymethyl)aminomethane) solution. Protein G column eluate was concentrated by ultrafiltration (Amicon, Oakville, ON) on PM30 membranes, then re-chromatographed on Protein G Sepharose and Protein A Sepharose. Concentrated eluate from Protein A Sepharose column was dialysed against 20 mM Tris HCl buffer (pH 7.7) and loaded onto an FPLC (Fast Protein Liquid Chromatography) Mono Q ion exchange column (Pharmacia). Bound protein was eluted with a NaCl gradient (Buffer A: 20 mM Tris-HCl, pH 7.7; Buffer B: 20 mM Tris-HCl, 500 mM NaCl, pH 7.7) at 35% Buffer B (175 mM NaCl). Concentrated eluate from Mono Q ion exchange chromatography was loaded onto an FPLC Superose 12 gel filtration column pre-equilibrated in phosphate-buffered saline (PBS: 30 mM phosphate, 150 mM NaCl, pH 7.4). Eluate fractions corresponding to the centre of the IgG peak were collected, pooled and concentrated by ultrafiltration.

Preparation of anti-HCMV monoclonal IgG Fab fragments

Purified monoclonal IgG (3 mg) was dialysed (2 x 4L) at 4°C against phosphate buffer (20 mM NaH₂PO₄, 10 mM tetrasodium ethylenediamine tetraacetate (EDTA-Na₄), pH 7.0). Dialysed IgG was added to an equal volume of papain-agarose (Pierce, Chromatographic Specialties, Mississauga, ON) washed and re-suspended in papain digestion buffer (phosphate buffer + 20 mM cysteine-HCl + 150 mM NaCl, pH 7.0). IgG and the papain-agarose slurry was incubated and mixed end-over-end at 37°C overnight. The slurry was centrifuged 5 min at 500xg and supernatant was filtered through 0.22 µm to remove any residual papain-agarose. Filtered protein was chromatographed on Protein A Sepharose as above and unbound material was collected, concentrated and dialysed against PBS, pH 8.0 (3 x 4L). Papain digest was evaluated by sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE (40)) on a Phast gel system (Pharmacia).

Biotinylation of anti-HCMV monoclonal IgG Fab fragments

Biotin N-hydroxysuccinimide reagent (Biorad, Mississauga, ON) was combined with purified Fab fragments in an equimolar ratio and incubated at 37°C for 1 h. The reaction mixture was loaded onto a GF-5 gel filtration column (Pierce) to separate unreacted biotinylation reagent from biotin-Fab fragments. Five drop fractions were collected and assayed for biotin-Fab fragment activity as follows. Aliquots (2 µl) from each fraction were dotted onto a Hybond C (Amersham, Oakville, ON) nitrocellulose membrane strip and air dried. The membrane was blocked with 1%

(w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS; 10 mM tris(hydroxymethyl)methylamine (Tris), 150 mM NaCl, pH 7.4) for 1 h at ambient temperature and incubated 30 min with streptavidin-horseradish peroxidase (SAV-HRP; Calbiochem, La Jolla, CA) diluted to 0.4 µg/ml in TBST (0.1%(v/v) Tween 20 (Calbiochem) in TBS). The membrane was washed 3 times with TBST and substrate solution (0.5 mg/ml 4-chloro-1-naphthol (Biorad), 20% methanol in TBS, 0.02% H₂O₂) was added. Colour development was stopped after 20 min and the membrane rinsed in water.

Immunoblot with anti-HCMV monoclonal antibody

Aliquots (20 µl, 8 x 10⁸ virus particles) of sucrose-density gradient-purified HCMV particles (ABI, Columbia, MD) were diluted with 30 mM PBS, 10%(w/v) SDS, 10% β-mercaptoethanol (βME) or a combination of these. The virions were incubated at room temperature or heated 3 min at 100 °C and 5 µl from each preparation was dotted onto three separate Hybond C nitrocellulose strips. After air drying, one membrane (A) was stained with amido black (0.3% (w/v) in 45% methanol, 10% acetic acid) and destained with distilled water. A second membrane (B) was blocked with TBST. The third membrane (C) was blocked with 1% BSA in TBS. Membrane B was incubated one hour with 1 µg/ml biotinylated concanavalin A (Vector, Dimension Labs, Markham, ON) diluted in TBST and washed 3 times with TBST. Membrane C was incubated for one hour with 0.4 µg/ml biotinylated monoclonal IgG Fab anti-HCMV diluted in TBST and washed 3 times with TBST.

Both membranes A and B were incubated 45 min with 0.5 µg/ml Sav-HRP and washed 3 times with TBST. Bound Sav-HRP was detected with substrate solution as described above.

Antibody and lectin western blotting of HCMV lysate

Sucrose density gradient-purified HCMV (5×10^8 particles/lane) were diluted in Laemmli reducing sample buffer (500 mM Tris, 8%(w/v) sodium dodecyl sulfate, 40%(v/v) glycerol, 0.04%(w/v) bromophenol blue, 130 mM dithiothreitol, pH 6.8) and electrophoresed in a 5-20% gradient gel (40). The viral proteins were electrotransferred (16 h at 250 mA) onto nitrocellulose membranes which were subsequently blocked with 1% BSA in TBS (membrane A) or TBST (membrane B). Membrane A was incubated for 1 hour at ambient temperature with 5% mouse serum in TBS, then incubated 1 hour with biotinylated monoclonal IgG Fab anti-HCMV (0.2 µg/ml in TBST). Membrane B was cut into strips corresponding to individual lanes which were incubated for 1 hour with 1 µg/ml solutions of biotinylated lectins (Vector) diluted in TBST. All the strips were washed 3 times in TBST and incubated 45 min with Sav-HRP conjugate (Jackson, Bio/Can Scientific, Mississauga, ON) diluted 1:10000 in TBST. Strips were washed 3 times with TBST and developed with the ECL chemiluminescent substrate system (41)(Amersham).

Immunoprecipitation of ^{35}S -labelled HCMV antigen with anti-HCMV monoclonal IgG

Confluent HFF (human foreskin fibroblasts), P5 - P12 (passage 5 -12), were infected with HCMV strain AD169 at an m.o.i. (multiplicity of infection) of 0.1. At three days post-infection 0.5 mCi ^{35}S -methionine was added to each T-75 flask (approximately $3\text{-}4 \times 10^6$ cells/flask). Ten days post-labelling the cells showed a maximal cytopathic effect. Cell culture supernatant was centrifuged 3000xg for 10 min to remove cell debris. Virions in the supernatant were centrifuged at 29000xg at 4°C for 1 hour through a cushion of D-sorbitol. The ^{35}S -labelled HCMV pellet was re-suspended in 1 ml of PBS and centrifuged at 14000xg at 4°C for 10 min. This step was repeated twice and the specific activity of the ^{35}S -HCMV measured on a Beckman (Beckman Instruments, Mississauga, ON) LS5000TD beta counter. The virus was aliquoted and stored at -70°C.

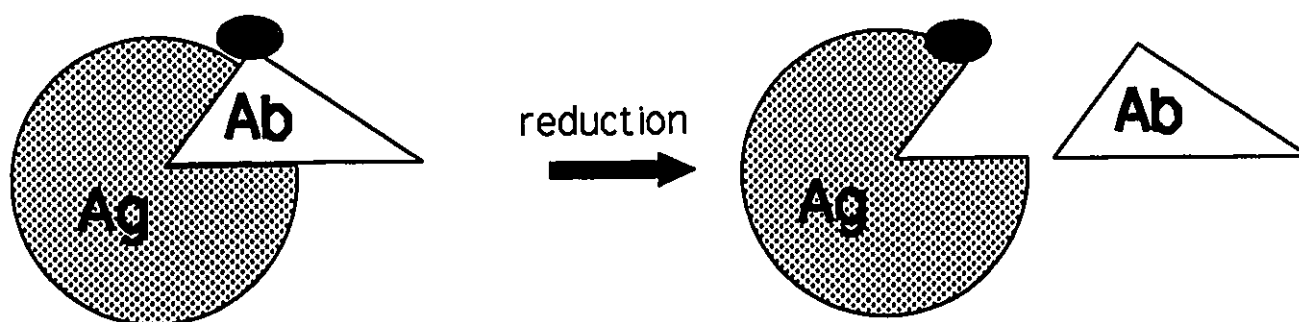
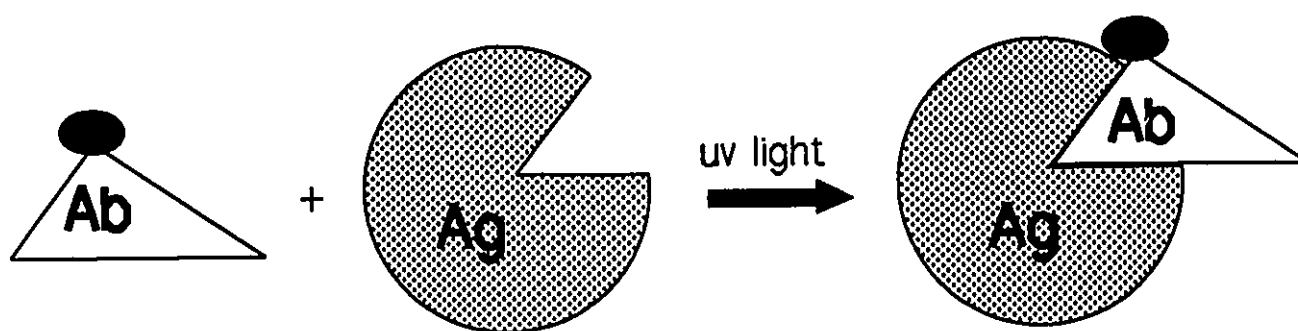
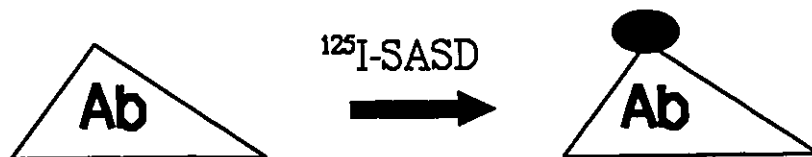
Aliquots of ^{35}S -HCMV (250000 cpm) were pre-incubated with 5 μl non-immune normal mouse serum for 2 h at 4°C. Pansorbin (Calbiochem) was pre-washed 3 times with immunoprecipitation buffer (IPB; 10 mM Tris, 150 mM NaCl, 0.1%(w/v) sodium dodecyl sulfate (Biorad), 0.1% BSA, 7 mM deoxycholic acid (Sigma, St. Louis, MO), 0.6 mM sodium azide (Sigma), pH 7.4) by centrifugation at 10000xg for 30 sec and re-suspension in IPB. Aliquots (100 μl /sample) of the washed Pansorbin were added to the ^{35}S -HCMV-mouse serum mixtures and incubated at 4°C for 2 h. Pansorbin was separated from the mixture by centrifugation (30 sec

at 10000xg). Protein G Sepharose (Pierce) was washed three times in IPB and diluted 1:10. CMVB1 monoclonal IgG Fab anti-HCMV (2.5 µg) was pre-incubated with 100 µl of Protein G Sepharose for 3 h at 4°C. The Protein G Sepharose - antibody complex was washed five times by centrifugation (1500xg for 2 min) with 1 ml of PBS containing 0.5%(v/v) Tween-20 and 1.0%(w/v) BSA. The ³⁵S-HCMV supernatant from the Pansorbin pre-adsorption step was added to the Protein G Sepharose and the mixture incubated overnight at 4°C. The Protein G Sepharose - antibody - antigen complex was washed 5 times with PBST (0.5%(v/v) Tween 20 in PBS) and re-suspended in Laemmli reducing sample buffer. The sample was heated and electrophoresed on 5-20% gradient gels. The gel was stained with 0.025%(w/v) R-250 Coomassie Brilliant Blue (Sigma) in 40% methanol, 7% acetic acid, and subsequently destained in 7.5% methanol, 10% acetic acid. The gel was soaked in Amplify fluorographic reagent (Amersham) for 30 min, dried and autoradiographed with X-OMAT film (Kodak, Eastview Photographic, Ottawa, ON).

Chemical crosslinking of antigen-antibody complexes

Sulfosuccinimidyl 2-(p-azidosalicylamido) ethyl-1,3'-dithiopropionate (SASD; Pierce) crosslinking reagent (50 mg) was dissolved in dimethyl sulfoxide (DMSO) under darkroom conditions and stored in light-proof vials of 25 µl aliquots at -30°C. All subsequent manipulations of this reagent were carried out under darkroom conditions in a specially adapted biocontainment hood (Figure 2.)

Figure 2. A schematic representation of a crosslinking procedure utilizing SASD. Antibody (Ab) is reacted with ^{125}I -labelled crosslinker. Antigen (Ag) is allowed to bind with antibody and complexes are then exposed to UV light. ^{125}I label is transferred to antigen by reduction of the complex.



A. Model system: albumin-anti-albumin complexes

SASD (2 µg) was radiolabelled with ^{125}I (10 µCi) according to the manufacturer's instructions. Briefly, 5 µl of SASD diluted in 10 mM phosphate buffer (PB; 10 mM sodium phosphate, pH 7.4) was mixed with 2.5 µl of 1.8 mM KI in PB and 10 µCi ^{125}I in amber reaction vials previously coated with Iodogen (Pierce) radioiodination reagent. The radiolabelling reaction was allowed to continue for 30 sec. The reaction mixture was removed from the vial and added to 225 µg anti-human albumin antibody (whole molecule, purified as described above; Cedarlane, Hornby, ON) previously dialysed versus borate buffer (100 mM sodium tetraborate/boric acid, pH 8.4). After 30 min the mixture was loaded onto an Excellulose GF-5 (Pierce) gel filtration column (exclusion limit of 5000 daltons) pre-equilibrated in 20 mM PBS. Fractions (5-6 drops) were collected and counted on a Beckman 5500B gamma counter. The fractions with high activity were pooled and an aliquot precipitated with 10% trichloroacetic acid (TCA) and counted. Aliquots (20 µg) of the ^{125}I -SASD-antibody were mixed with human serum albumin (Miles, Toronto, ON) aliquots (20 µg or 40 µg), human IgA1 (20 µg) previously purified in-house (42), or PBS. The reaction mixtures were incubated in borosilicate tubes at ambient temperature for 2.5 h, exposed to long wave UV light for 30 min and flashed ten times with a camera flash. Each sample was mixed with reducing or non-reducing sample buffer and electrophoresed on 4-12% gradient gels. Gels were stained with Coomassie Blue, destained, dried and

autoradiographed for 4 h.

