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THE UNIVERSITY OF ALBERTA

STUDIES ON THE STRUCTURE AND IMMUNOLOGY OF HUMAN HERPESVIRUSES

by

MARK E. REDMOND

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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DATED 10 Oct 1987

O'er ladies lips, who straight on kisses dream
which oft the angry Mab with blisters plagues,
Because their breaths with sweetmeats tainted are

Shakespeare "Romeo and Juliet" Act 1 Scene V

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled STUDIES ON THE STRUCTURE AND IMMUNOLOGY OF HUMAN HERPESVIRUSES submitted by MARK J. REDMOND in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Supervisor

External Examiner

Date 7th October 19 87

To my family.

ABSTRACT

Herpesviruses represent one of the major public health problems in the world today. It is probable that every individual carries one or more of the six human herpesviruses in a latent state. The maladies caused by these viruses in their active, replicating form range from mild or asymptomatic disease to recurrent skin lesions, keratoconjunctivitis, lymphoma, encephalitis or congenital defects in the newborn.

This thesis describes investigations into the structure and immunology of two members of the herpesviridae, human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1). In the case of HCMV the protein composition of the nucleocapsid has been analyzed, and it was determined that 4 out of more than 28 proteins present in the nucleocapsid are of cellular origin. One nucleocapsid protein of molecular mass 64 kD, was found to be a strong immunogen in mice. Evidence is presented that this protein is glycosylated and processed into proteins of 90, 27 and 14 kD during virion maturation.

The human immune response to HCMV has been examined through the generation of human monoclonal antibody secreting cell lines. These were obtained by exposure of human peripheral B cells to virions and subsequent transformation with Epstein-Barr virus. Several of the human IgG monoclonal antibodies produced were specific for HCMV proteins of virion molecules mass 66 and 95 kD, and neutralized virus.

In the studies involving HSV-1, synthetic peptides were used to map linear antigenic sites on the virion envelope glycoprotein D. Polyclonal antisera from rabbits immunized with peptide-carrier protein conjugates were analyzed for reactivity with HSV-virions in ELISA and neutralization tests. These results defined 7 surface sites on glycoprotein D, 4 of which are recognized by virus neutralizing antibodies. The neutralization sites of sequences 2-21 and 267-276 may be of importance in the development of an anti-HSV vaccine.

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LIST OF ABBREVIATIONS

AET	2-aminoethylisothiouronium bromide hydrobromide
AIDS	Acquired Immune Deficiency Syndrome
B Lymphocyte	Bursal lymphocyte
BB gly	(p-benzoylbenzoyl) glycine
BB lys	N^{ϵ} (p-benzoylbenzoyl) lysine
BOC	butyloxycarbonyl
bp	base pair
BSA	bovine serum albumin
CPE	cytopathic effects
CR	complement receptor
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
E+	AET Erythrocyte rosetting cell (T cell)
E-	non-rosetting cell with AET Erythrocytes (B cells, monocytes, *etc.*)
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked Immunosorbant Assay
Fc	complement binding region of an immunoglobulin molecule
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
gp	glycoprotein
HAT	hypoxanthine-aminopterin-thymidine medium
HCMV	human cytomegalovirus
HFL	human fetal lung
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

HSV	herpes simplex virus
Ig	immunoglobulin
I.P.	intraperitoneal
KLH	keyhole limpet hemocyanin
M_r	apparent molecular mass
MAC	membrane attack complex
MEM	Eagle's minimum essential medium
MHC	major histocompatibility complex
MOPC	MOPC 315.43 mouse myeloma cell line
NK	natural killer lymphocyte
NP-40	nonidet-P40 detergent
OK T3	monoclonal antibody specific for all T cells
OK T4	monoclonal antibody specific for T helper inducer cells
OK T8	monoclonal antibody specific for T cytotoxic/suppressor cells
OK IA	monoclonal antibody specific for all activated lymphocytes
PBS	phosphate buffered saline
pfu	plaque forming unit
PHA	phytohemagglutinin
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SP2	SP2/O-Ag14 mouse myeloma cell line
SRBC	sheep red blood cell
T lymphocyte	thymus derived lymphocyte
T_H	helper T lymphocyte
T_S	suppressor T lymphocyte
Tris	tris(hydroxymethyl)amino methane

CHAPTER I

OVERVIEW: HERPESVIRUSES AND IMMUNE RESPONSES

History and Classification. The word "herpes" has been used in medicine for over two millennia. In the time of Hippocrates (460 – 377 BC) the word *ερπης* meaning "a creeping" was used to describe conditions as diverse as eczema and cancer of the skin (Beswick, 1962). It was not until the nineteenth century that the modern notion of herpes began. Willan and Bateman restricted the use of the term "herpes" to conditions characterized by the appearance of localized groups of vesicles, a short self-limiting course of infection, and the absence of severe symptoms (Bateman, 1813). The species of herpes classified were zoster, labial herpes and genital herpes, also included were ringworm (*Herpes circinatus*) and erythema multiforme (*Herpes iris*). The latter infections were considered to be species of herpes as late as 1880. The break from nosological virus classification came with the introduction of new techniques of virus isolation and purification which, in the early 1950's saw the introduction of virus groupings on the basis of physicochemical data. Aiding the changes in virus classification was the introduction of negative staining for electron microscopy (Brenner and Horne, 1959). This had three immediate effects:

1. Virus particles could be characterized with respect to size, shape, surface structure and sometimes symmetry.

2. These properties could be determined for viruses irrespective of their host, be it plant, animal or bacterium.

3. Large numbers of virus strains could be examined.

By chance, herpes simplex virus was the first animal virus to be examined by this technique. Wildy *et al.* (1960) described two basic types of herpesvirus particles,

distinguished by the presence or absence of an outer coat or envelope. The naked particle or capsid showed varying degrees of angularity, some appearing hexagonal whilst others were almost spherical. The envelope in these negatively stained preparations was frequently distorted and hence had a variable shape and size. Most envelopes contained only one capsid, but occasionally two or more capsids were seen inside one envelope.

Whilst the virions of various herpesviruses cannot be differentiated by electron microscopic examination, they are readily differentiated on the basis of biological properties, immunological characteristics, and the size, base composition and genetic arrangement of their DNA's (Table 1). Roizman (1982) lists some 89 members of the family Herpesviridae with hosts ranging from fungi to man (Nahmias, 1974). Herpesviridae has been further divided into three subfamilies - the Alphaherpesviridae, the Betaherpesviridae and the Gammaherpesviridae.

Members of the Alphaherpesviridae are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells and capacity to establish latent infection primarily in the sensory and autonomic ganglia of the host. This subfamily includes the genera *Simplex-virus* (HSV-1, HSV-2, and bovine mammilitis virus) and *Poikilovirus* (pseudorabies virus, varicella-zoster virus and equine herpesvirus-1).

A non-exclusive characteristic of members of the Betaherpesviridae is a restricted host range. Their reproductive cycle is long and infection progresses slowly in culture. Infected cells frequently become enlarged or fuse together (cytomegalia). The virus can be maintained in latent form in secretory glands, lymphoreticular cells, kidney, lung and other tissues. The subfamily contains the genera, *Cytomegalovirus* and *Muracytomegalovirus*.

The experimental host range of the members of Gammaherpesviridae is limited to the family or order to which the natural host belongs. *In vitro* all members replicate in lymphoblastoid cells and some also cause lytic infections in epithelial and fibroblastic

TABLE 1.

Genomic properties of the human herpesviruses

Designation	Common name	G+C (mole %)	Genome Mol.mass $x10^{-6}$
Human herpes virus 1	Herpes simplex virus 1	67	96[a]
Human herpes virus 2	Herpes simplex virus 2	69	96[a]
Human herpes virus 3	Varicella-zoster virus	46	79-100[b]
Human herpes virus 4	Epstein-Barr virus	59	114[c]
Human herpes virus 5	Cytomegalovirus	57	145[a]
Human herpes virus 6	Human B-Lymphotropic virus		72-120[d]

[a] Characterized by inverted reiteration of sets of sequences internal to both termini, as well as reiteration of a subset of these sequences at both termini in the same orientation (Sheldrick and Berthelot, 1975; Wadsworth *et al*., 1975.).

[b] Characterized by inverted reiteration of a set of sequences internal to one terminus, as well as by reiteration of a subset of these sequences in the same orientation at the other terminus(Ben-Porat *et al*., 1979.).

[c] Characterized by multiple reiteration of one set of sequences in the same orientation at both termini as well as by internal tandem reiterations of other sets of sequences.(Raab-Traub *et al*., 1980)

[d] Genomic structure currently unknown. Human B-lymphotropic virus (HBLV) was first isolated from cultivated B cells in 1986 (Salahuddin *et al*.; Josephs *et al*.) Morphologically the virus resembles other herpesviruses, for example herpes simplex virus, but is serologically distinct from all other human herpesviruses.

cell lines. Viruses in this group are specific for either T or B lymphocytes. In the lymphocyte, infection is frequently arrested at either a prelytic or a lytic stage, but without the production of infectious progeny. Latent virus is frequently demonstrated in lymphoid tissue. The subfamily contains three genera, *Lymphocryptovirus* (Epstein-Barr virus, human B-lymphotropic virus), *Thetalymphocryptovirus* (Marek's disease virus) and *Rhadinovirus* (Herpes ateles, Herpes saimiri).

Virion Structure. The herpesvirion consists of four structural elements. An electron-opaque core; an icosadeltahedral capsid that encloses the core; an electron-dense, asymmetrically distributed material abutting the capsid designated as the tegument; and an outer membrane, or envelope, which surrounds the capsid and tegument (Figure 1).

The core of the native virion contains the viral DNA in the form of strands coiled around a proteinaceous torus (Furlong *et al.* 1972). Viral proteins have been reported within the core (Gibson and Roizman, 1972; Roizman and Furlong, 1974) and may constitute the toroid structure or be bound to the DNA as it is packaged within the capsid. All herpesvirus DNA consist of linear DNA duplexes. The structural organization of the DNA from HSV and HCMV is typical of alphaherpesviridae. These molecules consist of two segments, the long unique and the short unique segment bracketed by inverted repeat sequences. The long and short segments comprise approximately 82% and 18% of the genome respectively. The size of the HCMV DNA, approximately 230 kbp is considerably larger than that of other herpesviruses.

The capsid is composed of protein subunits (capsomers) which are icosahedrally arrayed, with five capsomers along each edge of the triangular facets of the icosahedron. In the notation of Casper and Klug (1962) the lattice has a triangulation number (T) of 16, with the 162 capsomers of herpes virus resulting from hexamer (150) and pentamer (12) clustering of the fundamental structure units.

The tegument is a term introduced by Roizman and Furlong (1974) to describe the material between the capsid and envelope. The thickness of the tegument may vary

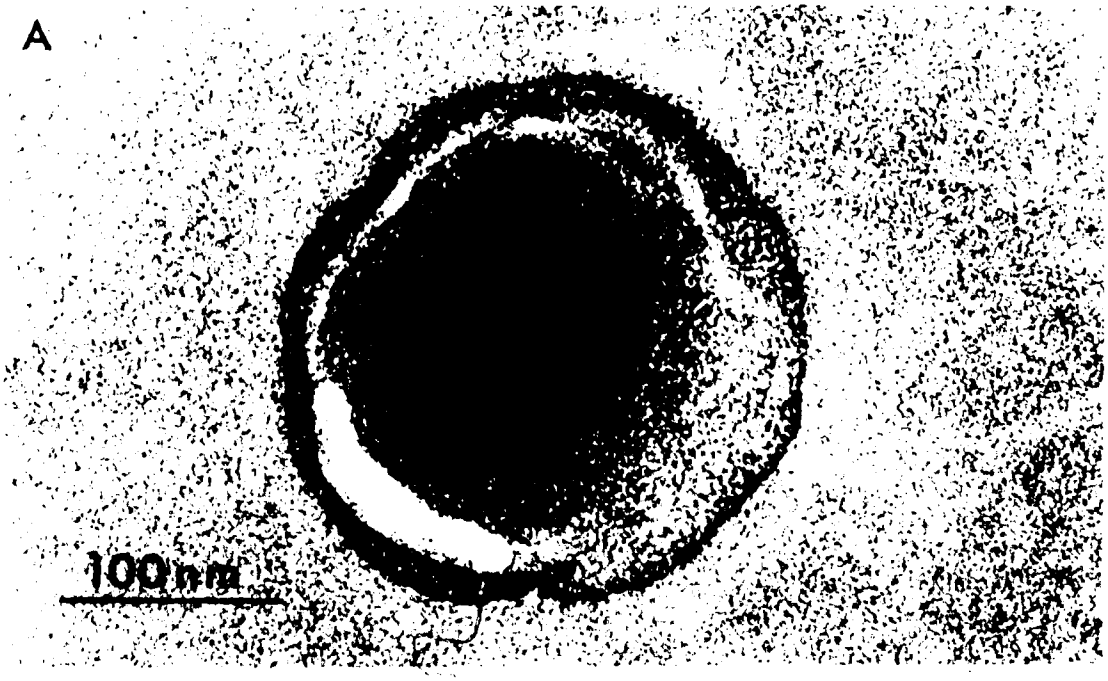
FIGURE 1

HERPESVIRUS STRUCTURE

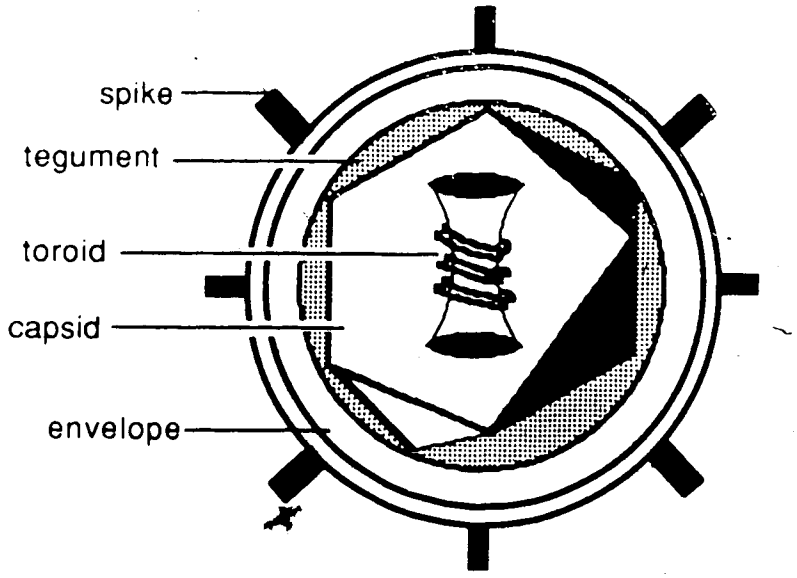
A) The photograph shows an electron micrograph of the virion of human cytomegalovirus; the envelope, tegument and capsid are clearly visible. The preparation was negativelv stained with 2% sodium phosphotungstate (pH 7.0).

B) A model of the herpesvirus virion illustrating the position of the major virion components. Based on Figure 1A and reference by O'Callaghan and Randall, 1976.

A



B



depending on the location of the virion within the infected cell; when the amount is variable, there is more of it in virions accumulating in cytoplasmic vacuoles than in those accumulating in the perinuclear space (Hong *et al.*, 1973). Available evidence suggests that the amount of tegument is more likely to be determined by the virus than by the host (McCombs *et al.*, 1971).

Electron microscopic studies on thin sections of the herpesvirion have shown that the envelope has a trilaminar appearance (Epstein, 1962). The envelope appears to be derived from patches of altered cellular membrane (Asher *et al.*, 1969). That the integrity of the envelope is essential for virus infectivity was demonstrated by the observation that infectivity was diminished by treatment with organic solvents or detergents (Spear & Roizman, 1972). The surface of the herpesvirus envelope contains numerous protrusions or spikes composed of proteins and/or glycoproteins, which are shorter than those appearing on the surface of many other enveloped viruses *e.g.*, Semliki Forest virus (Kondor-Koch *et al.*, 1983) and influenza (White *et al.*, 1982). These spike proteins have been found to play a role in the attachment/fusion processes between virion and cell surface at the initial stage of viral infection.

VIRAL MULTIPLICATION

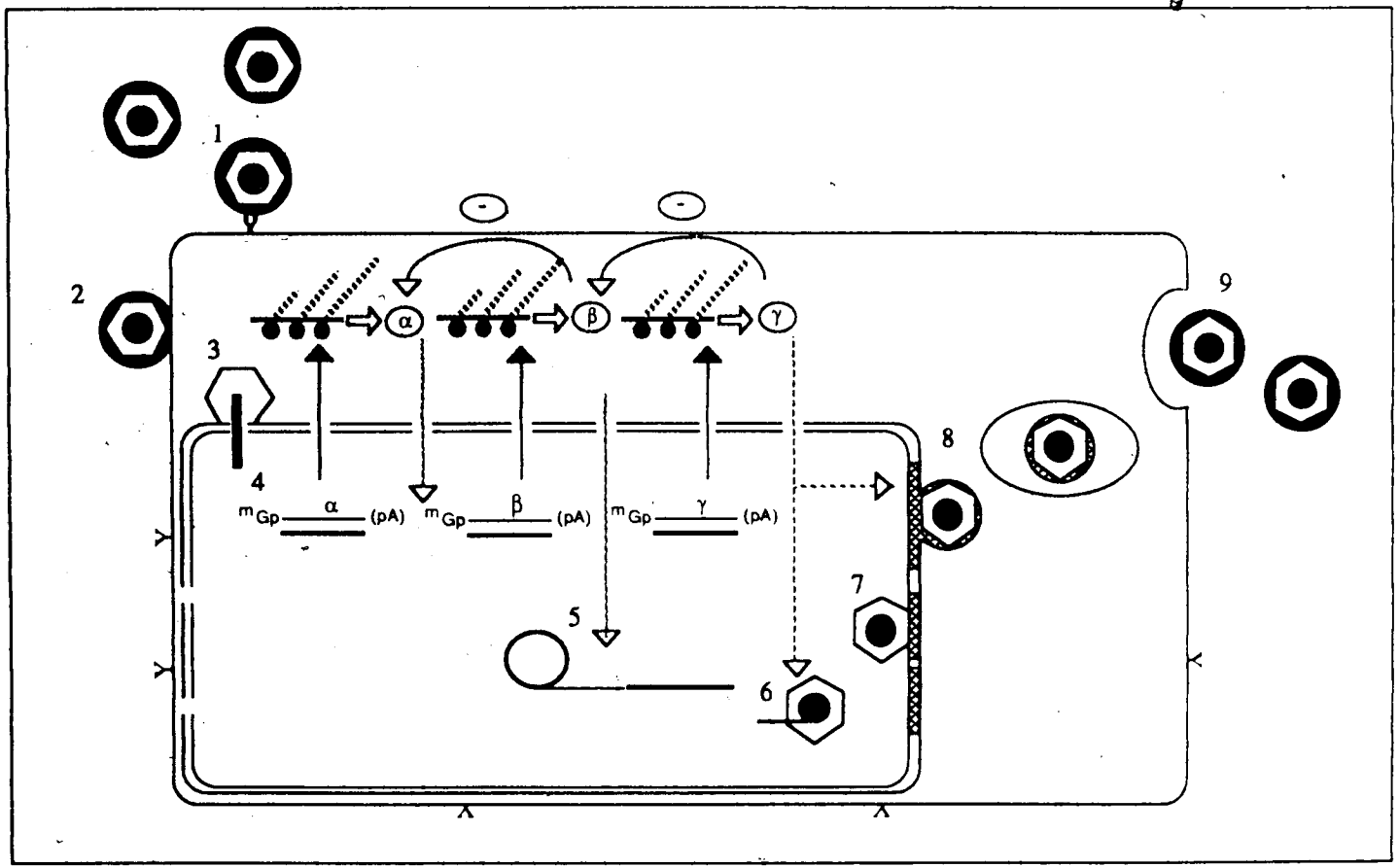
The infectious process follows a well-ordered sequence of events which can be summarized as follows. The virion attaches to the cell, the capsid penetrates to the nuclear membrane and the DNA is released into the nucleus. Different parts of the viral genome are then transcribed sequentially and translated into proteins. Structural viral components accumulate and are assembled into the infectious virus which is then released from the cells. This lytic cycle is illustrated in Figure 2.