B. Monoclonal IgG Fab anti-HCMV-viral antigen complexes

SASD (6 µg) was radiolabelled with ^{125}I (5 µCi), incubated with 170 µg of monoclonal IgG Fab anti-HCMV for 45 min and loaded onto a GF-5 column. Protein fractions exhibiting ^{125}I activity were pooled and an aliquot precipitated with 10% TCA. Reaction mixtures consisting of ^{125}I -SASD-antibody (50 µg), purified HCMV particles (50 µl, 1.5×10^9 particles), HCMV-infected MRC-5 lung fibroblast cell lysate (35 µg; Microbix, Guelph, ON), or PBS were incubated at ambient temperature for 2.5 h, then exposed to long wave UV light and 10 camera flashes. Samples were mixed with reducing or non-reducing sample buffer and electrophoresed on a 4-12% gel. The gel was stained with Coomassie Blue, destained, dried and autoradiographed for 24 h.

Characterization of antibody specificity by microtitre plate binding assay

To facilitate rapid measurement of anti-HCMV antibody binding to HCMV particles a microtitre plate format assay system was developed. Previously biotinylated Fab fragments of CMVB1 were utilized in this procedure.

A. Saturable binding of monoclonal IgG Fab anti-HCMV to HCMV particles and inhibition by non-immune mouse IgG

Purified monoclonal whole molecule IgG anti-HCMV was diluted to 5 µg/ml in PBS and incubated in 96-well microtitre plate wells (200 µl/well) overnight at 4°C. The plate was washed once with PBSA (PBS, 1%(w/v) BSA), blocked with PBSA and

washed 3 times. Purified HCMV particles were diluted in PBS and incubated in the antibody-coated wells (5×10^8 particles/well) for 1 h at ambient temperature. The wells were then gently washed 3 times with PBSA. Biotinylated monoclonal IgG Fab anti-HCMV was serially diluted ($\times 1/2$) from 800 ng/ml to 6 pg/ml and added in quadruplicate to microtitre plate wells (200 μ l/well). Unlabelled monoclonal IgG Fab anti-HCMV or non-specific mouse IgG Fab fragments were serially diluted ($\times 1/5$) from 20 μ g/ml to 50 pg/ml. Duplicate samples of these dilutions were mixed with 200 ng/ml aliquots of biotinylated monoclonal IgG Fab anti-HCMV and incubated 1 h at ambient temperature (200 μ l/well) with the HCMV particles bound to the microtitre plate. The plates were washed 3 times with PBSA and incubated with 0.4 μ g/ml streptavidin alkaline phosphatase conjugate (Sav-AP; Jackson) for 1 h at ambient temperature. The plate was washed 3 times with PBSA and 2 times with PBS and substrate added (0.5 mg/ml p-nitrophenylphosphate in 1.4M diethanolamine, 0.5 mM $\text{MgCl}_2 \cdot \text{H}_2\text{O}$, pH 9.8). Colour was measured spectrophotometrically at 405 nm on a Molecular Devices plate reader (Molecular Devices Corporation, Menlo Park CA).

B. Inhibition by HCMV, HSV-1 and HSV-2

HCMV particles diluted in PBS were also coated directly onto the microtitre plate in the absence of any anti-HCMV antibody. The plate was blocked and washed as described above. Purified HCMV AD169, HSV-1 (herpes simplex virus 1, MacIntyre strain; ABI) or HSV-2 (herpes simplex virus 2, G strain; ABI) particles were serially diluted ($\times 1/2$) in PBSA from 1.3×10^{10} particles/ml to 1.0×10^8

particles/ml and mixed with 0.4 µg/ml biotinylated monoclonal IgG Fab anti-HCMV. Triplicate samples of these solutions were incubated for 1.5 h at ambient temperature in the microtitre plate wells (100 µl/well). The wells were washed and incubated with Sav-AP and substrate as described above.

C. Inhibition by lectins and simple sugars

Serial dilutions (x 1/2, 20 µg/ml to 0.15 µg/ml in PBSA containing 0.1 mM CaCl₂, 0.1 mM MgCl₂, plus 0.01 mM MnCl₂ for concanavalin A) of purified lectins (Vector) were mixed with 0.4 µg/ml biotinylated monoclonal IgG Fab anti-HCMV and incubated in microtitre plate wells coated directly with HCMV particles. The binding assay was carried out as described above.

D-glucose, D-mannose and N-acetylglucosamine were serially diluted (x 1/2, 100 µg/ml to 1.56 µg/ml in PBSA) and mixed with 0.2 µg/ml biotinylated monoclonal IgG Fab anti-HCMV. Triplicate samples of the solutions were incubated in microtitre plate wells directly coated with HCMV particles. The binding assay was developed as described above.

Materials and Methods - Part B

Culture of human foreskin fibroblasts and virus production

HFF (P5-P15) were grown in Basal Medium Eagle (BME; Gibco, Burlington, ON) supplemented with 5% bovine calf serum, 14 μ M L-glutamine and 20 μ g/ml gentamycin in 175 cm² tissue culture flasks. Cells were grown to confluency (7 days) and infected with HCMV AD169 particles at a multiplicity of infection of 0.001. Infected cells were maintained in BME supplemented with 2% bovine calf serum (L-glutamine and antibiotic as above) until day 16 post-infection. At day 16 the fibroblast monolayers exhibited a high degree of cytopathic effect and were dislodged from the tissue culture flasks with vigorous pipetting. The culture medium and cell debris were centrifuged for 5 min at 1500xg. The cell pellet was retained and stored at -70°C and the supernatant was centrifuged at 25000xg for 1 h at 4°C. The virus pellet was stored at -70°C until 6-8 harvests had been completed.

Thawed virus pellets were re-suspended to homogeneity and layered onto a positive density (15-40% potassium tartrate) - negative viscosity (20-0% glycerol) gradient (43). The virus was centrifuged for 15 min at 200000xg at 4°C in a Beckman L8-80M ultracentrifuge and the separated virion or dense body bands collected with a syringe and needle. The virions and dense bodies were washed once with PBS by centrifugation (5 min at 10000xg at 4°C), aliquoted and stored

at -70°C.

Induction and detection of intracellular phosphorylation

A. Incorporation of ^{32}P -orthophosphate

Confluent HFF (P6-P9) in 6-well tissue culture plates were rinsed once and incubated overnight in serum-free BME. The cells were rinsed twice with phosphate-free Minimal Essential Medium (MEM; Gibco) and incubated with ^{32}P -orthophosphate (250-300 $\mu\text{Ci}/\text{well}$; Amersham) for 45 min. Fetal calf serum (PDI, Aurora, ON) was added to a final concentration of 10% to the positive control wells, purified HCMV particles (10^8 particles/well) were added to the test wells, and negative control wells were unsupplemented. The plates were incubated at 37°C for 5, 10, 30 and 60 min. At each time point the cells were cooled on ice and the supernatant medium was removed. Aliquots (300 μl) of lysis buffer (25 mM Tris, 5 mM tetrasodium EDTA, 1%(v/v) NP-40, 50 mM sodium orthovanadate (Aldrich, Milwaukee, WI), 10 mM sodium pyrophosphate (Fisher, Ottawa, ON), 1 mM phenylmethylsulfonylfluoride, 0.6 mM benzamidine (Sigma), 70 μM pepstatin A (Sigma), pH 7.4, 0.2 μm filtered) were added to the cells. Cell lysate was removed and stored on ice until diluted with sample buffer for analysis by SDS-PAGE or until immunoprecipitated with specific antibodies.

B. Detection of ^{32}P -orthophosphate-labelled cellular proteins

Whole cell lysate was diluted with 4x reducing sample buffer and electrophoresed

by SDS-PAGE on 1.5mm 5-20% gradient gels. The gels were dried and subjected to autoradiography using Kodak X-OMAT film. Alternatively, whole cell lysates were pre-adsorbed with Protein A Sepharose (15 mg/tube) for 30 min at 4°C and centrifuged for 10 min at 10000xg. The pre-adsorbed lysate was then incubated with 10 µl rabbit anti-Fos antibody (UBI, Lake Placid, NY), 5 µl rabbit anti-phospholipase C γ -1 antibody (UBI) or 6 µl mouse anti-phosphotyrosine antibody (Sigma) for 16 h at 4°C. Aliquots of Protein A Sepharose (3 mg/tube) were added to the lysates and incubated 30 min at 4°C. The Protein A Sepharose-antibody-antigen complexes were washed three times with IPB, once with 1M NaCl in TBS and 3 times with TBS. The complexes were suspended in 1x reducing sample buffer, heated 3 min at 100°C and electrophoresed by SDS-PAGE on 5-20% gels. The gels were dried and autoradiographed with X-OMAT film.

C. Detection of tyrosine phosphorylation by Western blotting

Confluent HFF monolayers in 6-well plates were stimulated with agonists and lysed at varying time points as described above. The stimulation was performed in the presence of normal phosphate concentrations without the addition of ^{32}P -orthophosphate. Whole cell lysates were electrophoresed and transferred onto nitrocellulose membranes as described above. The membrane was blocked with 1% BSA in TBST and incubated 1.5 h with monoclonal anti-phosphotyrosine (0.5 µg/ml in TBST). The membrane was washed 3 times and incubated with goat anti-mouse IgG horseradish peroxidase conjugate (0.02 µg/ml in TBST) for 1 h.

After washing 4 times with TBST and once with TBS the membrane was developed with the ECL substrate system.

Measurement of Intracellular cAMP concentrations

Confluent HFF in 6-well plates were incubated in serum-free BME overnight and incubated with positive control agonist (10% fetal calf serum in BME), negative control (serum-free BME) or HCMV diluted in BME (10^8 particles/well). At 10, 30 and 60 min the supernatant medium was aspirated from the monolayer and the cells rinsed once with ice cold PBS. Ice cold 65% (v/v) ethanol was added and the cells scraped from the wells. The cell suspension was kept on ice until all time points were completed, then were centrifuged at 2000xg for 15 min. The supernatants were dried in a vacuum centrifuge at 60°C and dissolved in 0.5 ml of 0.05M acetate assay buffer. Concentrations of cAMP were measured utilizing the ^{125}I scintillation proximity assay kit (Amersham) according to the manufacturer's instructions. Briefly, 10-fold diluted cell ethanol extracts or cAMP standards were incubated with mixing for 16 h at ambient temperature with ^{125}I -cAMP, rabbit anti-succinyl cAMP, and donkey anti-rabbit IgG-conjugated fluomicrospheres. Bound ^{125}I -cAMP was counted on a β -scintillation counter for 2 min and cAMP concentrations in the cell lysate samples were determined from a standard curve.

Measurement of membrane-bound Protein Kinase C activity

Confluent HFF monolayers in 6-well plates were incubated in serum-free BME

overnight, rinsed once with medium and incubated with positive control agonist (1 μ M 12-O-tetradecanoylphorbol 13-acetate (TPA) in BME), negative control (serum-free BME) or HCMV (10^8 particles/well). At 10, 30 and 60 min the supernatant media were aspirated from the wells and the cells were washed once with ice cold PBS. The cells were scraped from the wells in lysis buffer (1 mM NaHCO₃, 5 mM MgCl₂·H₂O, 100 μ M PMSF (phenylmethylsulfonylfluoride), pH 7.5) and stored on ice until all time points were completed. The cells were sonicated for 30 sec at 5 watts and centrifuged at 500xg for 5 min. The supernatant was removed from the tubes and centrifuged at 100,000xg in a Beckman L8-80M ultracentrifuge for 10 min at 4°C. The supernatants were discarded and the membrane pellets stored frozen at -70°C. Protein Kinase C (PKC) activity was assayed as previously described by others (44). Briefly, membrane pellets were re-suspended in assay buffer (50 mM Tris-HCl, 10 mM MgCl₂, 2 μ M CaCl₂, 200 μ M sodium orthovanadate, 200 μ M sodium pyrophosphate, 2 mM NaF, 200 μ M PMSF, pH 7.5) by vigorous pipetting. Protein content was measured with a colorimetric protein assay (45)(Biorad) and 3 μ g of membrane protein were used to assay PKC activity. Membrane protein (20 μ l), assay buffer (30 μ l), peptide substrate (FKKSFKL-NH₂, 9 μ M) and ³²P-ATP (adenosine triphosphate, 0.5 μ Ci/tube) were incubated for 10 min at 37°C. The reaction was stopped by addition of 10 μ l of 5% acetic acid. Triplicate aliquots (90 μ l) of the reaction mixture were pipetted onto 2 cm² pieces of P81 filter paper (Whatman, Fisher) which were subsequently washed twice with 5% acetic acid (10 ml/piece) by stirring gently for 5 min in a

large beaker. Radioactive peptide substrate bound to the P81 pieces was measured by liquid scintillation counting on a LKB 1217 β -counter (Pharmacia).

Results - Part A

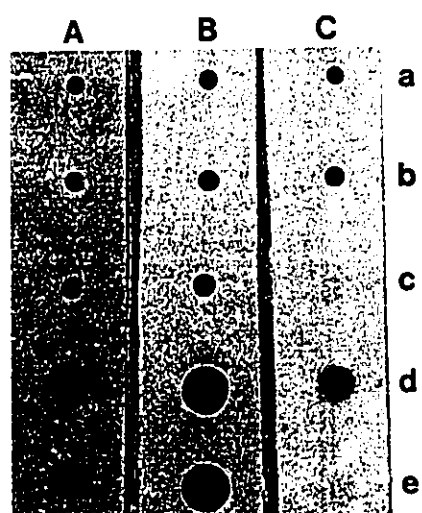
Preparation of anti-HCMV monoclonal IgG Fab fragments

Conventional methodologies were utilized to purify mouse IgG from ascites fluid and to prepare Fab fragments. Protein concentrations were determined spectrophotometrically by measuring absorbance at 280 nm. The overall yield of Fab fragments was 49 percent of initial IgG content on a molar basis. These purified fragments were subsequently biotinylated for use in several experimental procedures.

Immunoblot with anti-HCMV monoclonal antibody

The effects of disulfide reduction, denaturation and heating on the viral epitope targeted by the anti-HCMV monoclonal antibody were explored. Colour development on membranes A and B (Figure 3) confirmed that virions subjected to the different physicochemical treatments could be detected with amido black stain and with biotinylated concanavalin A. Reactivity with biotinylated monoclonal IgG Fab anti-HCMV (membrane C) was limited to untreated virions or virions treated with β ME only. Virions treated with SDS only were weakly detected with anti-HCMV monoclonal antibody, while virions subjected to both heat and β ME or β ME/SDS were not reactive at all with the monoclonal antibody.

Figure 3. Antibody and lectin blotting of HCMV particles. Purified HCMV particles were dotted onto nitrocellulose membranes. Membrane A was stained with amido black and destained with water. Membrane B was blocked with TBST and incubated with biotinylated concanavalin A. Membrane C was blocked with 1%BSA in TBS and incubated with biotinylated monoclonal IgG Fab anti-HCMV. Membranes B and C were incubated with streptavidin horseradish peroxidase and developed with substrate solution. HCMV particles were treated with (top to bottom) a) no physicochemical agents, b) 1.3M β -mercaptoethanol, c) 1.3M β -mercaptoethanol and heated at 100°C for 3 min, d) 1.3M β -mercaptoethanol and 10%(w/v) SDS and e) same as d) and heated 3 min at 100°C.



Antibody and lectin western blotting of HCMV

The relative reactivities of anti-HCMV monoclonal antibody and several lectins to viral lysate were examined. Biotinylated lectins exhibited differential and intense reactivity with many viral glycoproteins (Figure 4). Immunoblotting of viral lysate indicated two reactive bands with apparent molecular masses of approximately 70 kDa and over 200 kDa (lane 7). The weakness of the antibody interaction with the viral lysate was consistent with the dot blot results described above.

Immunoprecipitation of ³⁵S-labelled HCMV antigen

Native viral particles were reacted with monoclonal antibody to isolate the target antigen. Autoradiography of immunoprecipitated viral antigen electrophoresed on a 5-20% gel revealed a ³⁵S-labelled viral antigen migrating with an apparent molecular mass of 65-70 kDa (Figure 7). Under these immunoprecipitation conditions, no other viral antigen appeared to have been precipitated with the monoclonal IgG anti-HCMV.

Chemical crosslinking studies

Utilizing the model antigen-antibody system, covalently linked albumin-anti-albumin complexes could be observed by autoradiography of SDS-PAGE gels under non-reducing conditions (Figure 6, Panel A). Upon reduction, a ¹²⁵I-labelled species migrating at a position corresponding to the molecular mass of albumin was detected (Figure 6, Panel B). This confirmed the transfer of the ¹²⁵I-labelled

Figure 6. Antibody and lectin western blotting of HCMV lysate. Purified HCMV particles were electrophoresed by SDS-PAGE under reducing conditions in a 5-20% gradient gel. The separated proteins were transferred onto nitrocellulose membranes which was cut into individual lanes and incubated with 1 µg/ml solutions of biotinylated lectin: lane a), *Ulex europaeus* 1; lane b), *Ricinus communis* agglutinin 1; lane c), *Glycine max* agglutinin; lane d), *Triticum vulgaris* agglutinin; lane e), *Arachis hypogaea* agglutinin; lane f), *Concanavalin A*, or 0.2 µg/ml of biotinylated CMVB1 monoclonal IgG Fab anti-HCMV (lane g). All strips were subsequently incubated with Sav-HRP conjugate and developed with the ECL chemiluminescent substrate system. Arrows indicate bands reactive with CMVB1 in lane g). Molecular weight markers (kDa) are as indicated.

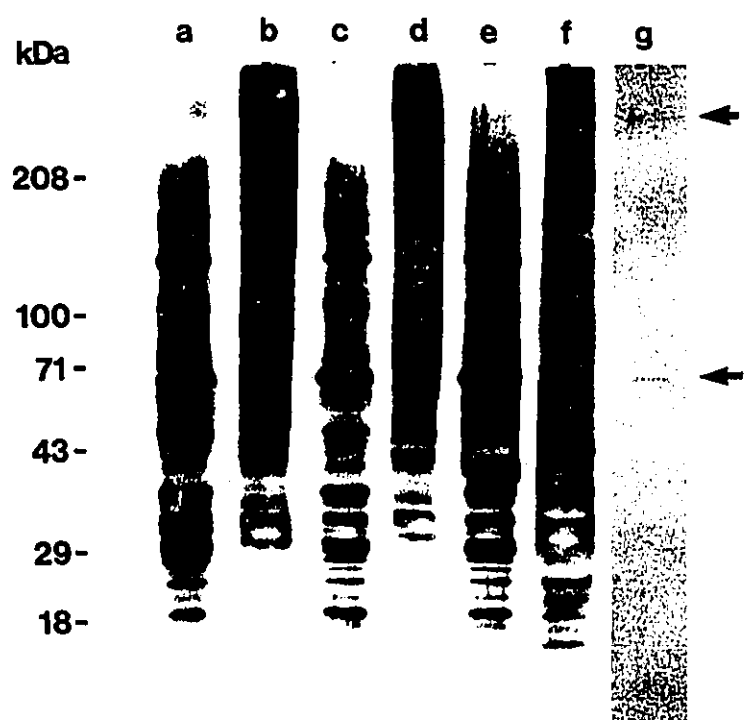


Figure 5. Immunoprecipitation of ^{35}S -labelled HCMV antigen with monoclonal antibody. Purified ^{35}S -methionine-labelled HCMV particles were incubated with Protein G-monoclonal IgG anti-HCMV antibody complexes overnight. The Protein G-antibody-antigen complexes were washed 5 times and electrophoresed on a 5-20% gel. The gel was soaked in fluorographic reagent, dried and autoradiographed for 12 days. Lane a), molecular weight markers (kDa), as indicated. Lane b), immunoprecipitated HCMV antigen.

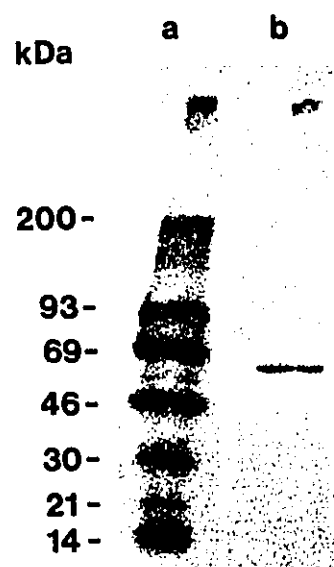
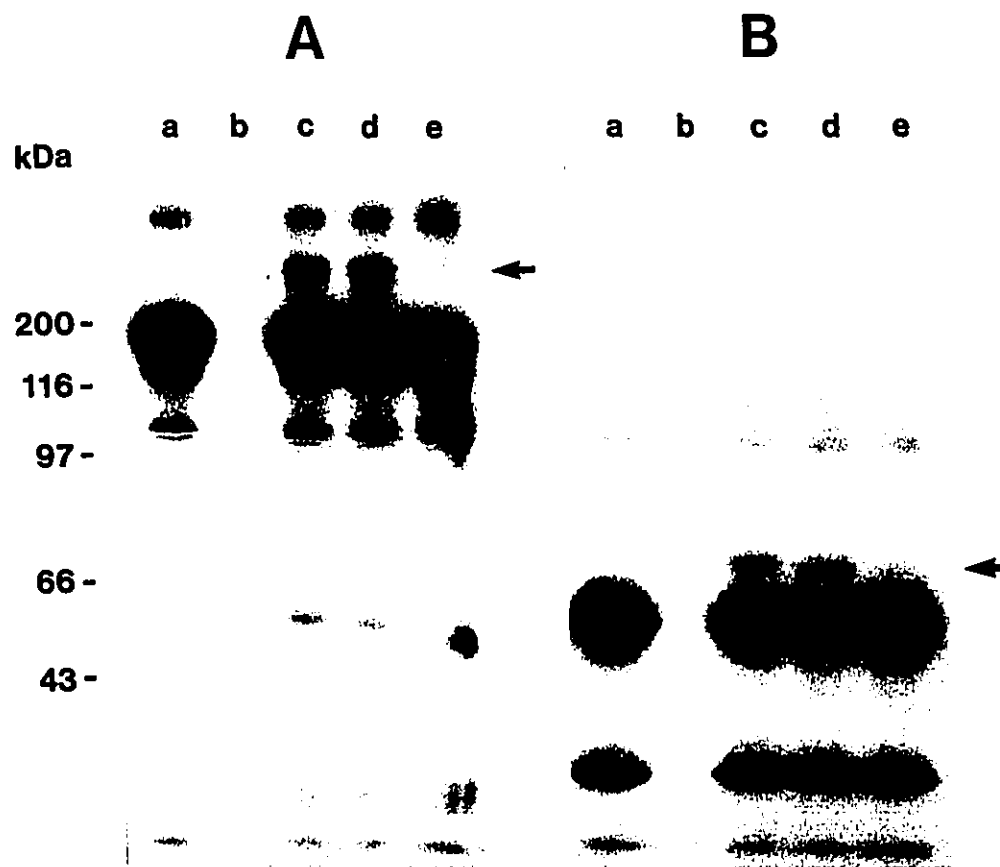


Figure 6. Chemical crosslinking of albumin to anti-albumin antibody with SASD. Whole molecule monoclonal anti-human albumin (20 μ g) was reacted with 125 I-labelled SASD crosslinking reagent and incubated with human albumin (20 μ g or 40 μ g) or an unrelated protein (human IgA1, 20 μ g). After a 1.5 h incubation SASD was photoactivated with UV light and camera flashes. Covalently-linked antigen-antibody complexes were electrophoresed by SDS-PAGE on 4-12% gels. The gels were dried and autoradiographed for 4 h. Panel A (non-reducing conditions): lane a) SASD-anti-albumin only, lane b) albumin only, lane c) SASD-antibody and 20 μ g albumin, lane d) SASD-antibody and (40 μ g) albumin, lane e) SASD-antibody and 20 μ g IgA1. Panel B (same as Panel A; reducing conditions). Arrows indicate position of 125 I-SASD-antibody-antigen complexes (Panel A) or 125 I-labelled antigen after reduction (Panel B). Molecular weight markers (kDa) are as indicated.



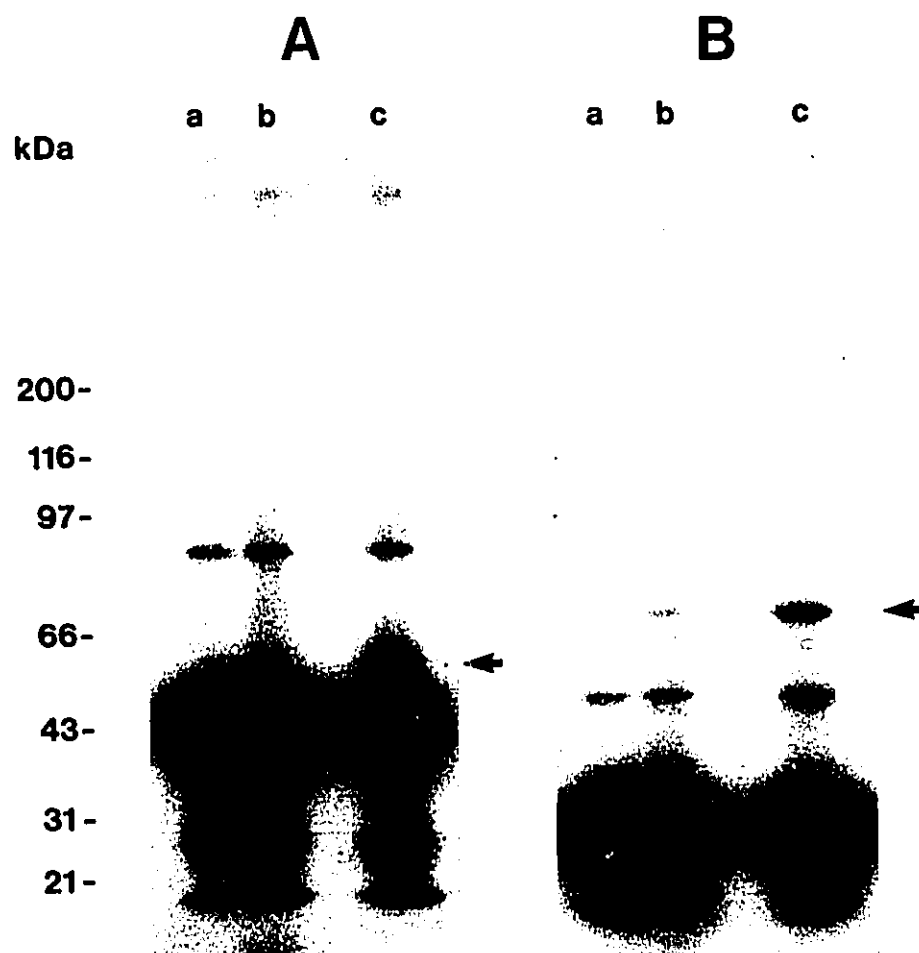
portion of the cross-linker to the antigen after reductive cleavage with dithiothreitol. Under non-reducing conditions no higher molecular mass complexes (antibody + antigen) were observed when specific antigen (albumin) was substituted with an unrelated protein (IgA1). As well, under reducing conditions no new ^{125}I -labelled species were observed with this negative control.

As a means of identifying the anti-HCMV antibody target antigen, SASD was used in cross-linking studies. Covalently cross-linked Fab fragment anti-HCMV-antigen complexes were detected by autoradiography after electrophoresis under non-reducing conditions (Figure 7, Panel A). A molecular complex migrating with an apparent molecular mass of 50-60 kDa was detected in samples consisting of SASD-monoclonal IgG Fab anti-HCMV incubated with whole virus particles or with HCMV-infected cell lysate (Figure 7, lanes b and c). Under reducing conditions, an ^{125}I -labelled species with an apparent molecular mass of approximately 70 kDa was observed in the samples derived from both the whole virus particles and the infected cell lysate. This observed molecular mass was consistent with the results of immunoprecipitation with the anti-HCMV monoclonal antibody.