Initiation of Infection. To initiate infection, the virus must attach to specific receptors on the cell surface. Only one herpesvirus receptor has been thusfar identified: the Epstein-Barr Virus (EBV) binds to the Complement 3d receptor, CR-2, on human

FIGURE 2

THE SEQUENCE OF EVENTS IN THE MULTIPLICATION OF HERPESVIRUSES

Virus attaches to a specific receptor at the cell surface (1) and is followed by the fusion of the viral envelope with the cell plasma membrane (2). The capsid then penetrates the cell and makes its way to the nuclear membrane (3) where the viral DNA is released into the nucleus (4). Transcription of the viral DNA and the coordinated synthesis of sets of proteins ($\alpha \rightarrow \beta \rightarrow \gamma$) required for DNA replication and for progeny virion construction. Replication of viral DNA is by a rolling mechanism (5) and occurs prior to assembly of the nucleocapsid. DNA is spooled into the newly formed nucleocapsid (6), which then move to the nuclear membrane (7) where they acquire their envelope by budding through areas of membrane modified by the insertion of viral envelope proteins (8). Virions are transported to the plasma membrane where they are released (9).


1
2
3
4
5
6
7
8
9
α
β
γ
mGp α (pA)
mGp β (pA)
mGp γ (pA)

lymphocytes (Yefenof and Klein, 1977, Nemerow *et al.*, 1985). The receptor binding protein(s) on the surface of the EBV virion is a glycoprotein of apparent molecular mass (M_r) 350 kD (Nemerow *et al.*, 1985). Observations that mono- or polyclonal antibodies to some glycoproteins are able to neutralize virus do not unequivocally implicate a receptor protein(s) inasmuch as these antibodies may merely sterically hinder attachment or inhibit other early viral functions.

Fusion of the viral envelope with the plasma membrane or an endosome membrane rapidly follows attachment. It has been shown that during HSV-1 infection, the envelope glycoproteins become transiently associated with the cell membrane prior to penetration (Jennings *et al.*, 1987). Expression of glycoproteins at the cell surface was observed immediately after infection and subsequently declined to undetectable levels over the next two hours after infection.

On the release of the capsid from the envelope, the capsid is transported to a pore in the nuclear membrane. With the aid of a viral function encoded in the capsid (shown by studies with temperature sensitive mutants defective in the release of viral DNA: Batterson *et al.*, 1983), viral DNA is released into the nucleoplasm, while the empty capsid remains outside the nuclear membrane.

Transcription takes place in three distinct stages. The first genes to be transcribed are the immediate-early or α-genes. It has been hypothesized that these code for proteins which produce the switch from restricted to extensive transcription. The β or early proteins have been defined as those proteins synthesized after the α-proteins but before viral DNA replication. It appears that these proteins are involved in the synthesis of DNA, *e.g.* the thymidine kinase of HSV. They also play a role in the shut-off of α and host protein synthesis (Honess and Roizman, 1974) whilst inducing the transcription of γ-genes. Most γ-proteins appear to be constituents of the virion, in HSV these comprise approximately half of the known gene products. Virion capsid proteins and envelope glycoproteins have been classified as γ-proteins. Studies on the maturation of

the glycoproteins following translation have identified a series of complex steps, involving multi-step glycosylation, acylation of some glycoprotein species, a process occurring predominantly within the Golgi apparatus (Johnson & Spear 1982; 1983), and insertion of the fully glycosylated species into the plasma membranes of infected cells (Heine *et al*., 1972; Spear *et al*., 1970).

Control of Protein Synthesis. The synthesis of herpesvirus proteins has been shown to be coordinately regulated and sequentially ordered (Honess and Roizman, 1973 and 1974). The rates of synthesis of α protein found in HSV 1 infected cells was highest between 3 and 4 hours after infection and thereafter declined. The rates of synthesis of β group proteins were maximal from 5 to 7 hours after infection and then similarly declined. The γ group proteins were made at increasing rates until at least 12 hours after infection. The conclusion that they were sequentially ordered was based on the requirements for their synthesis. The synthesis of α proteins required no prior infected cell protein synthesis; they were made immediately after withdrawal of cycloheximide or of puromycin added to culture medium at the time of infection. The production of β proteins required the prior synthesis of α proteins, since in cultures synchronized with inhibitors of protein synthesis, the synthesis of β proteins coincided with a rapid decline in the synthesis of α proteins. A similar relationship was observed between β and γ protein synthesis.

Few details are available concerning the mechanisms by which sequential synthesis of co-ordinately regulated groups of viral proteins is effected, and these relate mostly to the transition of α- to β-protein synthesis. Specifically, in cells treated with cycloheximide from the time of infection, transcripts arising from 10-14% of the viral DNA accumulated in the cytoplasm even though the nuclei accumulated transcripts made from at least 50% of the DNA (Kozak and Roizman, 1974). The transcripts retained in the nucleus were not transported into the cytoplasm even after the drug was withdrawn and α-protein synthesis began, and no synthesis of β-protein ensued unless new RNA

was synthesized. These data suggested that the synthesis of β protein required the participation of α-proteins in order for the β protein mRNAs to reach the cytoplasm.

The requirement of functionally active proteins in the turning on of β and γ protein synthesis and turning off of α protein synthesis was reported by Honess and Roizman in 1975. The amino acid analogues, canavanine (an analogue of arginine) or azetidine 2 carboxylic acid (an analogue of proline) were introduced at different times after herpesvirus infection and were found to selectively inactivate "on" and "off" regulatory functions. Specifically, one or more α protein is required to turn on the synthesis of the β and γ proteins since in the presence of α-protein containing one of the analogues, transition of synthesis to the later protein groups did not occur, in spite of the continued synthesis of α-protein comparable to maximal rates of untreated infected cells.

The requirement of functional proteins from the β- and/or γ-groups to shut off the synthesis of α-proteins is evident from the fact that amino acid analogues added at 3 hours post-infection, prior to the initiation of synthesis of β- and γ proteins results in the sustained simultaneous synthesis of all three groups, a phenomenon not seen in untreated infected cells. Since the mRNA specifying α-proteins appear to be relatively stable (Honess and Roizman, 1974), the shut off of α-protein synthesis is probably regulated at the translational level.

DNA Replication. Parental viral DNA penetrates the nucleus where the terminal repeat sequences of the DNA molecule is digested by double stranded DNA exonuclease(s) to form complementary cohesive termini. These structures have been reported by Jacob and Roizman, (1977), for HSV; Jean and Ben Porat, (1976), for pseudorabies virus, using electron microscopy. Replication is initiated chiefly on circular molecules at a fixed origin, giving the rise to theta-type structures. This mode of replication is transient and restricted to the first round of DNA replication. Thereafter, newly synthesized DNA is associated mainly with concatamers in which unit-sized molecules are linked in tandem

head-to-tail arrays, compatible with replication of DNA by a rolling circle mechanism (Gilbert and Dresser, 1968). Maturation of the precursor concatomeric DNA to unit-sized DNA is dependent upon protein synthesis and is linked to capsid assembly (Ben Porat *et al.*, 1976). Since all herpesviruses have a unique set of end sequences, site-specific cleavage of concatemeric DNA must occur.

Virion Assembly. Capsid structural proteins are transported from the cytoplasm to the nucleus of the cell, where with the aid of assembly proteins, the capsid structure is formed and a molecule of viral DNA is inserted. The nucleocapsid then moves to a region of inner nuclear membrane, into which virally encoded proteins have been inserted, and "buds" through this area. Envelopment can occur at sites other than the nuclear membrane. Epstein (1962) and Epstein and Holt (1963) have shown envelopment to take place either at the plasma membrane or from vesicles of the smooth surface endoplasmic reticulum. However, this type of envelopment has been found to be restricted to stages late in infection. Envelopment by budding from different cellular membranes is used by all the herpesviruses with the exception of *herpes saimiri*. *H. saimiri* uses an alternate mechanism in which the envelope is laid down around the nucleocapsid within the nucleus (Heine *et al.*, 1971). The enveloped particle, enclosed in the vacuole which sequesters it from the cell cytoplasm, moves to the cytoplasmic membrane where the vacuole releases the particle outside the cell.

IMMUNOLOGY OF HUMAN HERPESVIRUS INFECTIONS

By the age of 30 years, most individuals have undergone primary infections with all of the human herpesviruses (Shore and Feorino, 1981). Although differences exist in the clinical pattern of infection with the various viruses, some common factors affecting the severity and outcome of primary infections have been elucidated. The first is the age of the patient; children (excluding neonates) generally have clinically milder disease than adolescents or adults. This age-related difference is most striking for EBV and

VZV but has also been shown to hold true for HSV and HCMV. Whether this relationship between virus and host reflects a difference in the immune response of the child as opposed to the older individual or reflects a difference in the initial presentation of the infecting virus (size of virus inoculum or route of entry) is unknown. A second factor which influences infection is the patient's general immune status, regardless of age. Immunocompromised patients, especially those with primary or secondary deficiencies in cell-mediated immunity, can have severe or even lethal primary infections with herpesviruses. A third factor is the portal of entry, which would also influence the expression of the immune response. Thus, naturally acquired HCMV infection via aerosols or urine-oral contact (Sullivan and Hanshaw, 1982) is normally asymptomatic, whereas HCMV infection acquired by the transfusion of infected blood frequently gives rise to a mononucleosis-like syndrome.

The primary function of the immune system in primary virus infections is, of course, to limit the severity of disease and the spread of virus to the vital organs. A second function is to establish those immune factors (virus specific immune memory cells, serum antiviral antibody) that will limit the extent of future infection with the same agent, whether by the exogeneous route of reinfection or by endogenous reactivation of latent virus. That this occurs is indicated by the general observation that manifest primary infections are usually more severe or less well localized than clinically apparent reinfections or reactivations.

Immune Response. Defense against an invading herpesvirus depends on a number of different cell populations and humoral factors acting in concert to limit virus replication and to inactivate and remove free virions. The mechanisms involved in antiviral defense include both non-specific and specific immune response. Non-specific responses are induced immediately after infection begins and require no previous exposure to the foreign agent. Specific responses are not normally detected until three to five days after infection begins.

The first line of defense is provided by natural antibody, the complement system, antiviral lipoproteins and endogeneous natural killer (NK) cells (Cooper and Welsh, 1979). These factors act independently or coordinately to inactivate the virus. Macrophages (MΦ) present in the systematic blood circulation phagocytose virus particles, process them and present processed antigen on their cell surface (Figure 3). Helper T cells (T_H) recognize this antigen in conjunction with the major histocompatability (MHC) type II proteins, which are also present on the surface of the MΦ, and are thereby stimulated to produce the lymphokines necessary to in turn stimulate antibody-producing B-lymphocytes. These activated helper T cells also produce Il-2 which is essential for the clonal expansion of effector (cytotoxic) T-cells.

On the surface of each B-cell are several copies of a specific immunoglobulin molecule which act as surface receptors that recognize and bind to a specific circulating antigen. In the presence of both bound antigens and T-cell factors, the B-cell is activated, proliferates and undergoes terminal differentiation. The "plasma cells" so produced secrete antibody to combat the invading virus. Antibody molecules which bind to the virions serve to label them for phagocytosis by macrophages and eventual destruction. With the depletion of circulating virions, specialized T suppressor (T_S) cells are activated. These then act by an as yet obscure mechanism upon the activated B cells to differentiate them into memory B cells, which then exist in the circulation to guard against future infections.

Another branch of the immune response has been developed to deal with virus-infected cells. This cell-mediated response requires the expression of viral antigen at the cell surface (Figure 4). In the case of herpesviruses, this antigen may represent viral envelope proteins left at the cell surface after uptake of the nucleocapsid, or may represent progeny virion proteins. A third class of T-cell, the cytotoxic T-cell (CTL) recognizes the viral antigen in combination with HLA Class I protein and releases cytotoxic proteins into the target cell which then bring about cell lysis. Virus specific

FIGURE 3

THE HUMORAL IMMUNE RESPONSE TO INFECTION

This response mobilizes several cooperating populations of cells. Antigen is taken up by an antigen presenting cell, a macrophage (Mϕ) can serve in this role. The antigen is processed by the macrophage and is displayed on its surface. There in conjunction with a major histocompatibility (MHC) class II protein it is recognized by a T-helper cell (T_H), which is thereby activated. The T-helper cell then activates B cells; which carry antigen bound to their receptors. The activiated B cells proliferate and undergo terminal differentiation. Some of the progeny becoming memory cells, which provide response to future infections, whereas others develop into antibody-secreting plasma cells. The secreted antibodies bind to the antigen, thereby marking it for destruction by various other components of the immune system, including macrophages. The extent of B cell response is regulated by T-suppressor cells (T_S). A soluble factor secreted by T-suppressor cells blocks T-helper cell activity and so B cells that would otherwise be stimulated, fail to mature into antibody secreting cells.

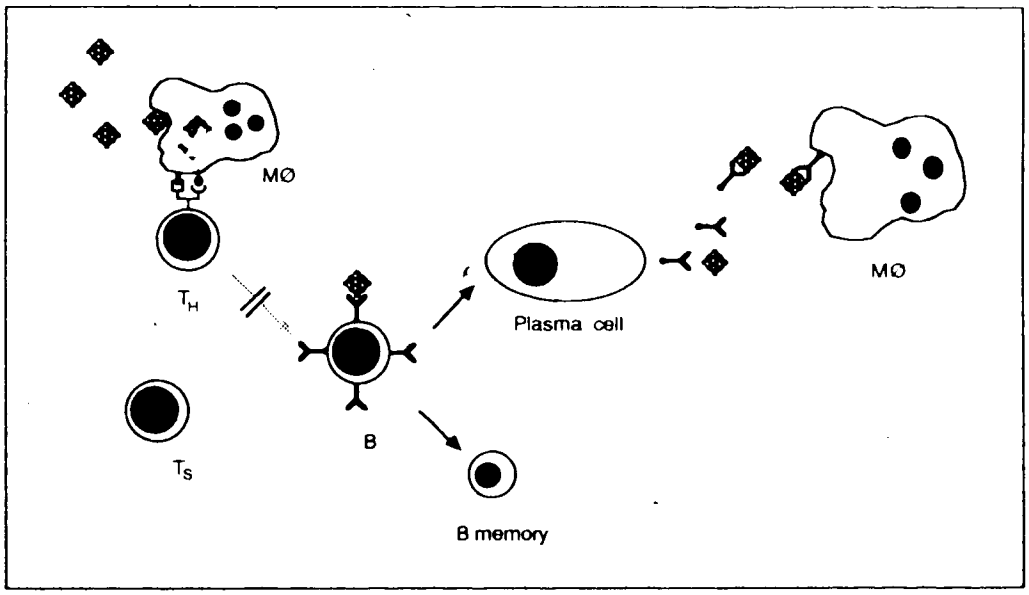
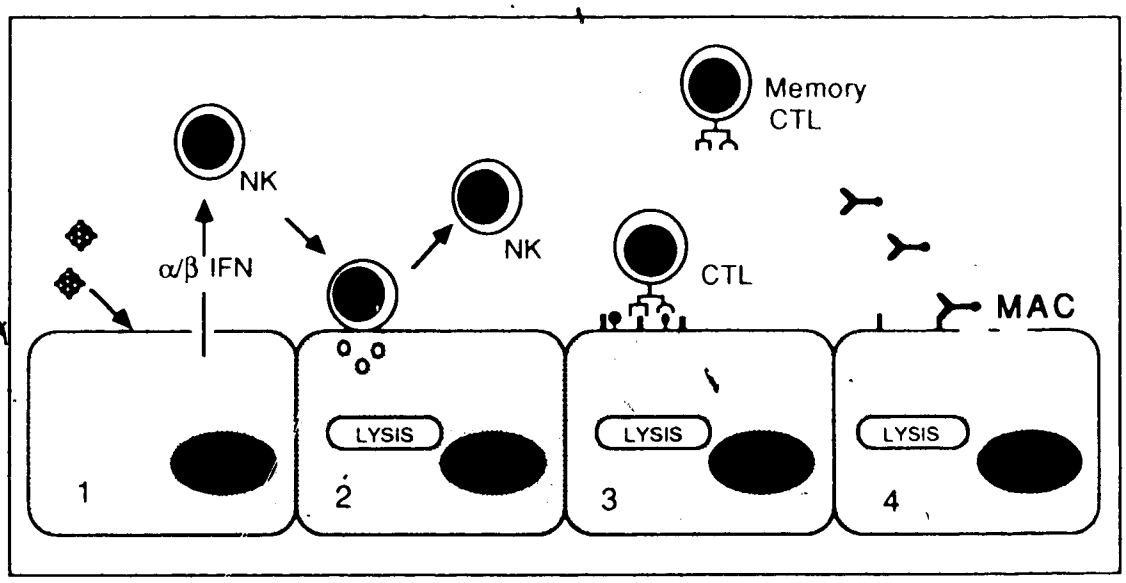

MØ
T_H
T_S
B
Plasma cell
MØ
B memory

FIGURE 4

THE CELL MEDIATED IMMUNITY TO VIRAL INFECTION

When a virus infects a cell (1), the cell responds by producing interferon (IFN). Interferon stimulates the activity of natural killer cells (NK), a class of cytotoxic T cell that does not require interaction with major histocompatibility complex proteins or immunoglobulin prior to the release of cell lysis proteins (2). Natural killer cells are therefore capable of destroying both infected and uninfected cells; it is interferon that confers a viral specificity on the natural killer cells by enhancing their cytotoxic effect on infected cells, while protecting uninfected cells from lysis. Cytotoxic T lymphocytes (CTL) recognize antigen positioned together with a class I major histocompatibility complex protein as a signal for cell lysis (3). They therefore act only on infected cells. Long-lived protection against similar infections is given by the development of memory cytotoxic T lymphocytes, which carry receptors of the same specificity as cytotoxic T cells. The presence of viral protein-antibody complexes on the surface of infected cells activates the complement system (4). With the formation of a membrane attack complex (MAC) the integrity of the cell membrane is lost and cell death follows.