HCMV-anti-HCMV microtitre plate binding assay

In order to characterize the anti-HCMV antibody a binding assay was developed to examine the specificity of the antibody. Biotinylated monoclonal IgG Fab anti-HCMV bound to purified HCMV AD169 whole virus particles coated onto microtitre

Figure 7. Chemical crosslinking of monoclonal IgG Fab anti-HCMV to viral antigen with SASD. Monoclonal IgG Fab anti-HCMV (50 µg) was reacted with ¹²⁵I-labelled SASD and incubated with 1.5 x10⁸ purified virions or HCMV-infected HFF cell lysate (35 µg) for 2.5 h. The SASD was photoactivated with UV light and camera flashes. Covalently-linked ¹²⁵I-labelled antibody-antigen complexes were electrophoresed by SDS-PAGE on 4-12% gels. The gels were dried and autoradiographed for 24 h. Panel A (non-reducing conditions): lane a) SASD-labelled monoclonal IgG Fab anti-HCMV only, lane b) SASD-antibody and purified HCMV particles, lane c) SASD-antibody and HCMV-infected HFF cell lysate. Panel B (same as Panel A, reducing conditions). Arrows indicate the position of newly formed ¹²⁵I-labelled SASD-antibody-antigen complexes (Panel A) or the ¹²⁵I-labelled antigen after reduction (Panel B). Molecular weight markers (kDa) are as indicated.



plates in a saturable manner (Figure 8). Non-biotinylated monoclonal IgG Fab anti-HCMV was able to block the biotinylated form of the antibody from binding to virions, while non-specific mouse IgG Fab fragments could not block the binding (Figure 9). Non-biotinylated anti-HCMV achieved 50% inhibition of the biotinylated antibody at a concentration of 6 nM.

Figure 8. Binding of biotinylated monoclonal IgG Fab anti-HCMV to HCMV particles. Purified whole molecule monoclonal anti-HCMV antibody was coated on microtitre plates at 5 µg/ml. The wells were blocked with PBSA and purified HCMV particles (5×10^8 particles/well) were incubated with the bound antibody. Biotinylated monoclonal IgG Fab anti-HCMV was serially diluted ($\times 1/2$) from 800 ng/ml to 6 pg/ml and incubated in quadruplicate with the microtitre-plate bound HCMV. After a 1 h incubation the wells were washed and incubated with 0.4 µg/ml streptavidin alkaline phosphatase. Substrate was added and bound conjugate was detected by measuring absorbance at 405 nm. Error bars indicate standard deviation.

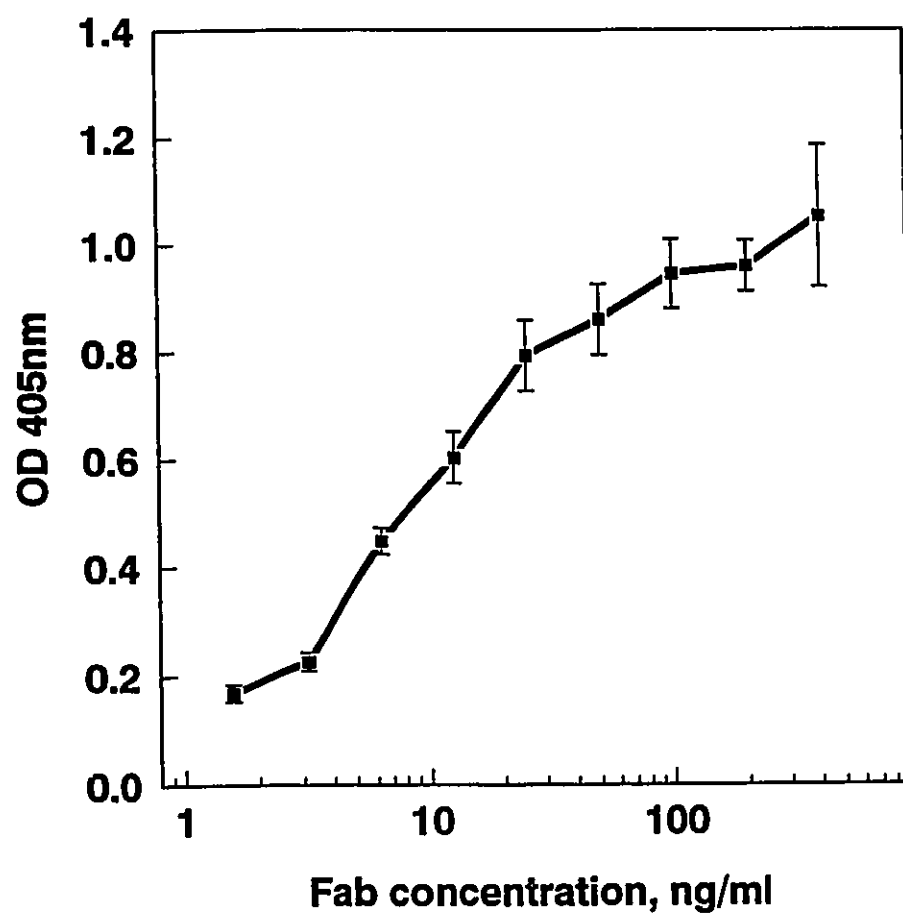
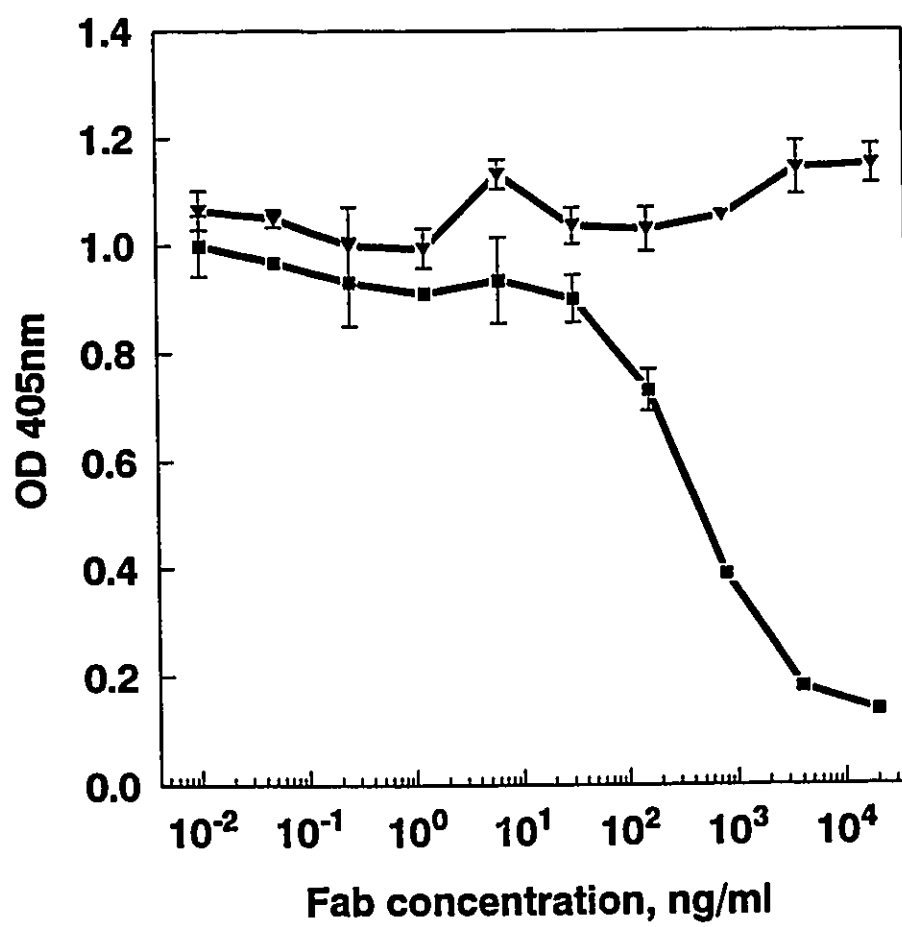


Figure 9. Inhibition of biotinylated monoclonal IgG Fab anti-HCMV by unlabelled homologous antibody or non-immune mouse IgG Fab. Purified HCMV particles were bound to microtitre plates coated with whole molecule anti-HCMV as described in Figure 8. Unlabelled monoclonal IgG Fab anti-HCMV or non-immune mouse IgG Fab fragments were serially diluted ($\times 1/2$) from 20 $\mu\text{g/ml}$ to 50 pg/ml . Duplicate samples were mixed with 200 ng/ml biotinylated monoclonal IgG Fab anti-HCMV and incubated in the wells for 1 h. Biotinylated anti-HCMV which bound in the presence of homologous unlabelled Fab (■) or non-immune IgG Fab (▼) was incubated with 0.4 $\mu\text{g/ml}$ streptavidin alkaline phosphatase. Substrate was added and bound conjugate was detected by measuring absorbance at 405 nm. Where no error bars are visible, standard deviation was too small to appear on graph.



Purified HCMV virus particles in solution phase were able to block the binding of biotinylated monoclonal IgG Fab anti-HCMV to virions coated onto microtitre plate wells (Figure 10). The binding of the antibody could not be blocked with the same concentrations of purified HSV-1 or HSV-2 particles.

The binding of biotinylated monoclonal IgG Fab anti-HCMV to HCMV particles coated onto microtitre plate wells could be partially blocked by concanavalin A lectin, but not by lentil lectin or by wheat germ agglutinin (Figure 11). At a concentration of 50 nM concanavalin A achieved a 50% inhibition of the anti-HCMV Fab fragment binding to virus particles. At concentrations up to 50 µg/ml (1 mM), neither D-glucose, D-mannose nor N-acetylglucosamine were able to inhibit the binding of the anti-HCMV antibody to virus particles (Figure 12).

Figure 10. Inhibition of monoclonal IgG Fab anti-HCMV binding to microtitre plate-bound HCMV by free HCMV, HSV-1 or HSV-2 particles. Purified HCMV (5×10^8 particles/well) were coated directly onto microtitre plate wells in the absence of any antibody. Purified HCMV, HSV-1 or HSV-2 were serially diluted ($\times 1/2$) in PBSA from 1.3×10^{10} particles/ml to 1.0×10^8 particles/ml and mixed in triplicate with $0.4 \mu\text{g/ml}$ biotinylated monoclonal IgG Fab anti-HCMV. The antibody which bound in the presence of free HCMV ($\blacktriangle$), HSV-1 ($\bullet$) or HSV-2 ($\blacksquare$) was incubated with streptavidin alkaline phosphatase. Substrate was added and bound conjugate was detected by measuring absorbance at 405 nm. Where no error bars are visible, standard deviation was too small to appear on graph.

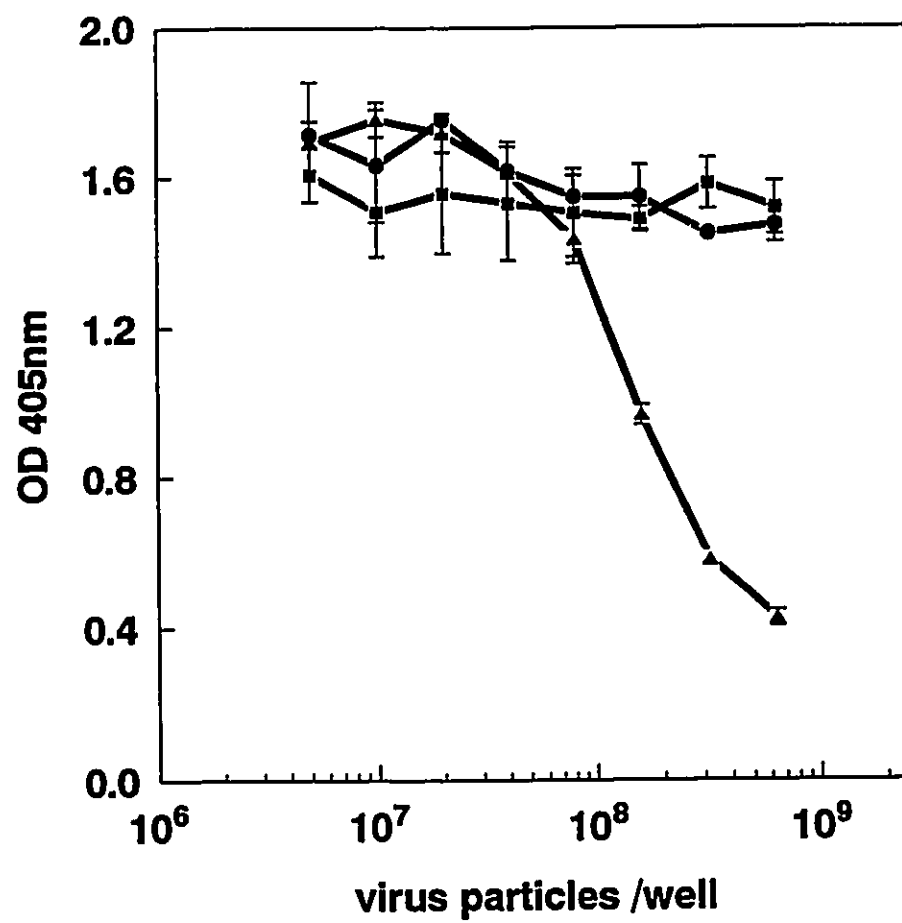


Figure 11. Inhibition of monoclonal IgG Fab anti-HCMV binding to microtitre plate-bound HCMV particles by lectins. Purified HCMV particles were coated onto microtitre plates in the absence of any antibody, as described in Figure 10. Wheat germ agglutinin (*Triticum vulgaris agglutinin*) (●), lentil lectin (*Lens culinaris lectin*) (▼), Concanavalin A (*Canavalia ensiformis lectin*) (■) or unlabelled monoclonal IgG Fab anti-HCMV (▲) were serially diluted ($\times 1/2$) from 20 $\mu\text{g/ml}$ to 0.15 $\mu\text{g/ml}$ and mixed with 0.4 $\mu\text{g/ml}$ biotinylated anti-HCMV antibody. Biotinylated anti-HCMV which bound in the presence of the lectin or unlabelled antibody was incubated with streptavidin alkaline phosphatase. Substrate was added and bound conjugate was detected by measuring at 405 nm. Where no error bars are visible, standard deviation was too small to appear on graph.