Memory
CTL
NK
NK
α/β IFN
CTL
MAC
LYSIS
LYSIS
LYSIS
1
2
3
4

CTL have been demonstrated for a number of viruses; HSV, EBV, HCMV, mumps and influenza (McMichael and Hildreth, 1983) and evidence has also shown specific memory CTL to exist following infection and so protect against future infections.

RESEARCH OBJECTIVES

In the course of the studies contained in this thesis, our initial objectives were to use mouse monoclonal antibodies to investigate the structure of the human cytomegalovirus virion (HCMV) and to explore the feasibility of employing these antibodies in a diagnostic assay for HCMV in urine. The work on HCMV structure is described in this thesis, and the diagnostic assay is still a possibility although the availability of HCMV-DNA probes for the detection of HCMV by DNA-DNA hybridization (Chou & Merigan, 1983; Spector *et al.*, 1984) has fulfilled the requirement for a rapid, sensitive and quantitative method for the detection of HCMV in clinical specimens.

While the HCMV project was in progress we were introduced to the field of human monoclonal antibody production by Dr. Larry Winger, then in the Department of Immunology at the University of Alberta. We have since produced human IgM and IgG antibodies to a number of structural proteins present in HCMV nucleocapsid and virion. In the analysis of the *in vitro* immune response to HCMV antigens we concluded that the production of antibodies by transformed lymphocytes is representative of the *in vivo* human B cell immune response.

Investigations into the use of synthetic peptides as potential vaccines were commenced in collaboration with Dr. R.S. Hodges of the Department of Biochemistry, University of Alberta. The amino-acid sequence of herpes simplex virus type-1 glycoprotein D was screened for surface sites using the "Surfaceplot" algorithm developed by Drs. J.M.R. Parker and Hodges. Since it is now widely believed that the entire accessible surface of a protein is antigenic (Benjamin *et al.*, 1984), the predicted surface sites are potential antigenic sites. Synthetic peptides to all of these sites were prepared and photochemically linked to carrier proteins by Natalie Strynadka and Dr. Parker in

Dr. Hodges' laboratory. When injected into rabbits all of the peptides elicited a good immune response. The resultant polyclonal antisera were screened for reactivity with isolated gD and HSV virions; neutralization was also investigated. These studies have led to the uncovering of three new neutralizing sites on gD-1. Recognition of the synthetic peptides by the human immune system was examined by ELISA assay using both HSV immune and non-immune sera. The B cell repertoire of non-immune B cell donors was also screened for lymphocytes carrying receptors for the synthetic peptides using analytical flow cytometry. We have found that such receptors are present in a number of donors, therefore the potential exists to produce antibodies against these synthetic peptides.

The outcome of the studies on synthetic peptide vaccines together with those on human monoclonal antibodies has led to the development of a theoretical model system for the *in vitro* analysis of synthetic peptide vaccines. From either nucleotide or amino-acid sequences, antigenic sites may be predicted and synthetic peptides of these sequences prepared. Using immune sera and flow cytometry analysis, antigenicity in humans may be confirmed. Human monoclonal antibodies made to these sites will enable neutralization capacity (if any) to be measured.

CHAPTER II

HUMAN CYTOMEGALOVIRUS I:
THE ORIGIN AND CHARACTERIZATION OF HUMAN CYTOMEGALOVIRUS NUCLEOCAPSID PROTEINS

INTRODUCTION

In 1904, Ribbert reported the observation of protozoan-like cells in the lungs, kidneys and liver of an 8-month fetus. The nuclei were large and eccentrically placed, and each nucleus contained a "central nuclear body" surrounded by an outer clear zone. A worker in Ribbert's laboratory, Lowenstein, went on to describe cytoplasmic as well as intranuclear inclusions in samples of infant parotid gland tissue and, not believing that these structures were normal or altered cellular features, concluded that the inclusions represented some type of protozoa; *i.e.* coccidia, sporozoa or amoeba. The term "cytomegalia" was introduced by Goodpasture and Talbot in 1921 to describe large mononuclear cells that reminded them of the intranuclear inclusions produced in varicella infections, and it was for this reason that they did not believe the inclusions to be protozoa. One year later this belief was confirmed when cytomegalic inclusions were observed in the kidney cells of infants. As was pointed out at this time, since such inclusions could also be found in the kidneys of stillborn infants, they were probably present before birth. Protozoa are not able to pass the placental barrier and therefore could not be the cause of this illness. Wyatt *et al.* (1950) suggested the term "generalized cytomegalic inclusion disease" on morphological grounds to describe the congenital infection. It was suggested that since these inclusion-bearing cells were invariably present in the renal tubules, it might be possible to identify them in the urine of afflicted infants. This was subsequently confirmed by Mercer *et al.* (1953) and Margileth (1955). Numerous attempts were made to isolate a virus from urine, but with the methods available these proved unsuccessful. The isolation of cytomegalovirus was

accomplished independently by Smith (1956) in St. Louis, by Rowe and co-workers (1956) in Bethesda, and by Weller (1957) in Boston. The comparison of the cytopathic changes produced by Rowe's virus, termed "AD169" and that of Weller's, strain Davis, showed the viruses to be pathologically identical. In 1960, Weller first applied the term cytomegalovirus to the viruses causing cytomegalic inclusion disease.

Clinical Spectrum of Human Cytomegalovirus Infection. Cytomegalovirus infections may readily be sorted into three generalized categories: 1) neonatal - those which are detected within the first few months of life; 2) acquired HCMV infections - may be asymptomatic or produce a mononucleosis syndrome; and 3) HCMV in immunosuppressed patients - renal, bone marrow and heart transplant recipients.

The primary characteristic of symptomatic congenital infection, cytomegalic inclusions, are in various organs. A typical case is a premature infant noted to be jaundiced within the first day of life. Enlargement of the liver and spleen (hepatosplenomegaly) is found at birth by abdominal palpation, and within hours discrete skin hemorrhages appear. Central nervous system depression and respiratory distress indicative of pulmonary involvement are evident within hours or days, and the infant usually dies. Those who survive are frequently severely brain-damaged, suffering from optic and auditory atrophy together with mental retardation.

Symptomatic acquired infection is marked by fever, often lasting 4-6 weeks, headache, muscle pain and mild hepatitis. Acquired HCMV is not commonly found in children and becomes more marked in adolescence. Across all age groups this form of the disease is often found post-transfusion, HCMV being carried in the polymorphonuclear leukocytes from sero-positive blood donors (Rinaldo *et al.*, 1979a). In 1982, following an assassination attempt, Pope John Paul II received a blood transfusion during treatment for gun-shot wounds; he subsequently contracted HCMV mononucleosis with the complications of adventious pneumonia, resulting from the immunosuppression which accompanies HCMV infection.

Immunosuppressed patients are at great risk from HCMV infection following renal, marrow and cardiac transplantation. There is the potential for a primary infection of HCMV seronegative recipient if the organ transplanted or blood transfused during the transplant procedure is from an HCMV positive individual. Given that the underlying condition of transplant recipients is generally poor (renal or other organ failure, leukemia) together with the use of antirejection therapy, the resistance to primary or reactivated HCMV infection is low.

An HCMV infection post transplantation is most of all characterized by acquired mononucleosis like symptoms which persist from 2-4 weeks. Only in very unusual circumstances does disseminated HCMV infection develop, and this can be fatal (Simmons *et al.*, 1977). More often it is the decrease in the number of circulating leukocytes associated with the mononucleosis that predisposes the individual to secondary bacterial or fungal infection, and it is these infections that contribute in a greater way to morbidity and mortality.

IMMUNOPATHOLOGY OF HUMAN CYTOMEGALOVIRUS INFECTIONS

There are many gaps in our knowledge about the pathogenesis of human cytomegalovirus infections. Sero-epidemiologic studies have demonstrated a wide variation in the age of acquisition of cytomegalovirus infection (Gold and Nankervis, 1976). Differences in geographic and socioeconomic factors account for these differences in age related prevalence of cytomegalovirus infections. In the Western Pacific islands of the New Hebrides, where large extended family units exist and the custom of mastication of food for infants by family members is practiced, 100% of infants have experienced cytomegalovirus infection by one year of age (Lang *et al.*, 1977). In the United States approximately 25-50% of 15-year old children have circulating cytomegalovirus antibody (Hanshaw and Dudgeon, 1978).

Most infections are probably acquired by aerosol or urine-oral (also fecal-oral) contact (Sullivan and Hanshaw, 1982), with oropharyngeal epithelial cell infection as the

primary focus. Infection of lymphoid tissue and leukocytes results in viremia and induction of a humoral and cell mediated response. The prolonged excretion of virus in the throat, feces, breast-milk and semen following infection increases the risk of spreading infection to susceptible persons in intimate contact. The cessation of virus shedding is not related to the acquisition of antibody, and little is known about the immunological mechanisms operating to control viral replication.

The specific lymphocytic cells which replicate cytomegalovirus are not known. Virus can be recovered from polymorphonuclear leukocyte rich fractions as well as mononuclear leukocyte rich fractions. Braun and Reiser (1986) have reported both helper and suppressor/cytotoxic T-cells to be susceptible to HCMV infection. The persistence of infection in the peripheral leukocytes of patients with HCMV mononucleosis has been documented up to 3 months following the onset of acute infection (Rinaldo *et al.*, 1977a). Infection and viremia is followed rapidly by the appearance of antibody; IgM and IgG neutralizing antibodies appear first and are followed by complement fixing IgG. Antibody alone does not appear to eliminate virus production by infected cells as is evident by the presence of viremia and viruria in congenitally infected infants and immunosuppressed adults with circulating HCMV antibodies.

In addition to antibody, cell-mediated immunity appears to be important in recovery from HCMV infection. Quinnan *et al.* (1980) reported that cytotoxic T lymphocytes, natural killer cells and cells involved in antibody-dependent cell-mediated cytotoxicity were less active in patients who died from HCMV infection following bone marrow transplantation. Cytotoxic lymphocytes may lyse infected cells early in the virus's replicative cycle before infection reaches surrounding fluids and may abort cell-to-cell spread of virus. Both mechanisms serve as highly effective ways of limiting infection.

HCMV infection often predisposes individuals to potentially severe superinfections with other opportunistic pathogens, fungi (*e.g. Aspergillus, Mucoraceae*), protozoa (*e.g. Pneumocystsis carinii*), or bacteria. This has been most clearly demonstrated in transplant recipients (Braun and Naukervis, 1978; Rubin *et al.*, 1977) but has also been suggested in other populations, including infants with cytomegalic inclusion disease (Seifert, 1984) and adults with mononucleosis (Ten Napel, 1980). How does HCMV increase susceptibility to opportunistic infections? Studies by Casali *et al.*, (1984), have shown antibody responses to be normal. Polymorphonuclear leukocyte function appears to be normal, as measured by phagocytosis, reduction of nitroblue tetrazolium (staining) and chemotaxis (Rinaldo *et al.*, 1979b). Rinaldo *et al.*, (1983) have studied NK cell cytotoxicity in mononucleosis patients and found these responses to be intact. It appears to be the abnormalities in the T-cell proliferative response, gamma interferon response to mitogens and antigens, and cytotoxic response to allogeneic (foreign) cells, which result in immunosuppression in HCMV infection (Rinaldo *et al.*, 1977b; Levin *et al.*, 1979). Immunosuppression appears to be linked to increased monocyte-macrophage suppressor activity (Carney and Hirsch, 1979) together with an inversion of the normal ratio of T helper to T cytotoxic/suppressor cells. Carney *et al.*, 1981 have analyzed peripheral blood T-lymphocytes from individuals in the acute and convalescent stages of HCMV mononucleosis using monoclonal antibodies directed towards T cell phenotypic surface marker proteins. The Ortho Pharmaceutica (OK) monoclonal antibody OK T3 binds to surface antigens on mature T cells, OK T4 surface antigens are helper/inducer T cells, OK T8 surface antigens on cytotoxic/suppressor cells, and OK 1A binds to all activated T cells. In these studies no net change in T cell number as shown by T3 binding analysis. It was found that acute HCMV is associated with a large absolute increase in $T8^+$ cells and a smaller decrease in $T4^+$ cells. Ia^+ cells were also increased fivefold. The preponderant $T8^+$, Ia^+ lymphocyte present during HCMV mononucleosis is virtually unresponsive to mitogen stimulation. The atypical lymphocytes characteristic of

this disorder fall mainly into this category.

Until the isolation of human immunodeficiency virus (HIV) by the groups of Luc Montagnier (Barré Sinoussi *et al*., 1983) and Robert Gallo (Popovic *et al*., 1984), HCMV was considered as a candidate as the causative agent of acquired immunodeficiency disease (AIDS) (Rapp and Robbins, 1984; Meyers, 1984). HCMV was linked to AIDS because AIDS is hallmarked by a reversal of the $T4^+/T8^+$ cell ratio, and also for the prevalence in AIDS patients of Kaposi's sarcoma, a cancer which has been associated with HCMV infection. Given today's greater understanding of the immunosuppressive effects of HIV, it is interesting to compare these two immunosuppressive viruses. T cells from HIV infected individuals examined by indirect immunofluorescence using OK T4 and OK T8 antibodies showed little or no change in the numbers of $T8^+$ cells; however $T4^+$ cells had fallen to 20% of normal values (Klatzman *et al*., 1984). The $T4^+/T8^+$ ratio is therefore inverted owing to the dramatic decrease in $T4^+$ cells. It is the lack of T helper cells that produces the general immunosuppression produced in HIV infection. In HCMV infection, the precise mechanism for immunosuppression is as yet undetermined, however, it is thought to involve the unresponsive $T8^+$ lymphocytes which proliferate during the acute stage of infection.

VIROLOGICAL DIAGNOSIS OF HUMAN CYTOMEGALOVIRUS INFECTION

The best method to diagnose HCMV infection is to isolate the virus. The virus may be recovered from many body fluids or from tissues obtained by biopsy or at autopsy. Human CMV has been isolated from urine, blood, throat washings, saliva, tears, milk, semen, feces and vaginal secretions. Almost all clinically significant HCMV infections are associated with viruria despite the fact that none produces symptomatic urinary tract infection or renal failure. Viruria at birth is the essential and sufficient finding for the diagnosis of congenital HCMV infection.

Samples of body fluids may be used to directly inoculate human fibroblast cultures. Cultures such as these may show cytopathic effects (CPE) in 3-4 days but

usually require 1-2 weeks, depending on the concentration of virus in the specimen. Fig. 5 shows a typical HCMV infection in tissue culture. Initially, foci of enlarged, rounded refractile cells appear in the monolayer. These foci will enlarge, coalesce, and may eventually destroy the monolayer, a process which may take 2-3 weeks. The cytopathology of HCMV is usually sufficiently characteristic for identification in the laboratory without further serological confirmation.

Given that the identification of HCMV by CPE of the virus is slow, we set out to develop a rapid diagnostic assay for HCMV in urine samples. This assay was to be based on a sandwich ELISA-capture type system. Such a test would utilize monoclonal antibodies directed against viral proteins to capture virions in the urine sample. Monoclonal antiboides conjugated with enzyme, for example alkaline phosphatase or peroxidase, would then be added to form a sandwich. The ELISA would then be completed by the addition of the appropriate enzyme substrate. The development of this assay system first required a sound understanding of the proteins found in HCMV, and this was the starting point of our studies.

HCMV PROTEINS

The HCMV proteins necessary for replication of the virus or for the maturation of the virion may also play a key role in the diagnosis and control of viral infection. *In vivo*, HCMV can potentially infect both productive (permissive) and non-productive (non-permissive) cells. In the permissive cell both early and late viral genes are expressed and the cytopathology of the infectious process normally leads to cell death. The viral genes are expressed sequentially and therefore have been grouped into three broad categories referred to as immediate early (IE), early and late (analogous to the α-, β- and γ-genes of herpes simplex viruses; see Chapter I). The IE genes are transcribed in the absence of prior protein synthesis; consequently these encode the first virus proteins to appear in the infected cell, normally within one hour of infection. Early genes require the *de novo* synthesis of the IE proteins. They appear in the cell

FIGURE 5

CYTOPATHIC EFFECTS OF A CLINICAL ISOLATE OF HCMV IN HUMAN FETAL FIBROBLAST CULTURES

Cytopathic effects of a clinical isolate of HCMV in human fetal fibroblasts. Note the rounded, refractile cells at the foci of infection. The isolate was derived from the throat washings of a patient at the University of Alberta Hospitals, admitted with severe retinitus. The washings were directly inoculated into fibroblast cultures. The foci of infection illustrated appeared 3 days after inoculation, indicating the presence of a high concentration of virus in the specimen. Lower concentrations of virus may require 2-3 weeks incubation before cytopathic effects develop. Magnification 220x.

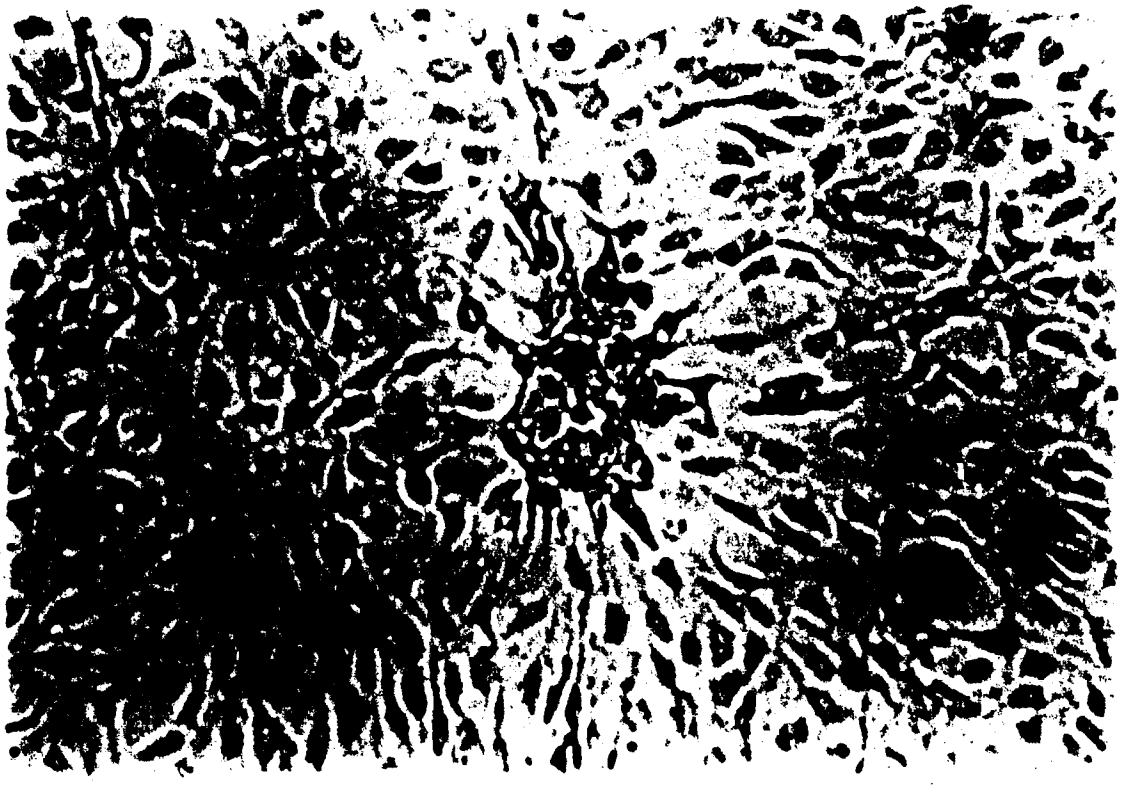


FIGURE 6

APPEARANCE OF HCMV INFECTED CELLS LATE IN INFECTION

In indirect immunofluorescence microscopy of HCMV infected cells in the late stages of infection (48-60 hrs) using antibodies directed against HCMV structural proteins visualized by FITC-labeled secondary antibodies, shows that cells have become rounded in appearance and contain a cytoplasmic inclusion (arrow) and a nuclear inclusion composed of cellulae (arrow-heads). (Magnification 875x.)

within four to six hours after infection but prior to the synthesis of viral DNA. In the permissive cell, viral DNA synthesis begins around 12 hours after infection. Newly synthesized viral DNA accumulates in the cell only very slowly, as do the late viral proteins. The latter reach their highest rates of synthesis by approximately 48 hrs after infection, and this rate may then continue for several days. Approximately 50-60% of the total protein synthesized is viral, the remainder is host protein. The continued production of host proteins has made the identification of viral proteins difficult, but approximately 50 such proteins have been recognized in infected cells, and 30 or more can be detected in virus particles (Hiala *et al.*, 1976; Gibson, 1981; Kim *et al.*, 1976; Sarov and Abady, 1975).

VIRUS PARTICLE ASSEMBLY

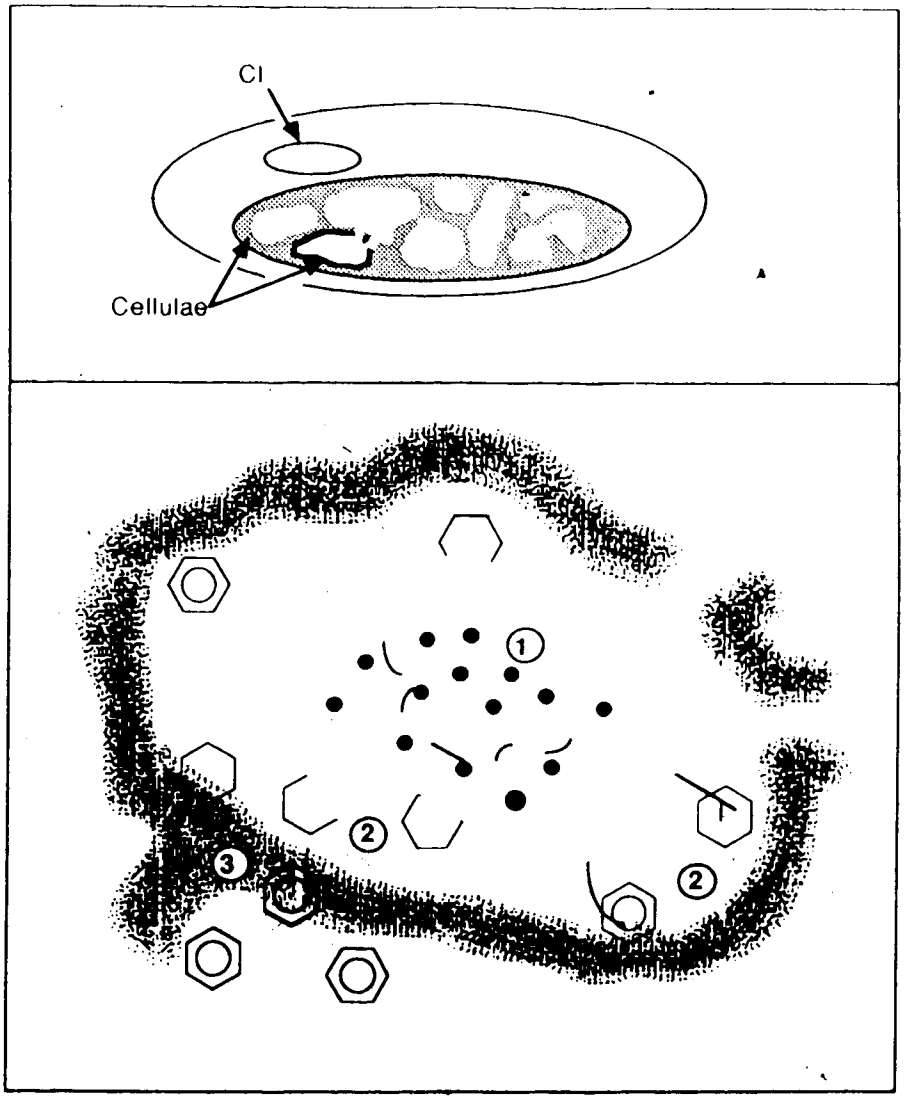
The assembly of virus particles has been reported to take place in the nuclear inclusions which appear in HCMV infected cells 48 hours post-infection (Figure 6). Cavallo *et al.*, (1981) have examined nuclear inclusions by electron microscopy and have found them to be composed of cellulae, (electron-luscent areas) surrounded by electron dense material. Fons and Albrecht (1986) examined the cellulae and found them to be composed of three zones. A central zone, devoid of any capsid structures but containing granular material and numerous small fibers. Around the periphery of the electro-luscent area was a zone containing incomplete capsids associated with fibrous materials. It was observed that the fibrous material showed a progression from loose association with incomplete capsids to a highly organized structure of tightly condensed fibres completely surrounded by capsid material. The third zone consisted of electron dense material containing well developed capsids similar to those observed budding through the nuclear membrane, acquiring envelope and so forming complete virions (Figure 7).

The architectural organization of the virion reflects the distribution of its structural constituents in the cell. Thus the viral DNA, which is situated in the core of the virion, is thought to accumulate in the centre of the cellulae (Huang *et al.*, 1973;

FIGURE 7

HCMV PARTICLE ASSEMBLY

In light microscopy of HCMV infected cells, the characteristic cytoplasmic (CI) and nuclear inclusions appear at approximately 48 hours post-infection. The nuclear inclusion as depicted in the upper panel of this figure is composed of numerous cellulae. In electron microscopic examination, the cellulae are found to be divided into 3 regions (lower panel). Region 1 contains granules and many small fibres, no capsid structures are found in this area. Region 2 contains incomplete capsids, many of which are associated with fibrous material. The third region (3) appears as a broad band of electron dense material containing well developed capsids similar in appearance to those found within the mature virion. The viral envelope is acquired as the capsid buds through the nuclear membrane. (These figures are based on the electron micrographs of Fons and Albrecht, 1986).


CI
Cellulae
1
2
3
2

Maeda *et al.*, 1979; DeMarchi, 1983). The capsid forms around the DNA containing core to the periphery of the cellulae. Between the sites of capsid formation and envelopment at the nuclear membrane (McGavran and Smith, 1965; Smith and DeHarven, 1973), the matrix protein present within the virus tegument is acquired. Weiner *et al.* (1985) have located the matrix protein in the nucleoplasmic region of the nuclei rather than the cellulae, therefore after capsid formation the capsid enters the nucleoplasm, acquires matrix protein and then buds from the nuclear membrane as the mature virion.

HCMV STRUCTURAL PROTEINS

There is general agreement between research groups that the virion capsid is composed primarily of a protein whose apparent molecular mass (M_r) is ~150 kD (Fiala *et al.*, 1976; Gibson, 1981; Farrar and Oram, 1986; Schmitz *et al.*, 1980). Irmiere and Gibson (1985) have reported the nucleocapsids of HCMV Strain AD169 to be composed of proteins with apparent molecular masses of 153,000 (major capsid protein), 34,000, 28,000 and 11,000 kD, while Farrar and Oram, (1986) reported the major AD169 nucleocapsid proteins to be 150, 64 and 35 kD.

Two matrix proteins of M_r 74 kD and M_r 69 kD have been assigned to the tegument region of the HCMV virion (Roby and Gibson, 1986). This assignment was made on the basis of their architectural location in the virion together with their biochemical similarity to cellular nuclear matrix or lamin proteins (Weiner *et al.*, 1985). The matrix proteins, which are made late in infection, are phosphorylated in infected cells and may be associated with the protein kinase activity observed when the viral envelope is disrupted, but which is not present within the nucleocapsid itself.

Identification of the glycoproteins of the HCMV virion has been carried out using radio-labeled glucosamine and mannose (Kim *et al.*, 1976; Stinski, 1977 and Pereira *et al.*, 1984). Also, Farrar and Oram (1986) labeled HCMV glycoproteins using the carbohydrate-specific procedure of oxidation followed by reaction with ^{3}H-borohydride.

These four groups of investigators have reported the presence of 14, 7, 17 and 5 glycoproteins, respectively. Very few of these share similar molecular masses (Table 2). Small variations may be explained for by the anomalous migration of glycoproteins in SDS polyacrylamide gels, but the larger variations in both molecular mass and number of glycoproteins can only serve as an indication of the complexity of the virion. Grouping of the HCMV glycoproteins into antigenically related families was achieved by the careful use of panels of mouse monoclonal antibodies (Pereira *et al.*, 1984). Four families of antigenically distinct HCMV glycoproteins designated as gA, gB, gC and gD have been identified (Table 3). By using the inhibitors of glycosylation, tunicamycin and deoxyglucose (Schwarz and Datema, 1980) in a series of pulse-chase experiments, Pereira and collaborators were able to propose a pathway for the maturation of one of these glycoproteins, gA, and so explain the multiple forms of glycoprotein found in the virion. This pathway is shown in Figure 8.

Details of the structure and processing of one HCMV glycoprotein have been given by Britt and Auger, (1986). They described the formation of a disulfide linked glycoprotein complex, gp 116-55, which is present in the viral envelope. The complex is formed by the cleavage of a glycosylated protein, gp 160, which in turn is derived from a precursor protein of molecular mass 150 kD. This pathway is also illustrated in Figure 8. Antigenic and structural analysis have indicated that gp 55 and gp 160 are dissimilar, suggesting potentially different structural roles for these two envelope proteins.

Several examples of cleavage in the processing of viral glycoproteins have been described for other herpesviruses. Hampl *et al.* (1984) have shown that one group of disulfide-linked envelope glycoproteins of pseudorabies virus is synthesized by cleavage of a higher molecular weight polyprotein precursor. Similarly, Van den Hurk *et al.* (1984) have provided extensive information on an envelope protein complex of bovine herpesvirus which was produced from a common precursor protein. Finally, proteolytic

TABLE 2.

Apparent molecular masses* of HCMV glycoproteins, as reported by four independent research groups.

Kim *et al.*, 1976 Strain C87	Stinski, 1977 Strain Towne	Pereira *et al.*, 1984 Strain AD 169	Farrar & Oram, 1986 Strain AD169
			250
			175-165
	160-148		
	145		
		142	
140			
		138	
	132		130
		130	
123-116			
	123-107		
	120		
		115	
105			
98			
			95 95
90	90		
84			
		80	
			67
66		66	
		58.5	
		56.5	
	55	55	
52			52
50		50	
		49	
48		48	
		46	
44			
		34	
		25	
22			
	16		

* Apparent molecular masses (as determined by SDS-polyacrylamide gel electrophoresis) are given in kD.

TABLE 3

Grouping of HCMV Strain AD 169 glycoproteins on the basis of immunological activity with a range of mouse monoclonal antibodies. Based on data from Pereira *et al.*, 1984.

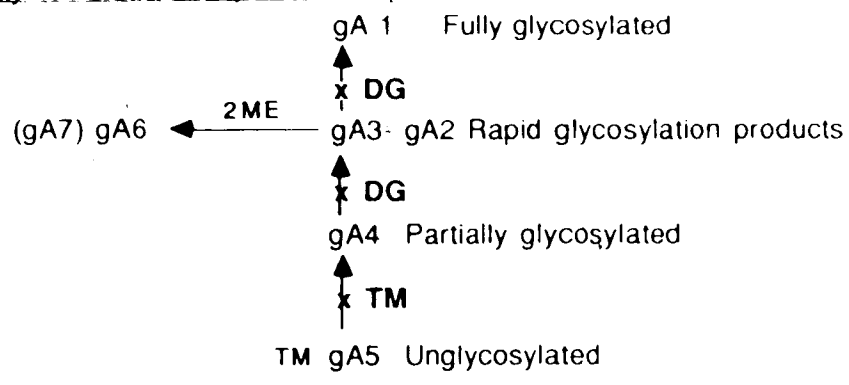
Form	Apparent M_R kD
gA1	160-148
gA2	142
gA3	138
gA4	123-107
gA5	95
gA6	58.5
gA7	56.5
gB1	123-116
gB2	80
gC1	66
gC2	55
gC3	50
gC4	46
gD1	49
gD2	48
gD3	34
gD4	25

FIGURE 8

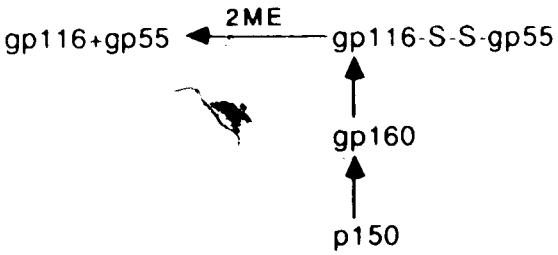
TWO PROCESSING PATHWAYS REPORTED FOR HCMV GLYCOPROTEINS

Inhibitors of glycosylation have been used to allow the accumulation of the intermediary forms of the glycoprotein. Tunicamycin (TM) an analogue of UDP-N acetylglucosamine, inhibits the synthesis of N-linked oligosaccharides at the first step in the lipid-saccharide pathway, the formation of dolichol-P.P-N-acetylglucosamine from UDP-N-acetyl-glucosamine and dolicholphosphate. The inhibitor 2-deoxyglucose, which effects the assembly of lipid-linked oligosaccharides as well as the elongation of oligosaccharide side-chains by mannose. The reducing agent 2-mercaptoethanol (2ME) was used in these studies to cleave inter protein disulfide bonds and so disrupt glycoprotein complexes.

Pathway 1: Pereira et al., 1984. (adapted from)



Pathway 2: Britt and Auger, 1986 (adapted from)



cleavage of an infected-cell protein of varicella-zoster virus was also shown to be involved in the synthesis of a virion envelope protein (Grose *et al.*, 1984).

The HCMV virion is therefore complex in terms of protein composition. We set out to characterize one part of this structure, the nucleocapsid, in terms of the number of protein components, their origin (host or virus coded), and their immunogenicity. The results indicated that there are in excess of 28 proteins in the nucleocapsid, of which 6 are major components and another 4 were present in significant quantities. Major nucleocapsid proteins of M_r 190 kD and 42 kD comigrated with cellular proteins of identical molecular mass and may therefore be of host origin. Monoclonal antibodies produced in mice and immunized with nucleocapsids reacted with a protein of M_r 64 kD in western blots of nucleocapsid proteins. This particular protein also bound convanavalin A, indicating that it is glycosylated.