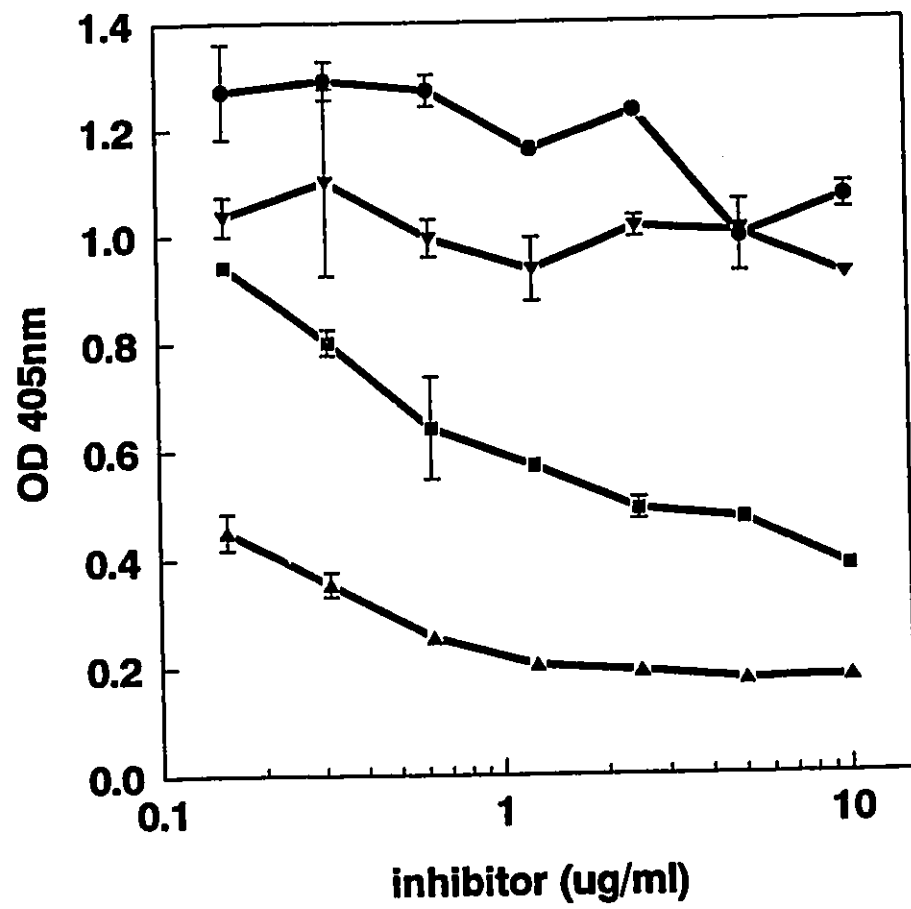
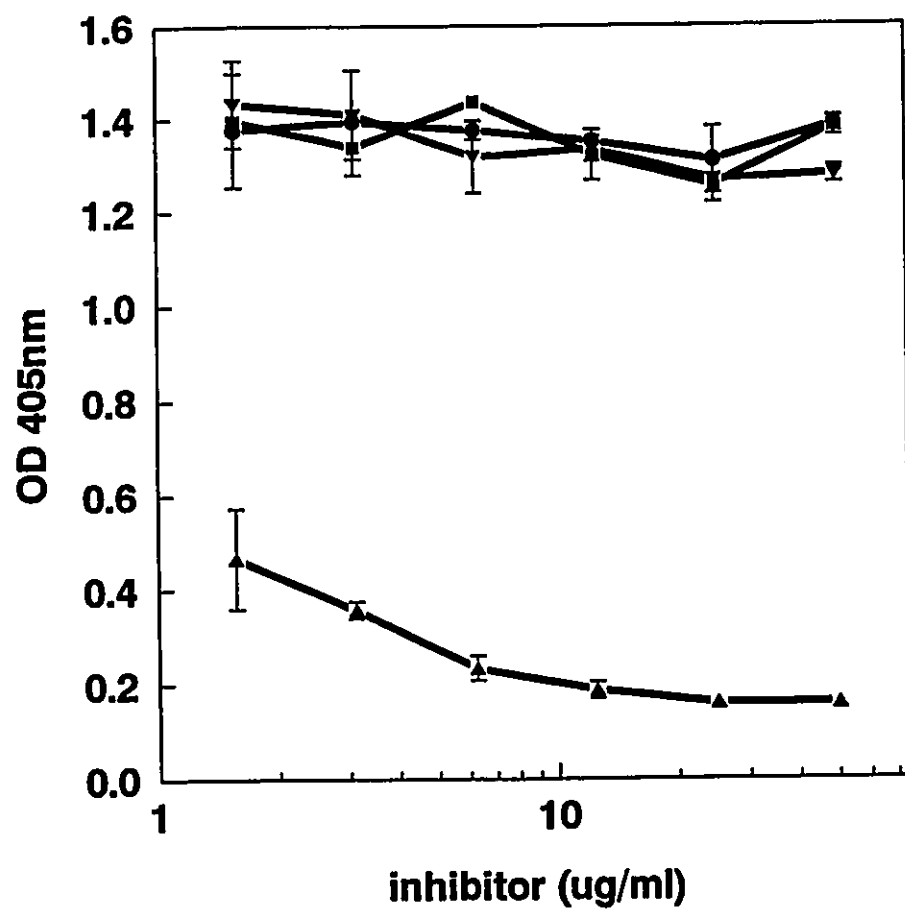


Figure 12. Inhibition of monoclonal IgG Fab anti-HCMV binding to microtitre plate-bound HCMV particles by lectin-specific sugars. Purified HCMV particles were coated onto microtitre plate wells as described in Figure 8. D-glucose (▼), D-mannose (●), N-acetylglucosamine (■) or unlabelled anti-HCMV antibody (▲) were serially diluted ($\times 1/2$) and mixed with 0.2 $\mu\text{g/ml}$ biotinylated monoclonal IgG Fab anti-HCMV. Triplicate samples of the solutions were incubated with HCMV coated in the wells, and bound biotinylated antibody was incubated with streptavidin alkaline phosphatase. Substrate was added and bound conjugate detected by measuring absorbance at 405 nm. Where no error bars are visible, standard deviation was too small to appear on graph.



Results - Part B

Detection of phosphorylated cellular proteins

Intracellular phosphorylation events induced by HCMV particles binding to the surface of fibroblasts were studied by examining ^{32}P -orthophosphate incorporation by phosphoproteins. Molecules involved in signal transduction processes, such as tyrosine phosphorylated receptors, PLC γ -1 and Fos were immunoprecipitated with specific antibodies.

The immunoprecipitation of phosphotyrosine-containing molecules with anti-phosphotyrosine antibodies indicated that tyrosine phosphorylation in cells stimulated with fetal calf serum was upregulated, but that no apparent differences existed between unstimulated cells and those stimulated with purified HCMV particles (Figure 13). Fetal calf serum stimulated cells contained higher levels of phosphoproteins migrating at 75 and 140 kDa at all time points, and contained transiently expressed phosphoproteins with apparent molecular masses of 16 and 20 kDa which appeared only at the 10 minute time point.

Tyrosine phosphorylation was also examined by Western blot analysis of whole cell lysates (Figure 14.). Tyrosine residues in HCMV-stimulated cells did not appear to be differentially phosphorylated relative to unstimulated cells. Fetal calf serum however, appeared to stimulate the phosphorylation of tyrosine in several

Figure 13. Immunoprecipitation of ^{32}P -phosphotyrosine-containing proteins from HFF cells stimulated with HCMV particles. Confluent HFF (P6-P9) in 6-well plates were incubated with 250-300 μCi /well of ^{32}P -orthophosphate for 45 min. Fetal calf serum (10% v/v final concentration) or purified HCMV particles (10^8 particles/well) were used to stimulate cells and negative control wells were unsupplemented. At 5, 10, 30 and 60 min the cells were cooled on ice and lysed with lysis buffer. Cell lysates were pre-adsorbed with Protein A Sepharose and incubated with 6 μl mouse anti-phosphotyrosine antibody for 16 h. Antigen-antibody complexes were separated from the lysate with Protein A Sepharose, were heated 3 min at 100°C and were electrophoresed by SDS-PAGE on 5-20% gels. The gels were dried and autoradiographed for 5 days. Molecular weight markers are indicated. Lane a) unstimulated cells, $t=5$ min, lane b) fetal calf serum-stimulated cells, $t=5$ min, lane c) HCMV-stimulated cells, $t=5$ min; lanes d) - f), same as a) - c), $t=10$ min; lanes g) - i), same as a) - c), $t=30$ min; lanes j) - l), same as a) - c), $t=60$ min. Arrows indicate proteins phosphorylated differentially in cells stimulated with fetal calf serum versus unstimulated cells. Molecular weight markers (kDa) are as indicated.

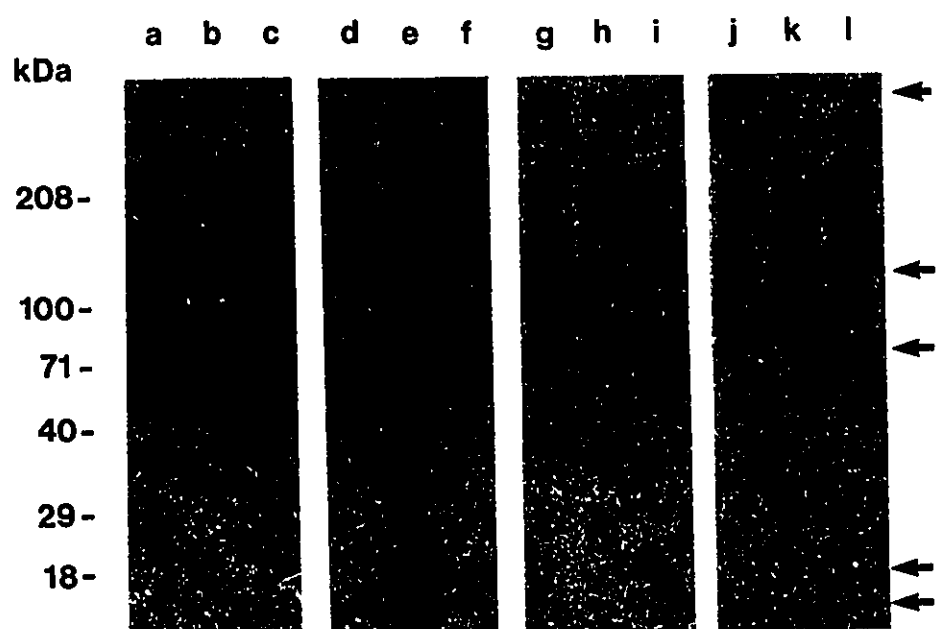
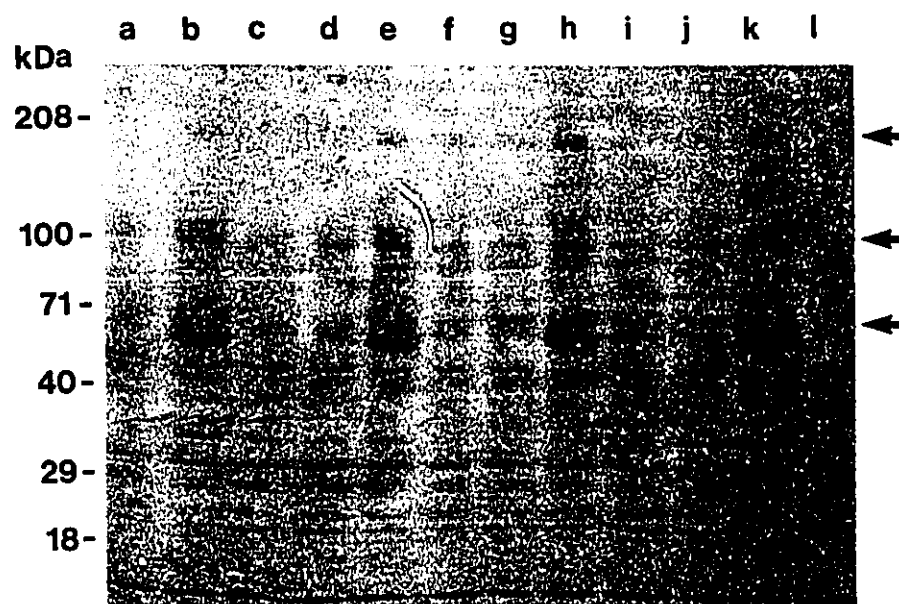


Figure 14. Identification of phosphorylated tyrosine residues in lysates of HFF cells by Western blot analysis. Confluent HFF monolayers in 6-well plates were stimulated with 10% fetal calf serum, HCMV particles or not stimulated. At the time points specified the cells were lysed, heated for 3 min at 100°C, electrophoresed by SDS-PAGE on a 5-20% gel and electrotransferred onto a nitrocellulose membrane. Phosphorylated tyrosine residues were detected with monoclonal anti-phosphotyrosine antibodies and horseradish peroxidase conjugated anti-mouse IgG. Blot was developed with chemiluminescent ECL substrate system. Lane a), unstimulated cells, t=5 min; lane b), cells stimulated with 10% fetal calf serum, t=5 min; lane c), cells stimulated with HCMV particles, t=5 min. Lanes d) - f), same as a) - c), t=10 min; g) - i), same as a) - c), t=30 min; j) - l), same as a) - c), t=60 min. Arrows indicate reactive bands present in fetal calf serum - stimulated cell lysates which were not observed in HCMV - stimulated or unstimulated cells. Molecular weight markers (kDa) are as indicated.



proteins. The relative molecular masses of these molecules is indicated with arrows on Figure 14.

Immunoprecipitation of Fos with specific antibodies indicated that the phosphorylation level of this molecule was not altered in response to HCMV particles, relative to unstimulated cells. Radiolabelled phosphoproteins immunoprecipitated with anti-Fos antibodies from cells stimulated with fetal calf serum demonstrated an increased level of ^{32}P incorporation in a 55 kDa molecular mass species (Figure 15.)

Radiolabelled PLC γ -1 immunoprecipitated with specific antibodies was not detected by autoradiography in cells stimulated with fetal calf serum, HCMV or in unstimulated cells.