MATERIALS AND METHODS

Cell Culture and Virus Preparation. Human fetal lung (HFL) cells (Flow 2000, Flow Laboratories Inc., McLean, VA) between passages 17 and 30 were grown in 175 cm flasks containing Eagle's Minimal Essential Medium (MEM) (Flow Laboratories) supplemented with 10% fetal calf serum (FCS; Gibco, Grand Island, NY). Cells were cultured at a temperature of 37° and relative humidity of 95%. HCMV strain AD 169 (American Type Culture Collection-VR 538) was used to infect sub-confluent monolayers of HEL cells at an input multiplicity of 1.

To obtain enveloped virus particles, the infection was allowed to proceed until the cytopathic effect involved approximately 90% of the cells. At this point the culture fluid was removed, centrifuged at low speed to remove cellular debris and then subjected to centrifugation at 100,000 g in a Beckman 70 Ti rotor for 1 hr to pellet the virus. The pellet was resuspended in phosphate buffered saline (PBS; Dulbecco & Vogt, 1954), containing 6% sucrose and 10 μg Bacitracin (Sigma Chemical Co., St. Louis, MO) per

ml, and layered onto a suspension of Percoll (Pharmacia, Dorval, Que.) of density 1.07 g/ml. The gradient which formed during centrifugation in a Beckman Ti70 rotor at 50,000 g for 30 min was fractionated, and the virus banding at a density of 1.07 g/ml of Percoll was removed. Density was calculated from refraction as measured on a Abbe refractometer (Model 302; Canlab, Edmonton). The presence of virus was confirmed by electron microscopy.

HCMV nucleocapsids were obtained by detergent treatment of nuclei isolated from HFL cells which had been harvested 60 hours after infection. This was essentially the method of Gibson (1981), except that the treatments with deoxyribonuclease I, urea and 20 cetyl ether (Brij 58) were omitted. Briefly, infected cells were harvested by scraping off from the walls of the tissue culture flask using a rubber policeman. The cells were washed with PBS pelleted at 500 g for 10 min and then resuspended in PBS containing 1% Nonidet-P40 (NP-40) (Sigma). The cells were homogenized (5 strokes using a Dounce homogenizer with a tight-fitting pestle) to ensure complete rupture of the cellular plasma membranes. Nuclei were pelleted from this suspension by centrifugation at 1000 g for 10 min. The nuclei were resuspended in PBS containing 0.5% sodium deoxycholate (DOC) (Sigma) and subjected to homogenization as above. Nucleocapsids were separated from nuclear debris by layering onto Percoll and centrifuging at 50,000 g for 30 min. The nucleocapsids banded at a density of 1.03 g/ml.

Total lysates of infected and uninfected cells were prepared by removing the growth medium from the cell monolayer, washing twice with PBS and then scraping the cells from the flask into 5 ml of PBS. The cells were then pelleted by low speed centrifugation and resuspended in 1.5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl and 1.5 mM $MgCl_2$. After allowing the cells to swell for 5 min, they were subjected to homogenization (10 strokes with a tight fitting pestle). NP-40 and DOC were then added, both at final concentrations of 1%, to solubilize plasma and nuclear membranes. Lysates were prepared for SDS-polyacrylamide electrophoresis as

detailed below.

SDS-Polyacrylamide Gel Electrophoresis. Samples of isolated nucleocapsids and virions were prepared for polyacrylamide gel electrophoresis by pipetting small portions directly into 63 mM Tris-HCl (pH 6.8), containing 2% SDS and 5% β-mercaptoethanol and boiling for 5 min. Proteins from cell lysates were precipitated by the addition of 100% tricholoracetic acid to a final concentration of 15%. After 30 min on ice, the proteins were collected by centrifugation (10,000 g, 15 min), the pellets washed twice with ice-cold acetone and then resuspended in sample buffer and boiled for 5 min.

The discontinuous system of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) described by Laemmli (1970) was employed, in which the separating gel contained 10% acrylamide. Slab gels were electrophoresed for 20 h at a constant current of 6.5 mA/gel and then fixed in methanol-acetic acid-water (4:1:5, v/v/v) overnight. For detection of radioactively labeled proteins, the gels were submitted to fluorography by treatment with EN'Hance (New England Nuclear), drying and exposing to X-omat AR films (Eastman Kodak Co., Rochester, N.Y.) at -70°. Silver staining of proteins was performed according to instructions with the Bio-Rad Silver Stain Kit. (BioRad Laboratories, Richmond, CA.). Scanning densitometry was performed on a Chromoscan 3 scanning densitometer (Joyce-Loebl, Gateshead, England).

Host Protein Identification and Analysis. Human fetal lung fibroblasts were seeded at a low density (3 x 10^6 cells) onto each of five 175 cm^2 tissue culture flasks. After 48 hrs, three of these flasks were radio-labeled by replacing the growth medium with medium containing 10% of the normal concentration of amino acids, 2% fetal calf serum and a mixture of ^{14}C-amino acids (NEC 445E New England Nuclear, Dupont Canada Inc., Lachine, Que.). Each culture was incubated with 5 μCi/ml of radioactivity in a total volume of 5 ml for a period of 24 hr. At 72 hours, the cells from one radiolabeled flask were harvested and lysed. Also at this time one of the hitherto

unlabeled flasks together with one labeled flask were infected with HCMV at a multiplicity of 1. At 108 hr the two unlabeled flasks were labeled as described above. After 132 hr all four remaining cultures were harvested. Nucleocapsids were isolated from the infected cells while a lysate was prepared from the uninfected cells. This labeling and infection protocol is summarized in Figure 9. Sample 1 represents the cellular proteins produced during the early growth phase, while sample 2 is representative of cellular proteins synthesized late in infection. Sample 3 was included to monitor labeled protein turnover. Samples 4 and 5 contained isolated nucleocapsids; sample 4 being labeled from 118-132 hours and sample 5 being labeled pre-infection to indicate host protein incorporation as well as proteins which incorporated label released by the degradation (turnover) of other proteins.

Generation of Mouse-Hybridoma Cell Lines

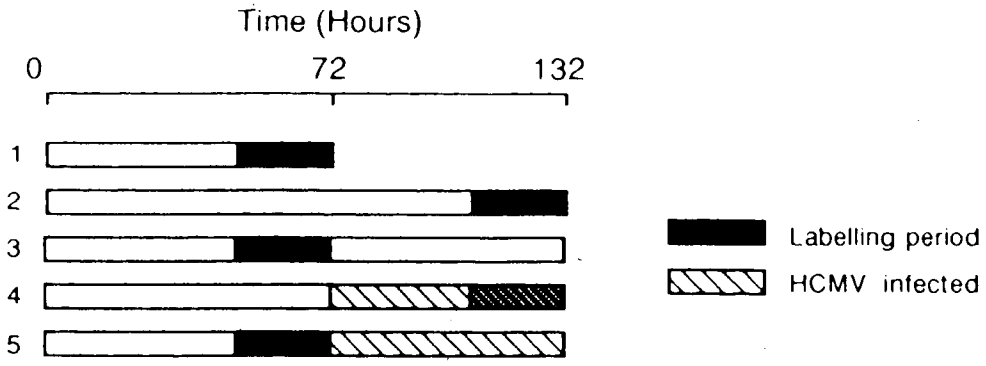
A. Myeloma Cell Lines. Fusion experiments were carried out using two different mouse myeloma cell lines; MOPC 315.43 obtained from Dr. T.R. Mosmann of the Immunology Department of the University of Alberta (Mosmann *et al.*, 1979; Mosmann and Williamson, 1980), and the SP2/O-Ag14 line, originally isolated by Shulman *et al.* (1978) obtained from Dr. J.S. Colter. Both of these lines carry the marker hypoxanthine-guanine phosphoribosyl transferase deficiency, together with resistance to ouabain (MOPC 315 line) or 8-azaguanine (SP2 line). SP-2 has the additional advantage in the SP2 line does not synthesize immunoglobulin chains; clone 43 of the MOPC 315 myeloma line does not synthesize heavy chains but does synthesize a small amount of a mutant γ2 light chain that cannot be secreted.

MOPC cells were grown at a cell density of 1-2 x 10^5 cells/ml in a growth medium consisting of RPMI-1640 containing 50 μM 2-mercaptomethanol and 5% fetal calf serum. One week prior to fusion, cells were transferred to media to which had been added 1.0 mM ouabain.

FIGURE 9

SCHEDULE OF ^{35}C LABELING AND INFECTION OF SAMPLES PREPARED TO IDENTIFY HOST AND HCMV NUCLEOCAPSID PROTEINS

Protein samples were prepared in the form of cell lysates or isolated nucleocapsids. Samples 1 contained cellular proteins labeled during the early growth of the cell culture, while sample 2 contained cellular proteins labeled in the later stages of culture. Sample 3 proteins were labeled during early growth but were not harvested until late in culture. Samples 4 and 5 contained isolated nucleocapsids, sample 4 being labeled late in culture (post-infection) and sample 5 was labeled early (pre-infection).


Time (Hours)
0
72
132
1
2
3
4
5
Labelling period
HCMV infected

Identification of the glycoproteins of the HCMV virion has been carried out using radio-labeled glucosamine and mannose (Kim *et al.*, 1976; Stinski, 1977 and Pereira *et al.*, 1984). Also, Farrar and Oram (1986) labeled HCMV glycoproteins using the carbohydrate-specific procedure of oxidation followed by reaction with ^{3}H-borohydride.

SP2 cells were maintained in suspension culture at a density of 3-8 x 10^5 cells/ml. Dulbecco's Modified Eagles Growth Medium (DMF) was supplemented with 10% fetal calf serum and 0.1 mM 8-azaguanine. The presence of 8-azaguanine was to ensure that the generation of revertants did not take place.

B. Immunization Protocols. In an initial study, mice were immunized by the injection of a purified preparation of HCMV nucleocapsids and virions which had been obtained by partial phospholipase digestion of intact virus. This preparation was judged by electron microscopic examination to contain approximately 50% free nucleocapsids and 50% intact or partially enveloped virions. In a subsequent study, a nucleocapsid preparation obtained by treating infected cells with detergents followed by purification on Percoll gradients was used as the innoculum.

Each mouse was injected by the intraperitoneal (I.P.) route with 100 μg of HCMV-protein (as estimated by the Bio-Rad Bradford Protein assay) suspended in 100 μl of Freund's complete adjuvant (Difco Laboratories; Detroit, MI) followed by booster injections (I.P.) at 7, 10 and 21 days of 50μg of HCMV proteins suspended in Freund's incomplete adjuvant. Fusions were performed 3 days after the final injection. A second immunization protocol contained the additional step of intraveneous and I.P. injection of 200 μg of capsid proteins in 100 μl of 0.85% saline on days 22 and 23.

C. Fusion Techniques. The derivation of antibody-producing cell lines from the fusion of mouse spleen cells to the MOPC 315 myeloma line followed the procedure of Longenecker *et al.* (1979). Hybrid cells were selected by growth in RPMI-1640 medium with 10% FCS; oubain (1 mM), and hypoxanthine (100 μM)-aminopterin (0.5

a feeder layer.

Clonality of the cell lines was ensured by sub-cloning cells from positive wells twice. Wells were tested for specific antibody secretion by ELISA using plates coated with the immunizing antigen. In the sub-cloning technique, cells were dispersed using limiting dilution methods into wells containing feeder layers of mouse blood or thymocytes depending on the monoclonal. Hybridomas found to be producing specific monoclonal antibodies were expanded step-wise to obtain sufficient antibody for characterization. Routine tissue culture of the expanded cell lines was carried out in RPMI 1640/DMF containing 10% FCS.

Classification of Immunoglobulin Secreted by Hybridoma Cell Lines. The immunoglobulin class of the monoclonal antibody produced by each cloned hybridoma line was determined by the Ouchterlony double diffusion technique (Ouchterlony, 1948), using agar gel plates supplied by Hyland Diagnostics, (Deerfield, IL). The central well was filled with hybridoma cell culture supernatant (7 μl) and surrounding wells with rabbit antisera (7 μl), specific for the different mouse immunoglobulins (Miles Laboratories, Inc., Elkhart, IN). The plates were incubated in a humidified chamber for 2-3 days at room temperature. Following incubation, the gel was separated from the plate and washed overnight in PBS. Immunoprecipitin bands were visualized by staining the gel with 0.2% Coomassie Brilliant Blue R-250 (Biorad) in 7% acetic acid - 45% methanol and destaining in 7% acetic acid - 40% methanol. In all cases (see Results), these cell lines produced IgM class antibody.

Antibody Purification. Monoclonal IgM antibodies were precipitated from culture supernatants (100-250 ml) by the addition of cold (4°) saturated ammonium sulfate to a volume ratio of 1:1 and stirring overnight at 4°. Precipitated antibody was collected by centrifugation at 24,000 g for 2 hr in a Beckman JA-14 rotor. After centrifugation the antibody was resuspended in 5 ml of distilled water and dialyzed for 24 hrs against

500 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1mM EDTA. Antibodies were then subjected to gel exclusion chromatography on Sephacryl S-300 in this buffer, and the eluant was monitored by measuring its absorption at 280 nm. Fractions of 4 ml were collected and those corresponding to the immunoglobulin M (M_r 9x10^5) were pooled. Purified antibodies were stored at -20°.

Protein Transfer ("Western Blotting"). SDS polyacrylamide gel electrophoresis was performed as described above. Gel replicas were prepared by electrophoretic transfer to nitrocellulose sheets (0.45 μm pore size, Schleicher and Schuell) in a Trans-Blot cell (Bio-Rad). Electrophoretic transfer in the electrophoresis buffer without SDS was carried out at a potential difference of 20 volts for 15 min, followed by 200 volts for a further 30 minutes. After transfer, the proteins which had been "blotted" onto the nitrocellulose were treated with 8M urea for 1 hr in order to remove SDS and aid in the renaturation of active sites. The nitrocellulose was dried between two pieces of Whatman 3mm filter paper and the antigen containing lanes excised. Remaining protein binding sites on the nitrocellulose itself were blocked with 0.3% Tween 20 in PBS, prior to overnight incubation with a solution of 0.05% Tween/PBS containing 5 μg/ml of the purified antibody. The nitrocellulose was then washed (3 x /30 min) with 0.05% Tween/PBS and incubated with biotinylated goat anti-mouse IgM (Sigma) for 2 hrs. Visualization of protein bound antibodies was achieved by using streptavidin-gold (Janssen Pharmaceutica, Beerse, Belgium) according to the manufacturer's directions.

Glycoprotein Detection. Glycoproteins were also detected on Western Blots of HCMV nucleocapsids and virions by employing a probe of biotinylated-Concanavalin A (Sigma). Visualization was with Streptavidin-gold following the methods described above.

Immunofluorescence Assay. Monolayer cell cultures were grown on glass coverslips to 70% confluency and infected with HCMV, at a multiplicity of 1. The cells were cultured for a further 72 hours, at which point the cells were washed with PBS (2x) and

fixed for 10 min in cold methanol (-20°). The cells were allowed to dry at room temperature and then stored at -20° until assayed. Uninfected cells were prepared as controls.

The indirect immunofluorescent assay (IFA) conditions were as follows. For each antibody, one HCMV infected and one uninfected control monolayer coverslip wefe incubated with a 1:20 dilution of monoclonal culture supernatant for one hour. After repeated washings with PBS, the cells were covered with fluorescein isothiocyanate-labeled antiserum to mouse IgM (Sigma) for a further hour incubation. Following a subsequent wash, the cells were mounted in a mixture of 1 part PBS and 9 parts glycerol. Observation and photography were performed on a Leitz Ortholux II fluorescent microscope using Kodachrome 400 ASA film.

RESULTS AND DISCUSSION

Nucleocapsid Protein Characterization. Nucleocapsids isolated from the nuclei of HCMV infected cells were found to contain at least 28 proteins by SDS PAGE under reducing conditions. Electron micrographs of this preparation of nucleocapsids, showed that the sample appeared to be free of contaminating cell debris (Fig. 10). We would therefore assume that the proteins we have observed are constituents of the nucleocapsids. Autoradiographs of SDS polyacrylamide gels containing nucleocapsid proteins labeled with ^{35}S-amino acids, indicated the presence of 5 major proteins of M_r ~250, 55, 42, 17 and 14 kD and 4 additional proteins M_r ~270, 190, 145 and 16 kD which were considered to be present in significant quantities within the nucleocapsid (Fig. 11).

The HCMV genome of 230 kbp is considerably larger than that of the other herpesviruses and is capable of encoding a large number of proteins. To put this into perspective, Equine herpes virus-1 with a genome of 143 kbp has been reported to encode 74 proteins (Gray *et al*., 1987). Although the HCMV DNA has the capacity to code for all of the nucleocapsid proteins we have detected, in addition to tegument and membrane proteins, we thought that such a large number of proteins present within the nucleocapsid might include cellular proteins incorporated into the nucleocapsid during assembly. Unlike other members of the herpesviruses, for example herpes simplex virus (Fenwick and Walker, 1978) and Epstein-Barr virus (Nonoyana and Pagano, 1972) HCMV does not completely shut off host protein synthesis. This renders difficult the task of identifying virus-coded proteins. According to Stinski (1978), protein synthesis following HCMV infection may be divided into two stages, an early stage that is 70-90% host specific and a late stage, coinciding with viral DNA synthesis at 45 hours post-infection, during which protein synthesis is predominantly viral, but 40-50% of the protein synthesized is still of host origin. We set out to determine the origin of nucleocapsid proteins by using a series of pulse-chase analyses. The labeling and

FIGURE 10

ELECTRON MICROGRAPH OF ISOLATED HCMV

NUCLEOCAPSID PREPARATIONS

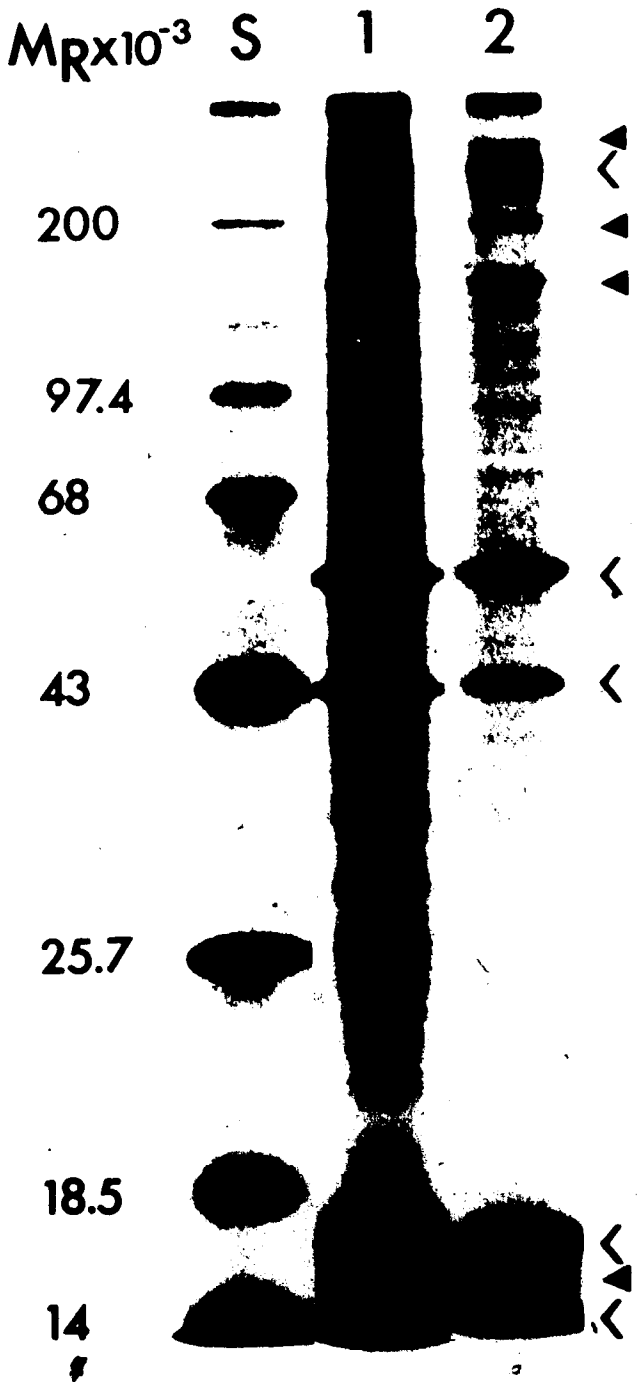
Nucleocapsids were isolated by the treatment of HCMV infected cells with Nonidet P40 and sodium deoxycholate followed by separation on a gradient of Percoll. This preparation was stained negatively with 2% sodium phosphotungstate (pH 7.0) and photographed in a Philips EM 420 electron microscope. The small spherical particles in the background are Percoll beads.


100nm

FIGURE 11

NUCLEOCAPSID PROTEINS LABELED WITH ^{14}C AMINO ACIDS

Lanes 1 and 2 are autoradiographs of the same gel sample exposed for 12 and 7 days respectively. Major nucleocapsid proteins are indicated by arrowheads whereas proteins indicated by arrows were considered to be present in significant quantity. The standard lane (S) contained ^{14}C-labeled high molecular weight marker proteins (from B R L).


$M_R \times 10^{-3}$
S
1
2
200
97.4
68
43
25.7
18.5
14

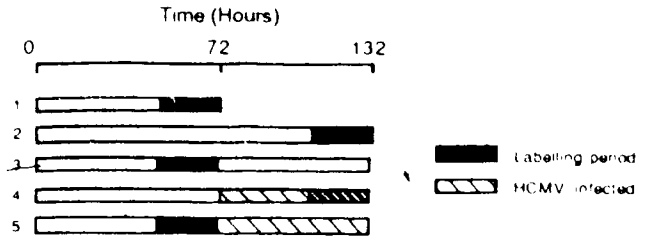
infection schedule is given in Figure 9. Comparison of the pulse-chase ^{14}C-labeled cellular lysates with ^{14}C-labeled nucleocapsids was made in order to determine the presence of coincidental proteins (Fig. 12). Scanning densitometry was used to accurately identify proteins of identical mobilities (within 1.5%) found in host lysates and nucleocapsids. A total of eight proteins were thus identified as being potentially host in origin (Fig. 13). The M_r of these proteins were 190, 98, 90, 64, 42, 47, 34, 26.3. The major nucleocapsid protein M_r 55 was found to be slightly larger than cellular proteins > M_r 54. We also noticed that one of the major capsid proteins, M_r 42 kD, comigrated with a host protein and therefore investigated this protein further. Losse *et al.* (1982) reported the "early functions" of HCMV to induce the loss of microfilaments, while actin synthesis appeared to be largely unchanged as estimated from the specific radioactivity of cytoplasmic actin. Since the M_r of SDS-actin is 43 kD we thought that the major HCMV capsid protein comigrating with a cellular protein may be actin. However, this was not so since Western blots of nucleocapsid proteins failed to react with polyclonal rabbit sera raised to SDS-actin. Another major protein M_r 190 was also of potential host origin but was not further investigated. Not all of the coincidental proteins found in samples 2, 3 and 4 were found in sample 5, the nucleocapsids isolated from prelabeled cells (Figure 12). From these studies we have concluded that the capsid proteins M_r 47, 36.5 and 26.3 are of viral origin. So, to summarize from Figures 11, 12 and 13: the major nucleocapsid proteins of viral origin are of apparent molecular mass ~250, 55, ~17 and 14 kD while the major nucleocapsid protein of M_r 42 kD is of probable host origin.

Specificity of Monoclonal Antibodies. The fusion of HCMV immunized mice spleenocytes with the mouse myeloma lines MOPC 314.43 and SP2/O-Ag14 resulted in the generation of eleven monoclonal antibody secreting cell lines. By Ouchterlony double diffusion assay, all these lines were found to secrete antibodies of the IgM class. The antibodies were characterized with regards to specificity using the Western blot

FIGURE 12

LABELING OF PROTEINS IN TOTAL LYSATES FROM CONTROL CELLS AND FROM ISOLATED HCMV NUCLEOCAPSIDS

The upper panel shows the labeling schedule for each sample. The lower panel shows the resolution of proteins on SDS-10% polyacrylamide gels. Solid arrows indicate coincidental proteins in samples 2, 3, 4 and 5. Hollow arrows indicate coincidental proteins in samples 2, 3 and 4 without a coincidental protein in sample 5. The star indicates a major nucleocapsid protein M_r 17kD labeled in sample 5 but absent from cellular lysates. The label in this sample represents ^{14}C amino acids returned to the amino acid pool protein by turnover of labeled cellular proteins. Densitometer scans of lanes 2, 3 and 4 are shown in Figure 13.


Time (Hours)
0
72
132
1
2
3
4
5
Labelling period
HCMV infected

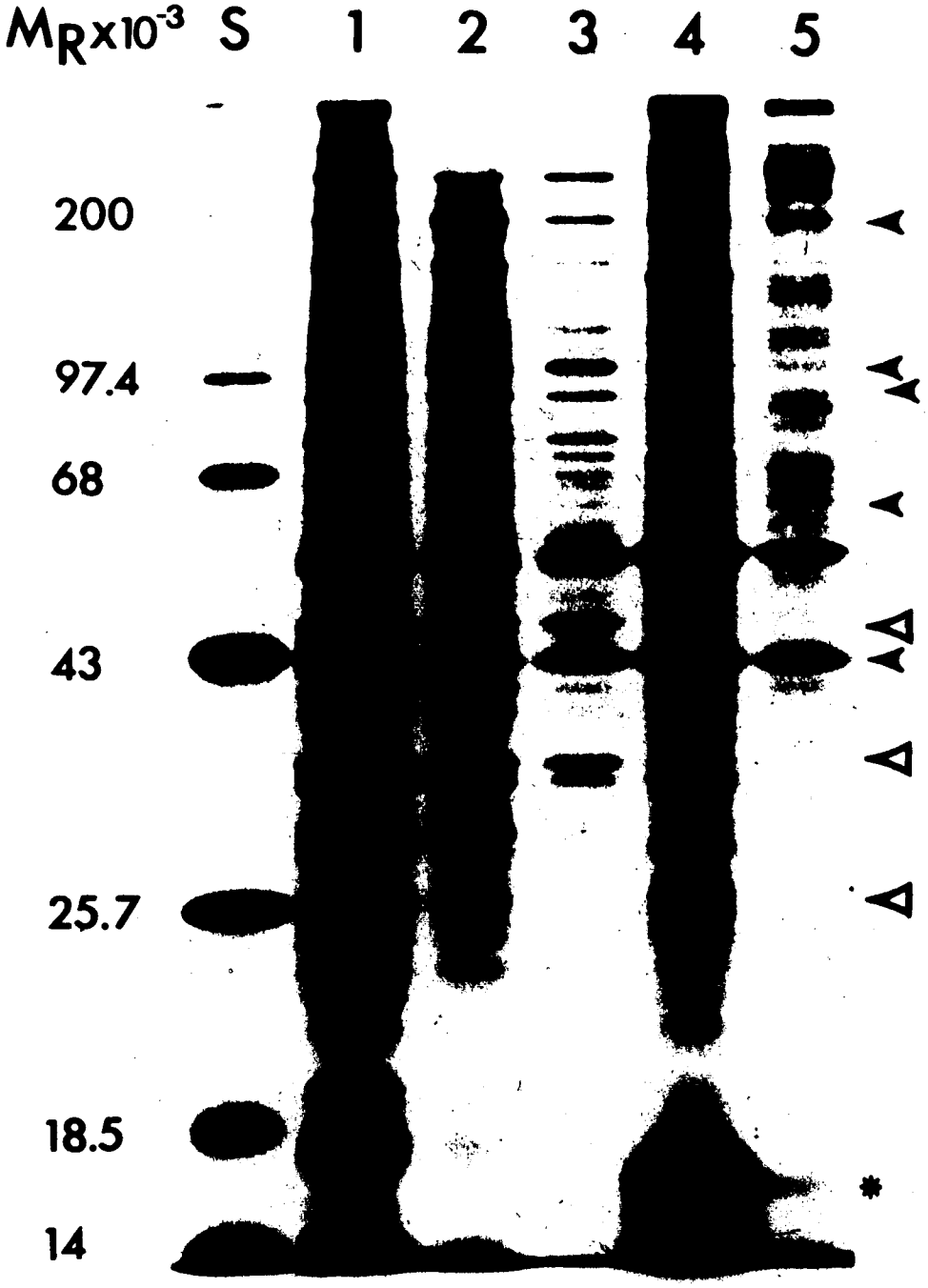
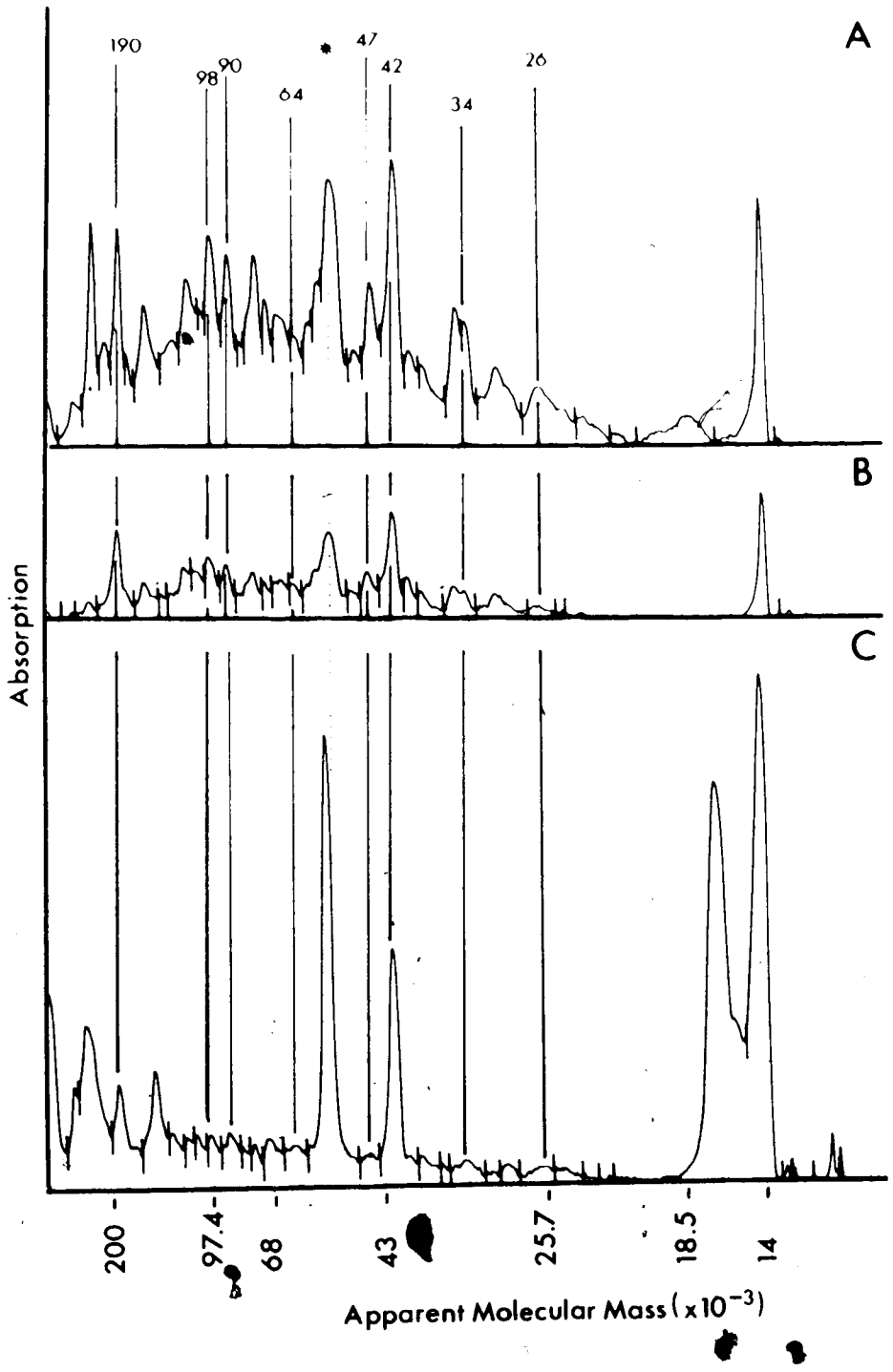

$M_R \times 10^{-3}$
S
1
2
3
4
5
200
97.4
68
43
25.7
18.5
14

FIGURE 13

SCANNING DENSITOMETER PLOTS FROM AUTORADIOGRAPHS OF

^{14}C-LABELED CELLULAR AND NUCLEOCAPSID PROTEINS

Scanning densitometer plots from autoradiographs of ^{14}C-labeled cellular and nucleocapsid proteins. Comigrating proteins are indicated by vertical lines and apparent molecular masses are indicated in Panel A. The star indicates proteins of approximate but not identical mobilities in SDS-Page. The samples were of equal protein concentration, as determined by the fluorometric protein assay of Bohlen *et al.*, 1973. Panel A corresponds to lane 2 of Figure 12, panel B to lane 3, and panel C to lane 4. Panel A shows cellular proteins labeled by incubation for 24 hours in media containing ^{14}C amino acids. Cells in this culture were harvested at the time of infection. The sample for Panel B shows the labeled cellular proteins present in uninfected cultures after a time period equivalent to that for the nucleocapsid production in HCMV infected cells. Note the decrease in overall protein labeling due to protein turnover during the 60 hour infection period. Panel C shows the isolated nucleocapsid proteins labeled for 24 hours prior to nucleocapsid isolation.


A
B
C
190
98
90
64
47
42
34
26
Absorption
200
97.4
68
43
25.7
18.5
14
Apparent Molecular Mass (x10⁻³)

techniques described in Materials and Methods. All of the monoclonal antibodies appeared to be of similar specificities; when incubated with nucleocapsid antigen, interaction with a protein M_r 64 kD was indicated. However, when incubated with virion antigen, the interaction with the 64 kD protein was less prominent and strong interaction with proteins M_r 90, 27 and 14 kD was observed (Figure 14). No interaction was found by Western blotting when either of the monoclonal antibodies was incubated with cellular proteins. In IFA analysis, all of the monoclonal antibodies showed strong overall fluorescence when incubated with HCMV infected cells (Figure 15). No fluorescence was observed with uninfected (control) cells.

These results indicate that the antibodies interact with a polypeptide of 64 kD present in the nucleocapsid, which is subsequently modified to polypeptides of M_r 90, 27 and 14 kD in the virion. As indicated in the following section, a glycoprotein present in the nucleocapsid comigrates with an apparent molecular mass of 64 kD. Virion glycoproteins were also observed with molecular masses of 90, 27 and 14 kD (see section below). One may propose a possible explanation for the shifts in antibody specificity observed with nucleocapsid and viral antigens to be due to both glycosylation and proteolytic processing of the 64 kD nucleocapsid antigen.

Glycoprotein Components. The presence of glycoproteins and their mannose containing precursors was investigated using Western blotting techniques and a biotinylated Concanavalin-A probe. With nucleocapsid antigens, a major glycoprotein of apparent molecular mass 64 kD was indicated; minor components of 150 and 17 kD were also found (Figure 16, lane N). The presence of glycoproteins or their precursors in the HCMV nucleocapsid has not previously been recorded. In preparations of HCMV virions, major glycoproteins of 125, 90, 62, 53 and 27 and 14 kD were detected (Figure 16, lane V). These glycoproteins fit well into the framework proposed by Pereira *et al.*, (1984) with each of these group classes, A, B, C and D being represented by one or two glycoproteins (see Table 3).

FIGURE 14

WESTERN BLOTS OF MONOCLONAL ANTIBODIES PRODUCED BY CELL LINES 10.5 AND 51.3 AGAINST NUCLEOCAPSID (N) AND VIRAL (V) ANTIGENS

Monoclonal antibody 10.5 showed strong interaction with a nucleocapsid protein M_r 64 kD and minor interaction with viral proteins M_r 90, 27 and 14 kD whereas antibody 51.3 showed minor interaction with the nucleocapsid protein M_r 64 kD and major interaction with the viral proteins M_r 90, 27 and 14 kD.