Autoradiography of whole cell lysates electrophoresed by SDS-PAGE indicated that incorporation of ^{32}P -orthophosphate into HFF phosphoproteins in unstimulated cells did not appear to differ from cells stimulated with purified HCMV particles (Figure 16). The lysate from cells stimulated with fetal calf serum contained several protein species with increased ^{32}P content migrating at apparent molecular masses of 19, 28, 33, 70, 105 and over 208 kDa. The degree of phosphorylation of some of the molecules displayed a temporal dependence, as evidenced by the appearance of the 33 kDa species after 10 minutes, and the weakened intensity of the high molecular mass band (over 208 kDa) after 10 minutes.

Figure 15. Immunoprecipitation of ^{32}P -labelled Fos from HFF cells stimulated with HCMV particles. HFF cells were stimulated with fetal calf serum or purified HCMV particles as described in Figure 13. Cell lysates were pre-adsorbed with Protein A Sepharose and incubated with 10 μl mouse anti-Fos antibody for 16 h. Antigen-antibody complexes were separated from the lysate with Protein A Sepharose, were heated 3 min at 100°C and were electrophoresed by SDS-PAGE on 5-20% gels. The gels were dried and autoradiographed for 4 days. Molecular weight markers (kDa) are as indicated. Lane a) unstimulated cells, t=30 min, lane b) fetal calf serum-stimulated cells, t=30 min, lane c) HCMV-stimulated cells, t=30 min; lanes d) - f), same as a) - c), t=60 min; lanes g) - i), same as a) - c), t=90 min. Arrow indicates position corresponding to known apparent molecular weight of Fos (55 kDa).

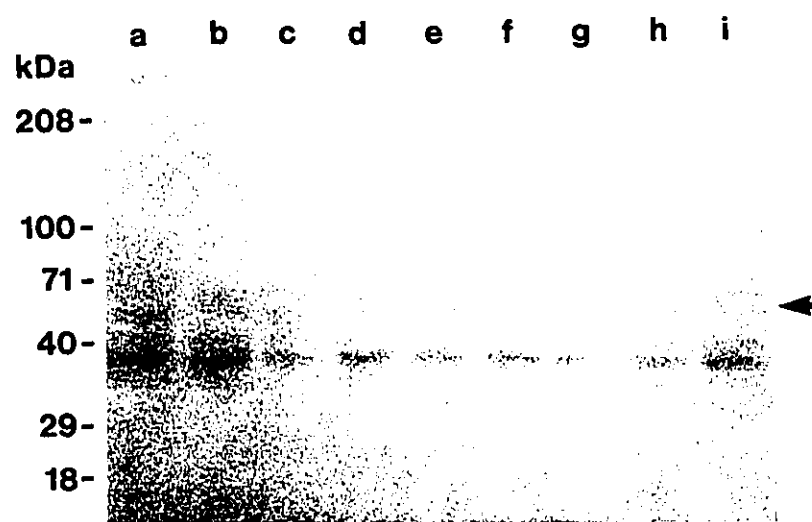
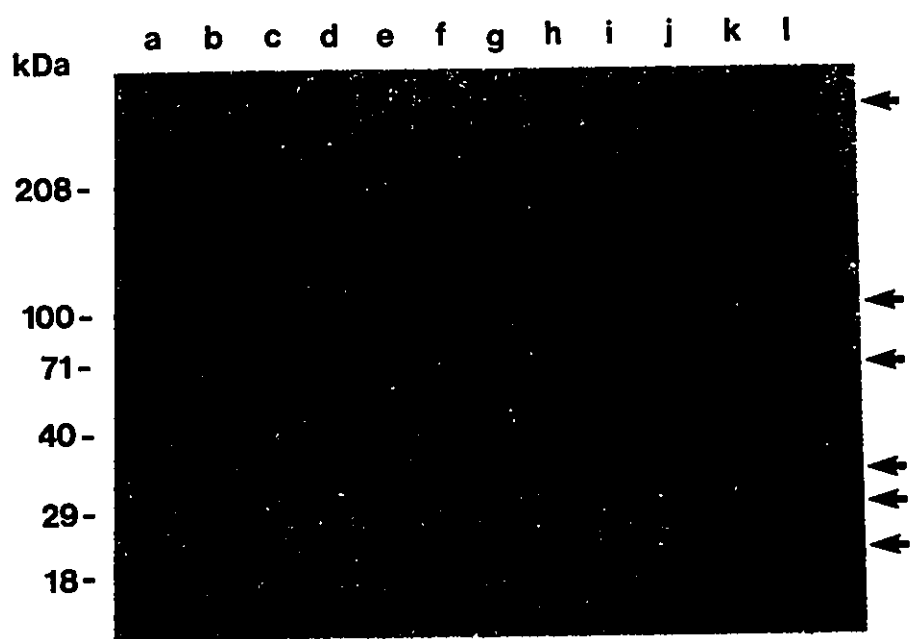


Figure 16. Detection of ^{32}P -labelled cellular proteins in HFF cells stimulated with fetal calf serum or purified HCMV particles. HFF cells were stimulated with fetal calf serum or purified HCMV particles as described in Figure 13. Cell lysate was diluted with 4x reducing sample buffer and electrophoresed on 5-20% SDS-PAGE gels. The gels were dried and autoradiographed 18 h. Molecular weight markers are indicated. Lane a) unstimulated cells, t=5 min, lane b) fetal calf serum-stimulated cells, t=5 min, lane c) HCMV-stimulated cells, t=5 min; lanes d) - f), same as a) - c), t=10 min; lanes g) - i), same as a) - c), t=30 min; lanes j) - l), same as a) - c), t=60 min. Arrows indicate proteins phosphorylated differentially in cells stimulated with fetal calf serum versus unstimulated cells. Molecular weight markers (kDa) are as indicated.



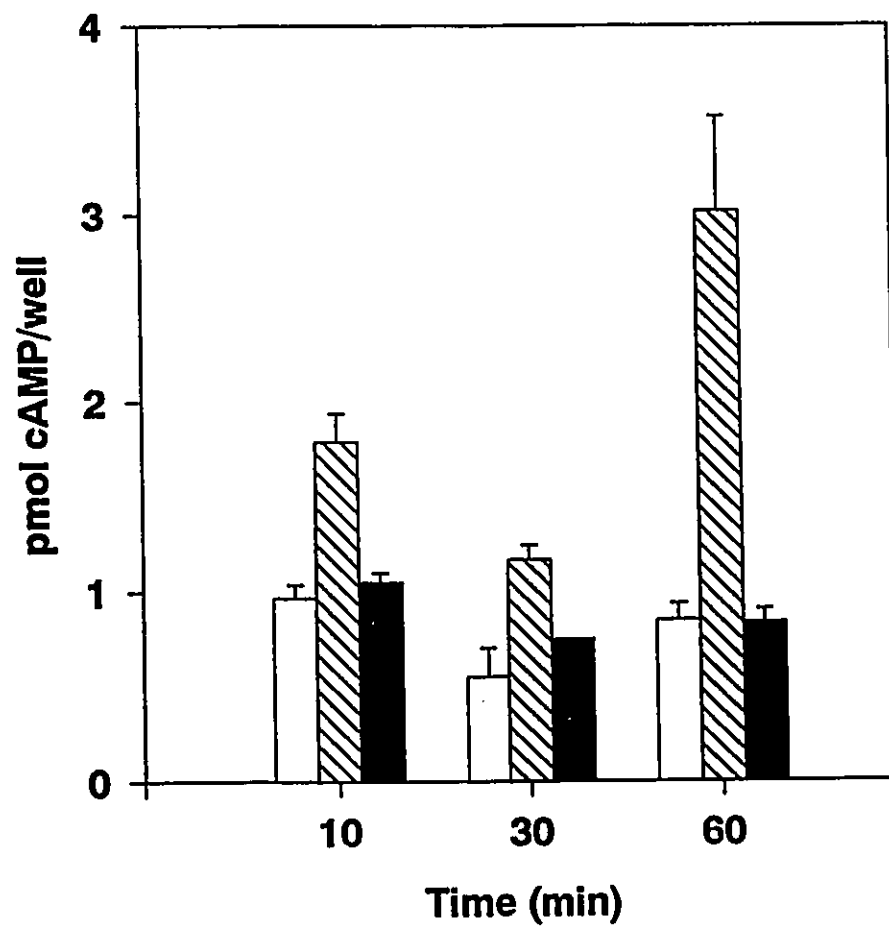
Intracellular cAMP concentrations

Cyclic AMP is an important secondary messenger molecule in many signal transduction pathways, and elevated cAMP levels in response to cell agonists indicate an activation process. Measurement of cAMP concentrations in ethanol extracts of lysates from cells stimulated with fetal calf serum, purified HCMV particles or unstimulated cells indicated that cAMP levels in HCMV-stimulated cells were 1.1-fold higher than in unstimulated cells after 10 minutes, 1.33-fold higher after 30 minutes, and 0.94-fold lower after 60 minutes (Figure 17). Cells stimulated with fetal calf serum contained cAMP concentrations 1.84-fold higher after 10 minutes, 2.2-fold higher after 30 minutes and 3.4-fold higher after 60 minutes. Absolute cAMP concentrations exhibited temporal variation as well. Levels of cAMP in unstimulated cells at 10 minutes were 0.95 pmol/well (6-well plate; confluent monolayer of HFF), 0.53 pmol/well at 30 minutes and 0.85 pmol at 60 minutes.

Membrane-bound Protein Kinase C activity

Protein Kinase C is also a central molecule in signal transduction pathways activated by growth factors, hormones and neurotransmitters. Membrane-associated PKC activity, as measured by ^{32}P incorporation into a peptide substrate, indicated that PKC activity in cells stimulated with purified HCMV particles increased 1.14-fold after 10 minutes, decreased 0.72-fold after 30 minutes and

Figure 17. Measurement of intracellular cAMP concentrations in HFF cells stimulated with HCMV particles. Confluent monolayers of HFF in 6-well plates were stimulated with 10% fetal calf serum (hatched bar), HCMV particles (solid bar) or were not stimulated (unfilled bar). At 10, 30 and 60 min cells were lysed, extracted with ethanol and cAMP was measured in triplicate by scintillation proximity assay (SPA, Amersham) on a β -counter (LKB). Where error bars are not visible, standard deviation was too small to appear on graph.



increased 1.69-fold after 60 minutes, relative to unstimulated cells. Cells stimulated with TPA exhibited a relative increase of 2.34-fold, 2.65-fold and 4.30-fold in PKC activity at 10, 30 and 60 minutes, respectively (Figure 18).

Figure 18. Measurement of Protein Kinase C (PKC) activity in HFF cells stimulated with HCMV particles. Confluent monolayers of HFF cells in 100 mm (55 cm²) cell culture dishes were stimulated with 1 μ M 12-O-tetradecanoylphorbol-13-acetate (TPA, hatched bars), HCMV particles (solid bars) or were not stimulated (unfilled bars). Membrane protein (3 μ g) was prepared from cell lysates and incubated with 9 μ M peptide substrate FKKSFKL-NH₂ and 0.5 μ Ci ³²P-ATP. Triplicate samples of reaction mixtures were pipetted onto P81 Whatman filter paper, which was washed three times and counted on a β -scintillation counter (LKB). Where no error bars are visible, standard deviation was too small to appear on graph.

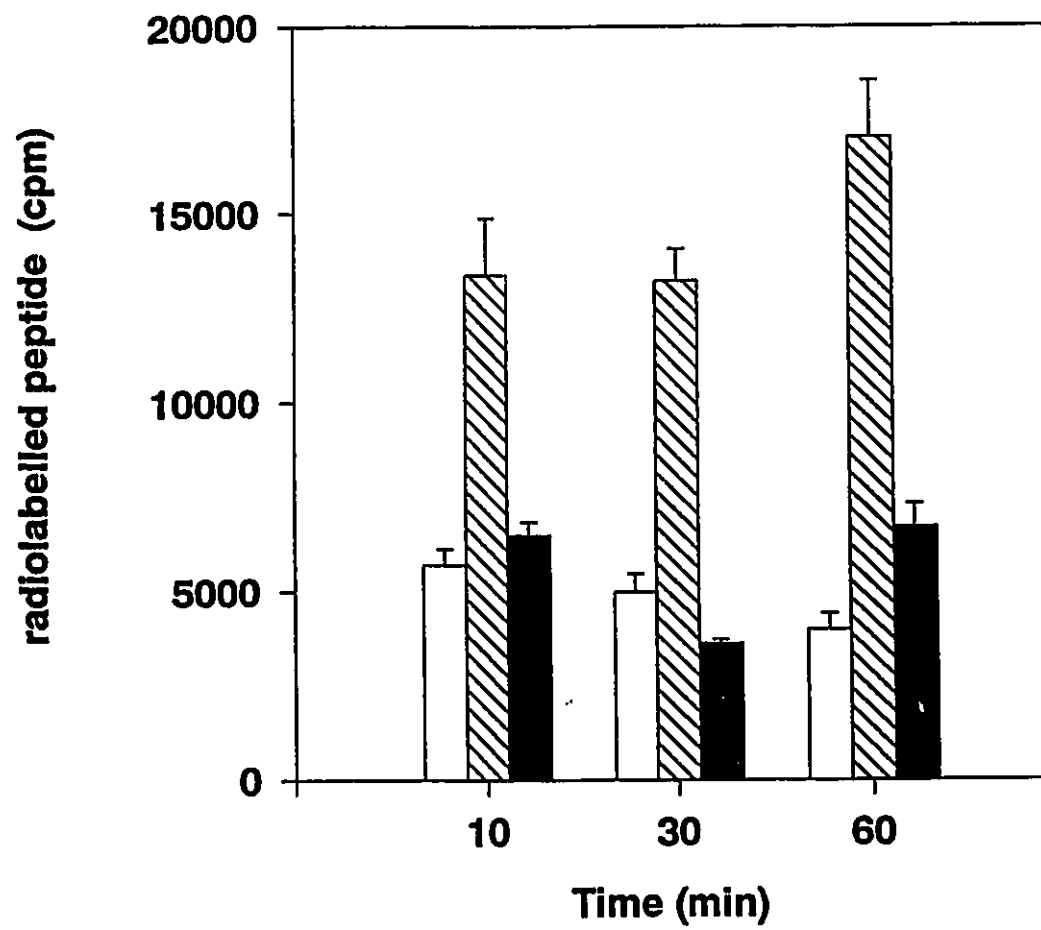
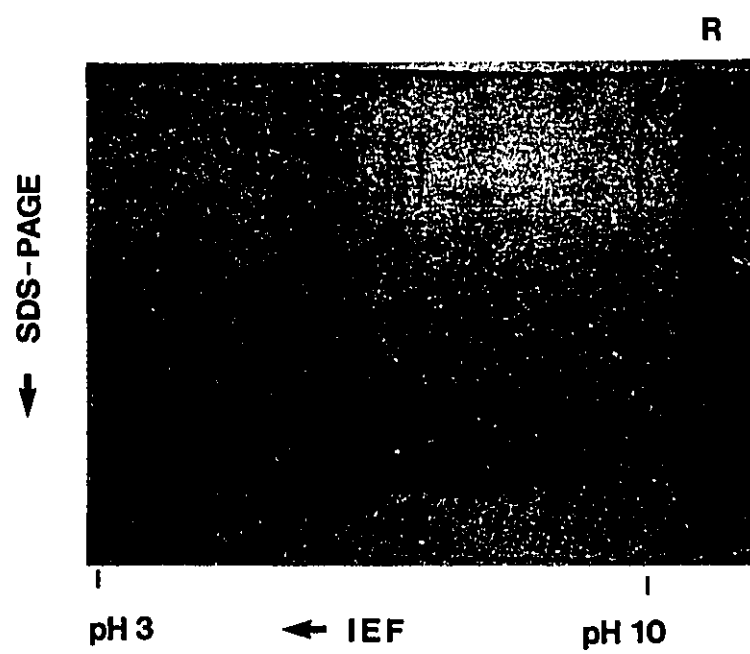


Figure 19. Analysis of HFF cell lysate by 2-dimensional electrophoresis. HFF cell lysate (25 μ l) was run on 2 mm isoelectric focussing (IEF) gels (pH 3-10) for 16 h at 400V, then for 2 h at 800V. The extruded gels were soaked in Laemmli sample buffer for 30 min, then electrophoresed onto 12% gels by SDS-PAGE. Gels were silver stained and dried. R) Reference, HFF lysate run on second dimension only.