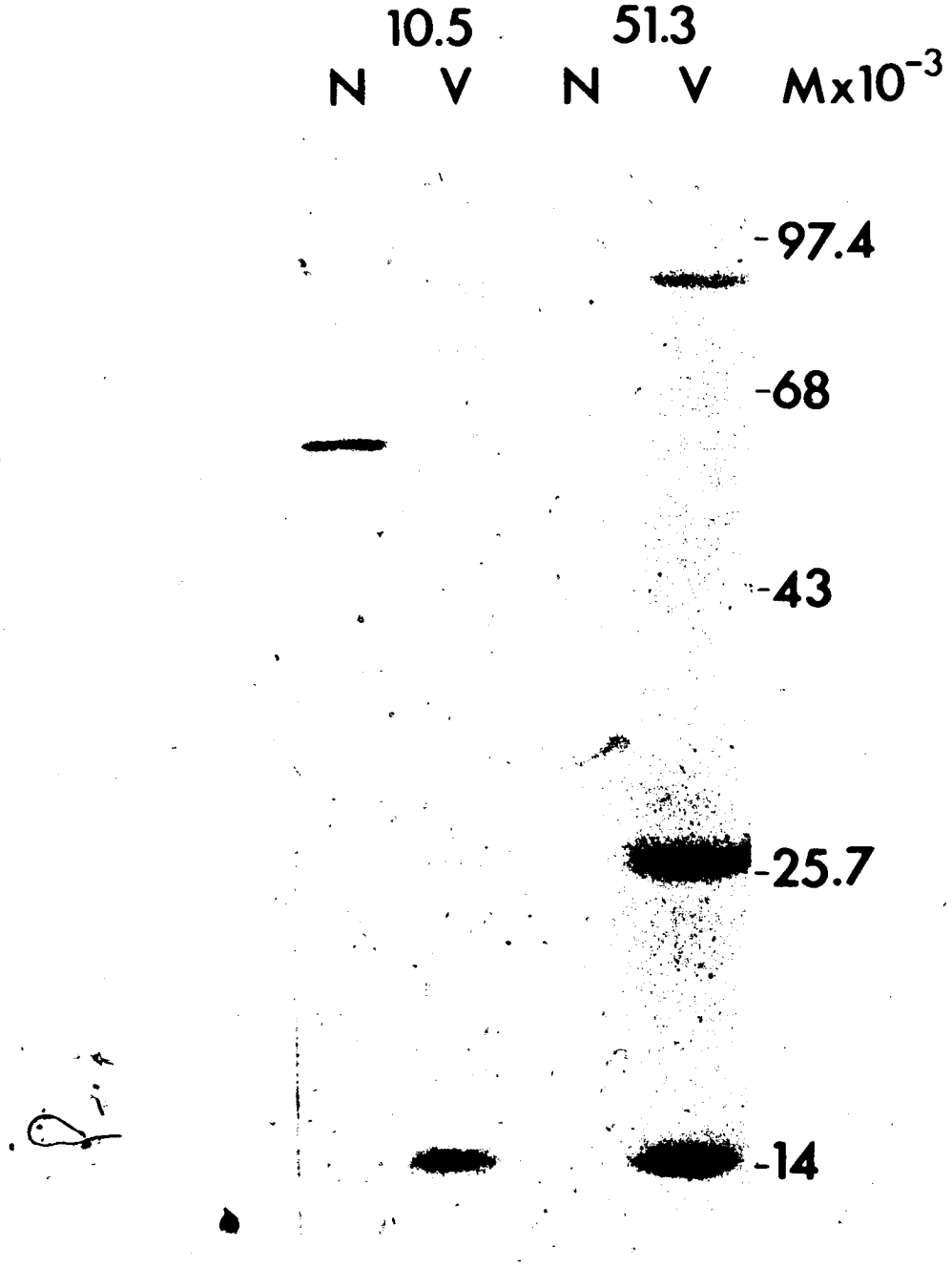

10.5
51.3
N V N V Mx10^{-3}
-97.4
-68
-43
-25.7
-14

FIGURE 15

IMMUNOFLUORESCENCE MICROGRAPHS OF HCMV INFECTED AND UNINFECTED CELLS FOLLOWING INCUBATION WITH MONOCLONAL ANTIBODY 10.5

Infected and uninfected monolayers of cells were incubated with mouse monoclonal antibody 10.5 and antibody binding was subsequently visualized using FITC-labeled goat anti-mouse antisera. Infected cells (Panel A) appeared to give a bright fluorescence over the entire cell, with nuclear and cytoplasmic inclusions clearly indicated. Uninfected control cells at the same magnification as the infected cells appeared dark with only a generalized background fluorescence, no nuclear fluorescence was visible. Panel B shows a lower magnification "survey" photograph of uninfected cells exposed for 1 minute. The exposure for panel A was 12 seconds. (Magnifications, Panel A 200x, Panel B 50x).

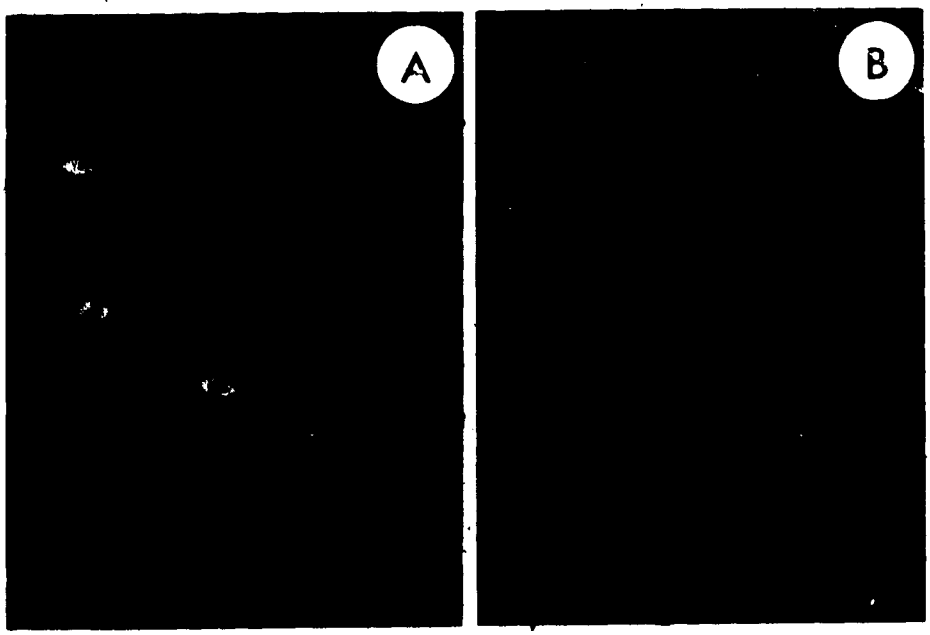
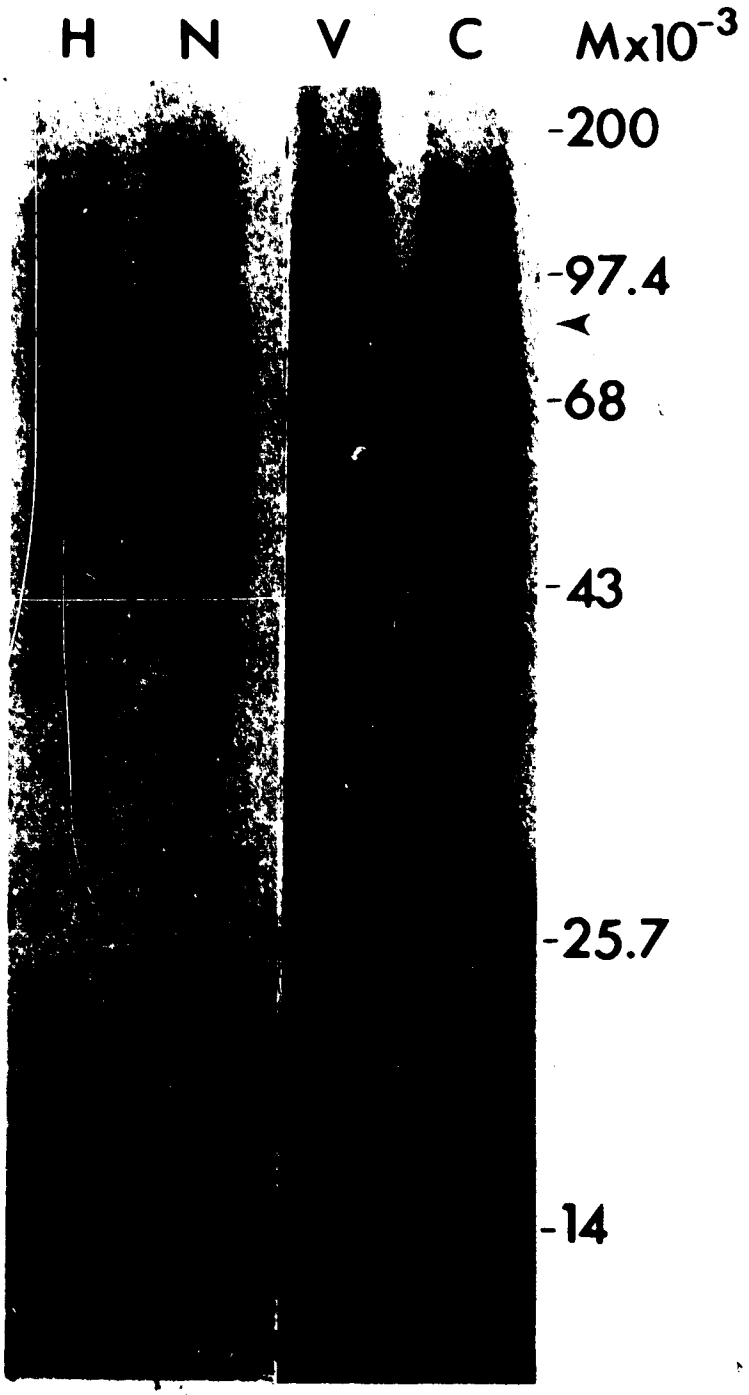

A
B

FIGURE 16

NUCLEOCAPSID AND VIRAL GLYCOPROTEIN DETECTED BY WESTERN BLOTTING

Glycoproteins were detected by the binding of biotinylated concanavalin A and subsequent visualization with streptavidin labeled colloidal gold. Concanavalin A binds specifically to α D-mannose and α D glucose. Lane H contained herpes simplex virus proteins as a control, lane N contained HCMV nucleocapsid proteins, lane V contained HCMV viral proteins and lane C contained cellular proteins. Western blotting clearly indicated the presence of a glycoprotein M_r 64 kD in the HCMV nucleocapsid. Arrow indicates viral glycoprotein M_r 90 kD.


H
N
V
C
Mx10-3
-200
-97.4
-68
-43
-25.7
-14

Summary of Nucleocapsid Proteins. Our studies have found nucleocapsids isolated from the nuclei of HCMV infected cells to contain approximately 28 proteins (Table 4), a number greater than those reported by Schmitz *et al.*, (1980) or Gibson, (1981). This may be attributed to increased sensitivity in detection (protein radiolabeling versus Coomassie Blue protein staining) used by Schmitz *et al.*, (1980) and increased concentration of radio-labeled amino acids (5 μCi/ml ^{14}C label versus 2 μCi/ml ^{14}C-label used by Gibson (1981). Schmitz *et al.*, (1980) concluded that nucleocapsids of HCMV strain AD 169 contained prominent proteins of M_r ~140, 86, 63, 51, 44 and 23 kD. Gibson (1981) reported that HCMV strain Colburn nucleocapsids (Type A capsids which closely resembled our nucleocapsids in electron microscopy) were composed of three proteins M_r 145, 34 and 28 kD. Although we agree that the 145 kD protein is likely to represent the major structural element of the nucleocapsid, we do not find large amounts of the other proteins in our nucleocapsid preparations.

Our studies indicate the presence of 5 major proteins and an additional 4 prominent proteins in the HCMV nucleocapsid. One protein M_r 64 kD which did not label well with ^{14}C amino acids but which did stain with silver (at late times in infection) reacted with all of our mouse monoclonal antibodies. This protein (or one with the same M_r) is glycosylated. It is possible that the 56 kD protein which did label prominently with ^{14}C-amino acids early in infection represents an unglycosylated precursor to the 64 kD protein. In herpes simplex virus, glycoprotein D M_r 60-65 kD has been reported to have an unglycosylated precursor M_r 52 kD (Spear, 1976).

In Western blots against viral antigen, the anti 64 kD nucleocapsid antibody reacted with 3 proteins (M_r 90, 27 and 14 kD) indicating a precursor-product relationship with the 64 kD protein of the nucleocapsid. We therefore have concluded that during virion formation the 64 kD protein is processed to yield the 90, 27 and 14 kD proteins. All of these proteins migrate with the same apparent molecular mass as glycoproteins found in the HCMV virion (Figure 16). One could conceive that the

TABLE 4.

Summary of the properties of HCMV nucleocapsid proteins.

	Apparent molecular mass (kD)	Major protein[1]	Host	Glycoprotein[2]	Reactive with antibody
1	270	x			
2	250	X			
3	190	x	X		
4	145	x		(150)m	
5	125				
6	115				
7	107				
8	98		X		
9	90		X		
10	85				
11	79				
12	73				
13	64			M	+
14	55	X			
15	47				
16	42	X	X		
17	39				
18	36.5				
19	34				
20	31.5				
21	30				
22	27				
23	26.3				
24	24				
25	23				
26	17	X		m	
27	16	x			
28	14	X			

[1] X denotes major nucleocapsid protein; x prominent protein.
[2] M indicates major glycoprotein; m minor glycoprotein.

64 kD protein is an initial translation product which is glycosylated to yield a 90 kD protein (this M_r may be overestimated owing to the anomalous migration of glycoproteins in SDS-PAGE; Weber and Osborn, 1978). The 90 kD protein may then be proteolytically cleaved to yield the 27 and 14 kD species.

Of the major proteins found in the nucleocapsid, only one of M_r 190 kD is potentially of host origin. The M_r 42 protein (which is not actin) is prominent in both viral and nucleocapsid preparations. Figure 17 shows this protein from a different preparation which was silver-stained for protein following SDS-PAGE. Similarly, the failure of the monoclonal antibodies to recognize host proteins discounts the possibility of the host 64 kD protein being identical to the nucleocapsid 64 kD protein. Our analysis has shown that isolated nucleocapsid contain preparations of cellular proteins, the role and composition of which remains unknown.

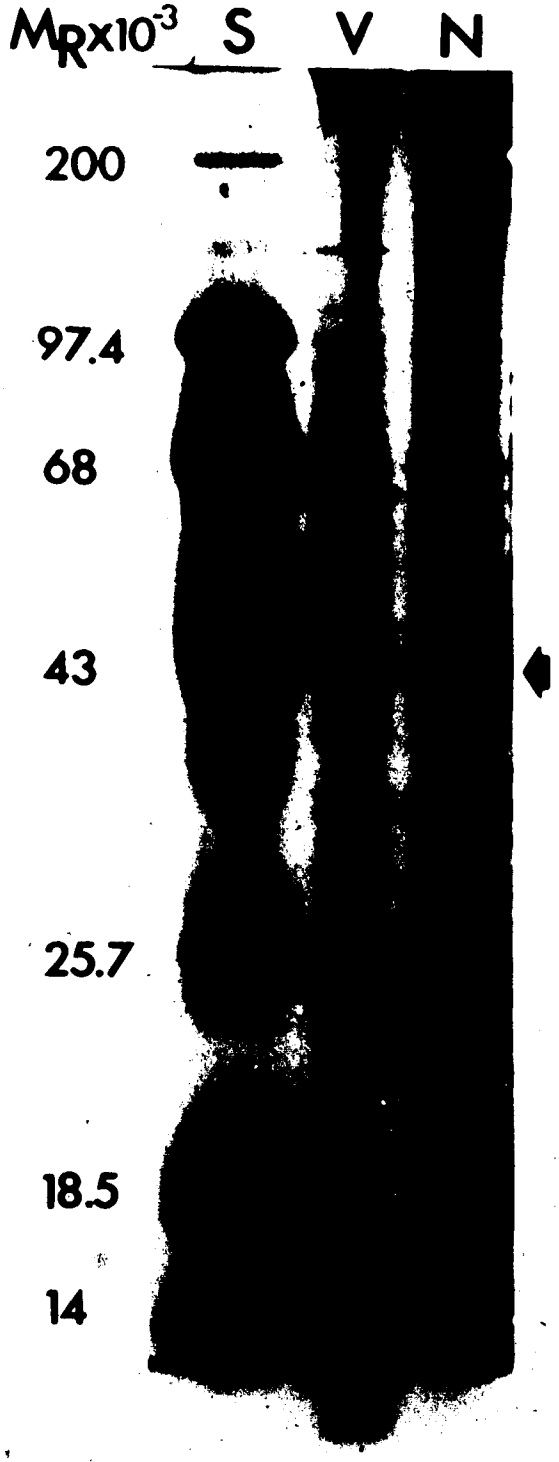
The discovery of glycoproteins in the nucleocapsid is interesting, since glycosylation is not a common modification of nuclear proteins. Farrar and Oram (1986) reported metaperiodiate labeling (indicative of glycosylated proteins) of nucleocapsid proteins with M_r of 35, 64 and 150 kD, but thought that this may have been due to non-specific oxidation of non-glycosylated proteins. The binding of concanavalin A to the M_r 64 kD protein found in our studies confirms the presence of a glycoprotein, or its high mannose precursor within the nucleocapsid. The recognition of this protein and its processed viral counterparts by all of our mouse monoclonal antibodies indicates that this glycoprotein may be of immunological importance.

One of the requirements for a diagnostic assay based on a capture-ELISA system is high affinity antibodies. Immunoglobulin M class antibodies are inheritantly less specific than those of the IgG class. Our lack of success in generating IgG class monoclonal antibodies from mice lead us to a different approach for monoclonal antibody production, that of producing human monoclonal antibodies by the transformation of B-lymphocytes with Epstein-Barr virus.

FIGURE 17

HCMV NUCLEOCAPSID AND VIRION PROTEINS SEPARATED BY SDS PAGE AND STAINED FOR PROTEIN WITH SILVER-CONTAINING REAGENTS

The arrow indicates a major nucleocapsid protein of M_r 42 kD which appeared consistently in nucleocapsid preparations. Protein standards were prestained high molecular marker proteins (BRL).