DISCUSSION

The primary objective of the work described above was to identify and characterize viral and cellular molecules which are essential to the interaction between HCMV virus particles and cell surfaces. The first set of experiments examined the molecular interaction between a strongly neutralizing mouse monoclonal antibody and HCMV surface glycoproteins. These studies defined the specificity and binding characteristics of the antibody, and subsequently explored the biochemical properties of the target antigen.

The second set of experiments sought to identify cellular proteins which may play a role in the intracellular signal transduction process induced by virus particle binding to the cell surface receptor. This approach focused on examining the phosphorylation state or activation of known secondary messenger molecules such as phospholipase C γ -1 (PLC γ), cyclic AMP (cAMP) or protein kinase C (PKC). Defining the cell signalling pathways induced by virion binding to the cell surface would shed light on the mechanisms by which the neutralizing monoclonal antibody was blocking the infection process.

The two sets of experiments are discussed separately below, and the conclusions are presented in the overall framework of the experimental objectives. Lastly, recommendations for future studies are provided.

Discussion - Part A

A mouse monoclonal antibody, designated CMVB1 was previously developed by others (46). This antibody exhibits potent complement-dependent HCMV neutralizing activity in a plaque reduction assay (30). The observation that the antibody requires complement to neutralize the HCMV particles suggests that viral membrane damage or steric effects could be central to the mechanism of neutralization. Nonetheless, the potency of the antibody reflects the importance of the target antigen in the overall infection process. Characterizing the viral antigen would facilitate exploiting the purified antigen or components of the antigen for development of subunit vaccines or in assay systems designed to detect neutralizing antibodies. Commercially available immune globulin preparations demonstrate no correlation between ELISA anti-HCMV antibody titre and *in vitro* virus neutralization assay titre (47). An assay capable of identifying plasma with high-titre neutralizing antibodies would theoretically produce the most clinically effective blood product.

Subjecting HCMV virus particles to the physicochemical elements of heat, disulfide reduction and denaturation with SDS revealed that the viral epitope targeted by CMVB1 was completely disrupted by boiling the virions, and partially disrupted by denaturation with SDS and reduction with β ME (Figure 3). Treatment with β ME only did not alter the ability of CMVB1 to bind to the viral antigen, suggesting that the presence of SDS sufficiently altered the tertiary structure of the epitope to

permit β ME to reduce the disulfide bridge and decrease the apparent reactivity of the antibody. Even in the absence of SDS, heating the virions completely eliminated antibody recognition when β ME was present. These results strongly suggested that the epitope recognized by CMVB1 was conformational and that biochemical techniques such as western blotting, which rely on SDS and β ME to facilitate virus sample preparation, would be unsuitable for antigen-antibody studies. Therefore the approach taken was to permit antibody to interact with the native, undisrupted viral structure.

Lectin binding to viral glycoprotein was explored as a possible route to purifying HCMV surface antigens. Western blotting techniques were used to explore the lectin-binding properties of HCMV viral glycoproteins (Figure 4). As expected, the viral lysate exhibited a high degree of reactivity with six different lectins. Reactivity of viral antigen with CMVB1 antibody was observed to be very weak, with bands barely detectable at approximately 70 kDa and over 200 kDa. This result confirmed the unsuitability of the electrophoresis and electrotransferring methodology for studying the CMVB1 epitope. Lectin binding to viral glycoproteins did not appear to be affected by disulfide reduction and denaturation. This agrees with the results of the dot blot assay.

Immunoprecipitation of ^{35}S -labelled viral antigen with CMVB1 yielded a single band with an apparent molecular mass of 65-70 kDa. Although this technique proved

satisfactory, the length of time required for each experimental procedure (approximately 14 days) was restrictive. Furthermore, several steps in the protocol required the presence of SDS, which may have disrupted the epitope prior to incubation with antibody. In previous experiments we have demonstrated that the native conformational state of the epitope is essential for antibody reactivity. Consequently an alternative approach was selected.

A novel chemical crosslinking reagent, SASD, was chosen to produce covalently-bound and radiolabelled antigen-antibody complexes. Initially, a model antigen-antibody system was used to define experimental conditions and to validate the methodology. Whole molecule monoclonal anti-albumin was reacted with the N-hydroxysuccinimidyl (NHS) group of the ^{125}I -labelled crosslinker. NHS reacts with primary amine groups at physiological pH and ambient temperature, permitting minimal physical disruption to the biological properties of the antibody fragment. Subsequent incubation with albumin or a negative control (IgA₁) permitted the formation of antigen-antibody complexes (Figure 6). The negative control (lane 3) confirmed the specificity of the albumin-anti-albumin complexes detected (Panel A, arrows). Upon reduction, a newly radiolabelled species appears at a position on the gel corresponding to the reduced form of albumin (Panel B). This model experiment confirmed the process of radiolabelling the SASD, covalently binding the ^{125}I -SASD to the antibody, preserving the activity of the ^{125}I -SASD-linked antibody, and the crosslinking the antigen to the antibody by photoactivating the

second reactive group on the crosslinker. Reduction of the albumin-SASD-anti-albumin complex successfully transferred the ^{125}I radiolabel from the SASD-antibody to the antigen (Panel B).

In order to avoid the possibility of a bivalent antibody molecules crosslinking two antigen molecules and thereby unnecessarily complicating the interpretation of the autoradiographs, purified monovalent Fab fragments were used in all the crosslinking studies. Monoclonal anti-HCMV Fab fragments were reacted with ^{125}I -SASD, incubated with whole virus particles and covalently bound to the viral antigen by photoactivating the aryl azide group on the SASD molecule. The nitrene group generated by irradiating the crosslinker with long wave UV light is short-lived ($t_{1/2}=10^{-2} - 10^{-4}$ sec) (48) and highly-reactive, but non-specific. Therefore the heavy and light chain components of the Fab fragment are crosslinked, as well as Fab fragment and antigen. The intramolecular crosslinking which occurs accounts for the heavily radiolabelled non-reduced Fab fragment ($M_r=45$ kDa) and reduced heavy and light chain fragments ($M_r=23$ kDa).

In the model albumin-anti-albumin crosslinking system, the covalently bound antigen-antibody complex appeared to migrate at a molecular mass which approximated the sum of whole IgG ($M_r=150$ kDa) and albumin ($M_r=68$ kDa). This was not observed in the HCMV-anti-HCMV crosslinking experiments. When ^{125}I -SASD-linked Fab fragments were incubated with whole virus particles and

photoactivated, a covalent complex migrating at an apparent molecular mass of approximately 60 kDa was observed. However, under reducing conditions, which transfers the ^{125}I radiolabelled onto the antigen component of the ^{125}I -SASD-Fab-antigen complex, a radiolabelled species appears at an apparent molecular mass of 70 kDa (Figure 7, Panel B). It is possible that the ^{125}I -SASD-linked antigen-Fab complex migrates at a lower apparent molecular mass than would be expected on the basis of the arithmetic sum of the Fab fragment ($M_r=45$ kDa) and the reduced form of the antigen ($M_r=70$ kDa). The presence of a crosslinker could conceivably contribute to a more compact tertiary structure and hence an altered mobility in a Laemmli buffer system. The newly radiolabelled species is not present in the sample containing ^{125}I -SASD-Fab fragment only (lane 9), and is present at a higher concentration in the HCMV-infected cell lysate sample (lanes 5 and 12). It is possible that the absolute amount of antigen present in $35\mu\text{g}$ of HCMV-infected cell lysate is much greater than in 1.5×10^8 virus particles (31), or that the epitope itself is more accessible to Fab fragment in the infected cell lysate, thereby increasing the effective concentration of antigen-antibody complexes.

The apparent molecular mass (70 kDa) of the antigen radiolabelled in the crosslinking experiments agrees with the results of the immunoprecipitation of ^{35}S -labelled viral antigen. This suggests that both techniques have identified the antigen targeted by the neutralizing antibody CMVB1 as a 70 kDa molecule. Molecular mass values estimated from SDS-PAGE gels are only approximations

and it is possible that the HCMV antigen identified by crosslinking and immunoprecipitation is gp58, the immunodominant component of glycoprotein B (23,26,31,33,49,50).

Recent studies have demonstrated that the immunodominant domain in gp58, designated AD-1 (31), is a continuous sequence of 70 amino acids. The domain contains two cysteine residues, but the addition of β ME does not alter the capacity of monoclonal antibodies to bind to AD-1 (49). The epitope structure appears to be maintained in the presence of SDS and β ME, although it is unclear whether antibody recognition is affected. The study of the epitope did not involve heating the antigen in the presence of β ME or SDS. The fact that the AD-1 epitope was unaffected or partially affected by disulfide reduction and treatment with SDS correlates with the results of the dot blot experiment described in Results. However, these observations provide only a limited degree of support for the identify of the 70 kDa antigen isolated by immunoprecipitation and chemical crosslinking.

To confirm that CMVB1 bound to viral antigen in a saturable and specific manner, a microtitre plate binding assay was developed. Commercially available purified HCMV virus was bound to CMVB1 Fab fragment-coated microtitre plate wells. Biotinylated CMVB1 Fab fragments saturated the binding sites (antigen) on the microtitre plate (Figure 8). An optimal concentration of CMVB1 was subsequently

used in all binding studies. The possibility that viral glycoproteins were interacting with a mouse IgG domain other than the antigen binding site of the Fab fragment was also examined. A non-antigen binding site-specific interaction would also appear saturable in the binding assay described above. Non-immune mouse IgG Fab fragments and non-biotinylated CMVB1 Fab fragments were used to examine any competitive inhibition effect. Homologous unlabelled CMVB1 Fab fragments effectively inhibited the biotinylated CMVB1, whereas non-immune mouse IgG Fab fragments exhibited no inhibitory effect (Figure 9). This observation verified the antigen-specific binding property of the CMVB1 antibody.

Given that one of the objectives of the research project was to characterize an HCMV antigen suitable for use in an assay selective for HCMV-specific neutralizing antibodies, the specificity of the antibody had to be established. It has been established by others that sequence similarity exists between HCMV glycoproteins and other herpesviruses (51). CMVB1 has previously been shown not to cross-react with other herpesviruses when measured by immunofluorescence studies on infected cells (46). This observation was verified by competitive binding assays with purified Herpes Simplex Virus-1 and -2 (HSV-1, HSV-2) particles. Biotinylated CMVB1 Fab fragments were competitively inhibited by solution-phase HCMV particles from binding to microtitre plate bound HCMV particles. Equal concentrations of HCMV, HSV-1 and HSV-2, spanning a 2.1 log₁₀ range were utilized to allow for possible inhibition at higher HSV-1 and HSV-2 concentrations

due to a lower affinity of CMVB1 for non-homologous antigen. Virus particles exhibited no inhibitory effect on the binding of CMVB1 to the solid phase HCMV particles in the microtitre plate wells (Figure 10).

The lectin-binding data, described above, indicated a broad range of viral glycoprotein reactivity to a panel of biotinylated lectins (Figure 4). To explore the possibility that a lectin might block CMVB1 binding to HCMV surface glycoprotein antigen, competitive inhibition assays using wheat germ agglutinin, lentil lectin and concanavalin A (ConA) were carried out. It was postulated that partial or complete inhibition of CMVB1 binding to viral glycoproteins by a lectin could permit further characterization of the neutralizing epitope targeted by CMVB1. Lectin inhibition of CMVB1 binding could suggest that carbohydrate moieties are part of the viral epitope, or that glycosylation sites on the target antigen are in close proximity to the epitope and therefore bound lectin interferes with CMVB1 through steric hindrance.

The lectins chosen represent a range of carbohydrate-binding specificities. Wheat germ agglutinin, lentil lectin and ConA recognize N-acetylglucosamine, alpha-linked mannose, and both alpha-linked mannose and glucose, respectively. Under binding assay conditions, only Con A demonstrated any dose-dependent CMVB1-inhibiting properties (Figure 11). This suggests that carbohydrate moieties on the viral glycoprotein targeted by CMVB1 are sufficiently close to the neutralizing

epitope to cause glycoprotein-bound ConA lectin to partially obstruct the antibody Fab fragments. Since mouse IgG contains no carbohydrate on the heavy and light chain domains present in Fab fragments (52), the inhibitory effect of ConA must be assumed to be due to lectin-viral glycoprotein binding.

To establish whether carbohydrate moieties on the viral glycoprotein were components of the structure of the CMVB1 epitope, sugars specifically recognized by wheat germ agglutinin, lentil lectin, and ConA were evaluated for possible inhibitory effects on CMVB1 antibody. D-glucose, D-mannose, and N-acetylglucosamine were not found to exhibit any inhibitory effect on CMVB1 Fab fragment binding to viral glycoprotein. This suggested that those three carbohydrates did not directly constitute a part of the CMVB1 epitope. Since ConA binding to viral surface glycoprotein did exhibit an inhibitory effect on the binding of CMVB1 to its antigen, the carbohydrate to which the lectin bound was probably close to, but not part of, the epitope. This would suggest that ConA bound to a glycoprotein region and interfered with CMVB1 antibody binding by sterically hindering the Fab fragment. One instance of a carbohydrate-dependent neutralization epitope in HCMV has previously been reported (34). The immunodominant AD-1 domain of gp58 is also known to contain two N-linked glycosylation sites (49).

On the basis of the apparent molecular mass of the viral antigen identified by immunoprecipitation and crosslinking, there is a reasonable correlation with the published molecular mass of HCMV glycoprotein gB (gp58/130). The use of whole virus particles in the crosslinking studies and binding assays ensures that native surface molecules on the viral surface are the target antigen for CMVB1. The ability of ConA lectin to partially inhibit CMVB1 binding to its epitope strongly suggests that carbohydrate moieties are associated with the viral antigen. The sensitivity of the viral epitope to heat but not to disulfide reduction and only partially to denaturation also correlate with the published physicochemical properties of AD-1, the immunodominant domain on the gp58 (49). Considerable consensus exists that gp58 is the immunodominant viral antigen targeted by neutralizing antibodies (33,53,54).

Within the gp58 molecule, a 70 amino acid sequence (residue 565 to residue 635) termed AD-1 is believed to form a conformational epitope targeted by the majority of neutralizing antibodies (31,49). Human sera with high neutralization titres (>1:128) by plaque reduction assay exhibited a 96% positivity rate with an ELISA using a recombinant gp58 fusion protein encompassing the AD-1 domain (29). Sequence analysis of 33 clinical isolates has revealed a high degree of conservation of the AD-1 epitope (54). Previous evaluation of CMVB1 had shown that the antibody recognized 15 clinical isolates when tested by immunofluorescence and did not recognize HSV-1, HSV-2, Varicella Zoster virus

(VZV), or Epstein-Barr virus (EBV) (46).

Although the data described above does not conclusively identify a viral epitope for CMVB1, there is considerable correlation with the observed properties of the AD-1 epitope on gp58. The novel approach of chemical crosslinking supports the previously reported molecular mass approximations (46). In addition, the binding assays developed as described above confirmed the HCMV specificity of CMVB1 and the glycoprotein nature of the viral antigen. Future studies could include sequence analysis of the CMVB1 target antigen isolated through chemical crosslinking or immunoprecipitation. This would permit a definitive identification of the viral molecule. It is noteworthy that others have pursued the development of an immunoassay selective for HCMV-neutralizing antibodies with purified gp58 antigen (29). The rationale provided by those investigators is very similar to that defined at the outset of the work described in this thesis.

DISCUSSION - PART B

The second series of experiments described above (Part B) employed molecular approaches to examine the interaction between virus particles and host cells. However, the biochemical events investigated have generally been considered to be signal transduction events and therefore associated with cell responses to growth and differentiation factors or hormones (reviewed in (55)).

The intent of these studies was to identify any HCMV-mediated intracellular signalling event which could be exploited as a tool for the further characterization of anti-HCMV therapeutics, including neutralizing antibodies. A signal transduction pathway activated by HCMV particle binding to cells could be used as a gauge to measure neutralizing activity of HCMV-specific antibody. Characterization of such pathways could potentially assist in identifying antiviral therapeutics as well.

It is outside the scope of this thesis to discuss signal transduction events in detail. Generally, the signalling process is initiated by an extracellular ligand binding to a cell surface molecule, subsequent cytoplasmic amplification and transmission of the signal by secondary messenger molecules, and culminates in the activation of nuclear proteins which regulate gene expression. In this hierarchical view of events, activation at the cell membrane initiates "downstream" signal transduction processes, which ultimately lead to cell activation and proliferation.

Intracellular signalling pathways which lead to growth and proliferation responses such as those induced by growth factors or hormones are broadly classified as transmembrane receptor tyrosine kinase, phosphatidylinositol-calcium cascade or cyclic AMP (cAMP)-mediated (55). Others have shown that HCMV infection generates cellular physiological responses similar to those observed for growth factors (56). Furthermore, viral particles induce rapid upregulation of messenger ribonucleic acid (mRNA) for cellular oncogenes *myc*, *jun* and *fos* (18,35). These observations suggested that HCMV particles were activating a cell-signalling pathway at the cell membrane which was leading to a downstream mRNA upregulation. The relatively rapid (20 to 60 min post-exposure) increase in mRNA levels even when HCMV particles inactivated by UV irradiation were used as agonists further implicated a cell membrane-bound molecule(s) in the initiation of a signalling process. In order to probe which of the three types of signalling pathways might be involved, a central molecular species from each was investigated for evidence of activation.

Tyrosine phosphorylation occurs only rarely in normal tissue, and phosphotyrosine residues account for only 0.03% of all phosphorylated amino acids (57). Tyrosine kinase substrates typically exhibit a 10-fold increase in phosphate content upon activation. This includes receptor tyrosine kinases, which frequently autophosphorylate upon ligand binding. Receptors for insulin, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) exhibit ligand-induced

autophosphorylation (reviewed in (39)). Other known substrates for receptor tyrosine kinases are phospholipase C γ -1 (PLC γ -1), phosphatidylinositol-3-kinase (PI-3K) and ras GTP-ase activating proteins, all of which are secondary messenger molecules.

In this context, any cellular proteins which are phosphorylated specifically by tyrosine kinases activated by HCMV particle binding could be secondary messengers or cell receptors. Several putative receptors for HCMV have been identified, including a 30 kDa and 93 kDa species, but the identity of the molecule remains unknown (58-62). In order to identify any newly phosphorylated tyrosine residues arising from HCMV stimulation of cells, ^{32}P -labelled phosphotyrosyl-proteins were immunoprecipitated from HFF lysates stimulated with HCMV particles. No differences were observed between cell monolayers stimulated with serum-free medium or with HCMV (Figure 13). Cells stimulated with fetal calf serum showed marked differences, with several molecular species demonstrating an increase in ^{32}P content. There were transiently ^{32}P -phosphorylated species present at 10 minutes which were undetectable earlier or later, demonstrating the temporal nature of phosphorylation/dephosphorylation. Dephosphorylation by phosphatases is known to play a critical role in the overall regulatory balance (63,64).

Detection of tyrosine-phosphorylated proteins in stimulated cells was also undertaken by Western blotting (Figure 14). Phosphotyrosine residues identified with this approach could not be distinguished as the products of de novo phosphorylation. Unlike the previously described experiment, in which only tyrosine residues with newly-incorporated ^{32}P could be identified, the immunoblotting procedure identified all existing phosphotyrosine residues. Nonetheless, no significant differences were observed between unstimulated cells and cells stimulated with HCMV. Several phosphotyrosyl-proteins were detected in the fetal calf serum-stimulated cells.

Immunoprecipitation of ^{32}P -labelled phosphotyrosyl-protein and Western blot analysis of whole cell lysates with anti-phosphotyrosine antibodies failed to identify a tyrosine kinase substrate phosphorylated specifically in response to cell stimulation by HCMV particles. The results suggest that under these experimental conditions HCMV does not initiate any detectable signal transduction process through tyrosine kinase activation.

A second major signalling pathway relies upon fluctuations in cAMP levels. Cyclic AMP concentrations are elevated by adenylate cyclase, which is regulated by a membrane bound G-protein complex. When the inactive form of the complex interacts with a receptor-ligand complex, a GTP molecule is released, and the α subunit of the G protein binds to adenylate cyclase (55). The activated adenylate

cyclase converts adenosine triphosphate (ATP) to cAMP, which in turn activates several cAMP-dependent protein kinases (PKAs). The exact nature of the PKA substrates remains unclear. Nonetheless, intracellular cAMP levels can be measured directly, and this approach was taken to detect possible HCMV-induced activation of a cAMP signalling pathway.

Intracellular cAMP concentrations in HCMV-stimulated cells were compared to cAMP levels in unstimulated cells or cells treated with 10% fetal calf serum (Figure 17). No significant differences between the cAMP levels in unstimulated and HCMV-stimulated cells were observed, while the fetal calf serum treated cells demonstrated increases ranging from 84% to 340%. Absolute cAMP levels appeared to drop in both stimulated and unstimulated cells after 30 minutes, and the reason for this is unclear. At 60 min the cAMP levels rebounded to approximately the same concentrations as measured at 10 min, excepting the fetal calf serum-stimulated cells, which exhibited a dramatic increase, likely due to the presence of growth factors. Given that cAMP levels in HCMV stimulated cells are nearly identical to, or within the experimental error range of, the unstimulated cells, there does not appear to be any evidence of virus-specific induction of cAMP-mediated signal transduction pathways.

The third major signalling pathway investigated is the phosphatidylinositol-calcium cascade. Ligand-receptor complexes, acting through an intermediary G protein,

activate membrane-bound phospholipase C- γ , which cleaves phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (65). DAG dramatically alters the affinity of protein kinase C (PKC) for Ca^{2+} ions, thereby activating the enzyme. PKC, a serine-threonine kinase, is known to act on many substrates (65) and is believed to play a central role in pathways regulating gene expression (55).

Initial attempts to detect an activation of this pathway centred on PLC- γ . Activation of PLC- γ is believed to occur through phosphorylation (39), and immunoprecipitation of ^{32}P -labelled PLC- γ from stimulated cell lysates was attempted. The signal strength of immunoprecipitated radiolabelled enzyme proved to be too weak for useful evaluation, and an alternative approach was sought. Utilizing an assay system developed by others (44), membrane-bound PKC activity was measured directly. Since only membrane-associated PKC is active, this procedure avoids erroneous results generated from inactive cytosolic PKC being rendered active under assay conditions.

PKC activity in HCMV-stimulated cells after 10 and 30 minutes was nearly the same or lower than activity in unstimulated cells (Figure 18). However, after 60 minutes, PKC activity in HCMV-stimulated cells was 1.7-fold higher than in unstimulated cells. PKC activity increases of 1.6 to 1.7-fold are considered a positive indication of cell stimulation (J. Whitfield, personal communication). These

results, therefore, suggest that HCMV particles induce a PKC-dependent cell signalling pathway within 60 minutes of exposure to the virus. These results are preliminary and require additional data to verify that the phosphorylation detected in the assay system can be blocked by PKC inhibitors. Interestingly, others have shown that PKC inhibitors can block the entry of enveloped viruses, including HSV-1 into human embryo fibroblasts (66). Furthermore, one of the substrates for PKC is believed to be Fos, the protein product of the oncogene *c-fos* (reviewed in (67)), although evidence for its phosphorylation by PKA is strong. Boldogh et al showed that mRNA levels for Fos in cells stimulated with purified HCMV or fetal calf serum were increased 12-14 times over levels in mock infected (unstimulated) cells. This upregulation occurred within 20-60 minutes of exposure to HCMV. Fos is known to repress its own transcription through phosphorylation of its C-terminus, and stimulation of cells with TPA or fetal calf serum increases the phosphorylation of Fos. Temporally, this suggests that Fos is phosphorylated through a mechanism which acts in response to increased transcription, since mRNA levels are upregulated quickly.

Immunoprecipitation of ^{32}P -labelled Fos from cell lysates indicated that Fos was phosphorylated in response to fetal calf serum but not in response to HCMV particles (Figure 15). This effect was observed at 30 and 60 minutes post-exposure to agonist. Increased phosphorylation of Fos in cells stimulated with fetal calf serum would agree with a regulatory mechanism for downregulation of

transcription. However, despite published reports of increased Fos mRNA after stimulation with HCMV (35), phosphorylation of Fos was not observed in HCMV-stimulated cells.

The increase in PKC activity observed in HCMV-stimulated cells suggested that newly phosphorylated substrates for PKC might be detectable. The wide range of known PKC substrates (65) dictated a more general approach to identification of molecular species phosphorylated in response to HCMV stimulation. Whole cell lysates of HFF monolayers stimulated in the presence of ^{32}P radiolabel were electrophoresed. While increased phosphate incorporation was observed in cells activated with fetal calf serum, no proteins appeared to be phosphorylated differentially in cells stimulated with HCMV particles. The large number of phosphoproteins observed under these experimental conditions led to the use of a two dimensional gel electrophoresis system in an attempt to exploit the greater resolution obtained (Figure 19)(68). However, the limited number of samples which could be evaluated and the relative complexity of comparing gels rendered this approach inadequate.

Summary

The data described above suggest that HCMV induced cell activation does not operate through tyrosine kinase or cAMP-mediated pathways, but does lead to increased PKC activity. Although increased phosphorylation of Fos was not observed, these preliminary data offer several possibilities for future experiments. The specificity of the PKC assay results would require additional assays in the presence of PKC inhibitors. Defining a kinetic profile of PKC activation by including several additional timepoints would assist in correlating the temporal components of downstream c-fos mRNA level increases and Fos phosphorylation. Developing an alternative approach to the study of PLC- γ phosphorylation could confirm this pathway as the route of DAG generation and subsequent PKC activation. Confirmation of PLC- γ involvement would implicate a G-protein intermediary between a cell surface receptor and the phospholipase.

It would also be intriguing to study the effect of HCMV neutralizing antibodies on the cell activation process. Those antibodies which simply prevent virus from binding to cell receptors would presumably also prevent any cell signalling from occurring. Those antibodies which do not block binding but still neutralize may not prevent the signal transduction process, but must interfere in a subsequent step which is essential to productive infection.

The work described above has provided some additional clues with respect to the

process of HCMV infection in fibroblasts. Although these experiments utilize laboratory strains of virus adapted to a cell line with limited clinical relevance, the observations noted may permit some inferences to be made to the issue of HCMV infection. The methodologies utilized in the characterization of HCMV-neutralizing antibody CMVB1 may also prove useful in the study of other virus-cell systems.

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