$M_R \times 10^{-3}$
S
V
N
200
97.4
68
43
25.7
18.5
14

CHAPTER III

HUMAN CYTOMEGALOVIRUS II:
THE SELECTION AND CHARACTERIZATION OF HUMAN MONOCLONAL ANTIBODIES TO HCMV

INTRODUCTION

In 1975, Kohler and Milstein demonstrated that it was possible to fuse myeloma cells, derived from a malignancy of B-lymphocytes, to B-lymphocytes from an immunized animal to produce a cell line that grew continuously and secreted antibody continuously. Thus the technology of hybridomas and monoclonal antibodies was born. The significance of this discovery and the subsequent advances made by its application to the fields of the biological sciences and medicine, were marked in 1984 by the awarding of the Nobel Prize in Medicine to Kohler and Milstein.

One limitation of the fusion technology devised by Kohler and Milstein has been the availability of cell lines to which lymphocytes can be fused. Although rodent myeloma lines are available (mouse, rat and guinea pig) human myeloma lines are more difficult to obtain (Table 5). The desire to obtain human monoclonal antibodies has been driven by the search for antibodies of greater specificity than those obtained through rodent fusions. The immunization of rodents with human tumor material has demonstrated that the antibodies produced emphasize certain cell surface components that delineate the "foreignness" of the animal species rather than the differences between tumor and normal cells. A lack of fine specificity to different molecular polymorphisms found in certain structures, *e.g.* human HLA, has also been observed. Human monoclonal antibodies have the additional benefit of direct use on patients for diagnostic or therapeutic purposes without fear of sensitization or immune response.

In order to produce human monoclonal antibodies, human B-cells have been immortalized by the fusion of human lymphocytes with drug resistant mouse (Teng

TABLE 5.

Human cell lines for hybridization.

Cell line	Ig secreted[1]	Reference
U-266	IgE	Nilsonn *et al.*; 1970
RPMI-8226	λ	Matsuoka *et al.*; 1967
UC 729-6	IgM	Glassy *et al.*; 1983
UC 727-HF2	None	Abrams *et al.*; 1983
LICR-LON-HMy2	IgG	Edwards *et al.*; 1982
GM 4672	IgG	Croce *et al.*; 1980
SKO-007	IgE	Olsson & Kaplan;1980
RH-L4	IgG (not secreted)	Brodin *et al.*; 1983
8226 AR/NIP4-1	None	Pickering & Gelder;1982
WIL2/729HF		Denis *et al.*; 1983

[1] Ideally, fusion partners should not express or secrete immunoglobulin. In hybridomas made by the combination of non-ideal-partners, there is often a decrease in specific antibody production due to irrelevant recombination of the parental heavy and / or light chains. (L.Winger, personal communication.)

et al., 1983), human myeloma (Edwards *et al.*, 1982), or lymphoblastoid cell lines (Olsson & Kaplan, 1980). The transformation of human B-lymphocytes with Epstein-Barr virus (EBV) has also been used successfully. The first report of the successful immortilization of specific antibody producing cells was by Steinitz *et al.*, (1977). Since that time monoclonal antibodies directed against a wide variety of antigens have been produced by this method. These include antibodies to hapten molecules (Kozbor *et al.*, 1979; Steinitz *et al.*, 1979), autoantigens (Steinitz *et al.*, 1980; Kamo *et al.*, 1982), viral (Crawford *et al.*, 1983a; Seigneurin *et al.*, 1983), bacterial (Rosen *et al.*, 1983; Steinitz *et al.*, 1984; Kozbor and Roder, 1981) or protozoal (Lundgren *et al.*, 1983) antigens, blood group antigens (Crawford *et al.*, 1983b), or tumor-associated antigens (Irie *et al.*, 1982).

Each system for producing human monoclonal antibodies has problems. In the fusion system, as pointed out earlier, there is a shortage of human fusion partners. Most heterohybridomas, mouse-human or rat-human, have proved unsuccessful due to the chromosomal instability of the fusion; human chromosomes are preferentially eliminated (Shay, 1985). The EBV-transformation system too has associated problems. Cell lines produced in this fashion are often short-lived and antibody production is often low (Bird *et al.*, 1981). Recently, Roder *et al.* (1985) have combined the two techniques and have fused EBV-transformed cell lines with human plasmacytomas. The resulting technique uses the EBV system to expand the rare antigen specific B cells in the peripheral blood prior to fusion. These fusion lines have been found to be extremely stable and produce good yields of antibody. It is likely that the Roder system will become the method of choice in the future.

Epstein-Barr virus (EBV) is a ubiquitous herpes virus that infects most adult individuals worldwide, and can usually be identified in peripheral blood (Henle and Henle, 1979). Unlike other human blood cells, B-lymphocytes express an EBV-specific surface receptor, CR-2, (Yefenof and Klein, 1977; Nemerow *et al.*, 1985) and are

accordingly a primary target for infection with EBV. Infection with an immortalizing strain of EBV, for example B95.8, induces a fraction of immunocompetent peripheral blood cells to proliferate and secrete IgG (Bird and Britton, 1979). This process is dependent on the infection of the B lymphocytes with biologically active virus and does not require either prior immunity of the lymphocyte donor to EBV (Tosato *et al.*, 1982) or the presence of T-cells or accessory cells (Kirschner *et al.*, 1979). The immunocompetent lymphocytes can be specifically selected and immortalized (Steinitz and Tami[illegible]82; Crawford *et al.*, 1983a,b) with high efficiency in a limiting dilution transformation system (Winger *et al.*, 1983). Approximately 48 h after *in vitro* exposure to the virus, B-cells begin to proliferate and after 3-4 days they become activated into immunoglobulin (Ig)-secreting cells. Eventually a proportion of these B-cells become transformed into immortal cell lines that express a B cell phenotype and secrete Ig. It is still unclear exactly when during development cells of B lineage acquire a receptor for EBV. It was recently reported that "immature B cells" (cells that have not as yet undergone gene rearrangement; Katamine *et al.*, 1984) and "pre-B cells" (surface Ig-negative cells expressing cytoplasmic μ-chains) can be immortalized by the virus (Hansson *et al.*, 1983) suggesting that EBV receptor expression is an early event in B-cell development.

EBV is capable of activating resting B-lymphocytes into Ig-secreting cells, but there is no evidence that infection of immature B cells with this virus facilitate their further differentiation. For example, peripheral blood B cells committed to IgM production do not undergo Ig class switch upon EBV infection (Yarchoan *et al.*, 1983). In addition, EBV-immortalized cells with Ig genes in germ-line configuration have not been observed to rearrange their Ig genes (Katamine *et al.*, 1984). Conversely, EBV immortalization of mature B cells has not been associated with their acquisition of a more immature phenotype. For example, expression of a variety of cell surface markers including membrane Ig, C3b receptor, and major histocompatability complex antigens is

comparable in EBV-immortalized B cells with those of normal B cells and B blasts (Nilsson and Klein, 1982).

In spite of much study, the mechanism and molecular basis for B cell immortalization by EBV is only now becoming clear. Wang *et al.* (1987) have described how one protein, the EBV virion glycoprotein gp 350/220 is responsible for attachment to, penetration of and release from B lymphocytes by the EBV virion. Glycoprotein gp 350/220 is a highly specific ligand for CR-2 (Nemerow *et al.*, 1987). Binding to the CR-2 receptors results in aggregation of CR-2 receptor proteins (capping). The surface Ig were reported to cocap with CR2 (Wang *et al.*, 1987) and subsequently be endocytosed. The cocapping of surface Ig may be a key step in the stimulation of proliferation and final differentiation of B cells (Eisen, 1980).

Infection with EBV results in the persistence of the virus genome as an episome or integrated into the B cell DNA. The expression of viral genes is highly restricted; only five gene products are regularly expressed during latent infection. One of these, EBNA-2 (EBNA-Epstein-Barr Nuclear Antigen) is required for B cell enlargement and proliferation to high cell densities. EBNA-2 has been characterized as a direct or indirect transactivator of expression of the B lymphocyte surface protein CD 23. CD 23 is the receptor protein for B cell growth factor (IL-4), which is required for B cell activation and proliferation (Yokato *et al.*, 1986; Noelle *et al.*, 1984; Noma *et al.* 1986). Following EBV-transformation, B lymphoblasts release a soluble factor which mimics the B-cell factors produced by mitogen stimulated T cells (Gordon *et al.* 1984). The virally-transformed cells then utilize this activity to sustain their own growth.

Detailed analysis of EBV-DNA demonstrated considerable similarities in fragments generated by restriction endonuclease cleavage of transforming (immortalizing) and nontransforming strains of EBV (Heller *et al.*, 1981), and it was not until 1985 that Skare *et al.* were able to localize the transforming sequence of EBV. Although a large percentage of B cells are able to bind EBV (Graves *et al.*, 1975; Aman *et al.*, 1984),

only a small proportion are transformed as a result of infection (Henderson *et al.*, 1977; Zerbini and Ernberg, 1983). Investigators have endeavored to determine the nature of the transformable subset (Aman *et al.*, 1984; Robinson *et al.*, 1979; Einhorn and Klein, 1981, Chan *et al.*, 1986; Koome and Reading, 1987) and have, in general, concluded that the primary target for EBV transformation lay in the resting B cell subset in peripheral blood, tonsils or umbilical cord. With activation and entry into S-phase, the number of susceptible B cells decreases. The reasons for the loss of the capacity for transformation are presently unknown, but the results of Aman *et al.*, (1984), suggest that it is not due to a loss of the EBV-receptor or the ability of the virus to penetrate the cell. However, other work indicates that *in vitro* activation of B cells by the addition of anti-IgM antibody leads to the loss of CR-2, the receptor for EBV (Boyd *et al.*, 1985). These authors report that CR-2 expression declines in culture from 74% on day 0 to 9% by day 4 in response to anti-Ig stimulation. Consonant with these findings is the conclusion that the enrichment of antigen specific B lymphocytes prior to EBV transformation is optimally carried out by selection of the desired cells from the resting B cell subset (Winger *et al.*, 1983) rather than attempting the propagation of these cells via *in vitro* immunization. The introduction of antigen to B lymphocytes will result in activation and loss of susceptibility for EBV-transformation.

We have applied EBV transformation to a simple B cell selection technique, which increases expression of Ig producing transformants, as well as initial success in generating, from a random sampling of blood donors, transformants producing either IgM and IgG capable of binding to human cytomegalovirus (HCMV). Since a large proportion of the world adult population has been exposed to HCMV (Krech, 1973), this antigenic system appeared to be appropriate for a systematic study of the available (primary) and immune (secondary) human antibody response to herpes type viruses, and so may contribute to the development and in vitro testing of specific vaccines.

MATERIALS AND METHODS

Antigen Preparation. The preparation of HCMV virions and nucleocapsids has been described in Chapter II of this thesis.

Lymphocyte Preparation. Referring to Fig. 18, segment 1, lymphocytes were prepared following the protocol of Winger *et al.* (1983). Briefly, lymphocytes were purified by density gradient separation on Ficoll-Hypaque (Pharmacia). T cells were depleted from the B-lymphocytes by erythrocyte rosette formation with sheep red blood cells (SRBC) treated with 2-aminoethylisothiouronium bromide hydrobromide (AET; Sigma) (Kaplan & Clark, 1974). Rosettes were separated into positive (E+; T cells) and negative (E-; B-cells and monocytes) fractions by centrifugation through Percoll. E- cells at the interface were harvested and the E+ cells in the pellet were discarded. Recovery of E- cells was 10-30% of the input peripheral blood mononuclear cells. The E- cells typically comprised about 50% B-cells and 50% monocytes.

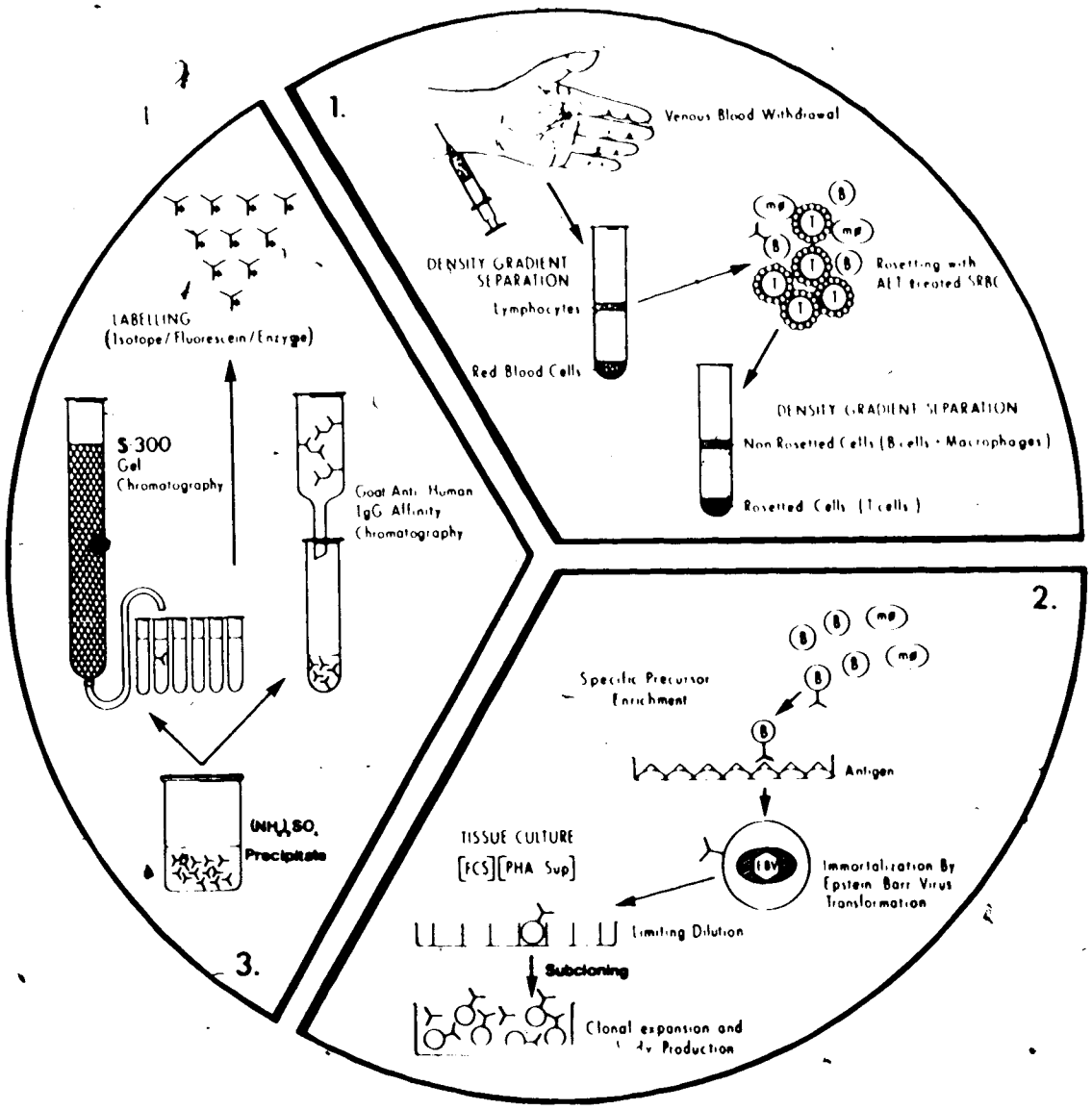
Specific Precursor Cell Enrichment - Transformation. Referring to Fig. 18, segment 2, HCMV antigens in PBS were allowed to adhere overnight to each of the wells of a Costar 6-well culture plate (protein concentration for coating was 5-10 μg/ml). The antigen-carrying plate was then washed 3 times with PBS, and the remaining protein binding sites blocked by incubating with 10% FCS for 1 hour at 4°. Maintaining the temperature at 4°, 5 x 10^6 E- cells in 2 ml RPMI-1640 (Flow Labs) containing 5% FCS were added to each well and allowed to settle for 2-3 hours. The plate was then gently swirled, and any unbound cells removed by aspiration. The wells were washed by slowly adding cold RPMI-1640/5% FCS down one side of the well and gently swirling. One wash was found to remove about 95% of E- cells. The bound cells were subsequently removed by vigorous direct pipetting with RPMI-1640/5% FCS.

Up to 10^6 of these E- cells were resuspended in 1 ml of Epstein-Barr Virus (EBV) supernatant containing 10^6 transforming units/ml. The EBV supernatant used was

FIGURE 18

SCHEMATIC DIAGRAM OF PROCEDURES EMPLOYED FOR THE PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES

Details are given in the text


1.
Venous Blood Withdrawal
DENSITY GRADIENT SEPARATION
Lymphocytes
Red Blood Cells
Rosetting with AET treated SRBC
DENSITY GRADIENT SEPARATION
Non Rosetted Cells (B cells + Macrophages)
Rosetted Cells (T cells)
2.
Specific Precursor Enrichment
Antigen
TISSUE CULTURE
[FCS][PHA Sup]
EBV
Immortalization By Epstein Barr Virus Transformation
Limiting Dilution
Subcloning
Clonal expansion and Ab Production
3.
LABELLING
(Isotope/Fluorescein/Enzyme)
S 300
Gel
Chromatography
Goat Anti Human
IgG Affinity
Chromatography
(NH4)2SO4
Precipitate

from the producer line B95.8, and was obtained from Dr. R. Lau of the Showa University Research Institute, St. Petersburg, FL. Transformation was accomplished during a minimum of 4 hr incubation at 37°. Suspensions were then centrifuged at 250 *g* for 5 min, the supernatant removed and the cell pellet resuspended in RPMI-1640/20% FCS at the desired concentrations for limiting dilution culture.

Feeder layers consisting of surplus EBV cells were irradiated at 15-20 gray (Gammacell, Atomic Energy of Canada, Chalk River, Ontario) in RPMI-1640/20% FCS, containing garamycin (60 μg/ml), and cultured at a concentration of 10^5 cells per well in 96-well flat-bottomed microtiter plates (Linbro; Flow Labs).

Specific precursor-enriched cells EBV were cultured with the feeder layer at limiting dilutions (10^4-10 cells per well) in a total volume of 200 μl of culture medium. After 9-11 days, 100 μl of supernatant was removed and replaced with 100 μl of fresh medium containing 10% FCS. All subsequent culture work was then carried out using 5% FCS. After one further week, the feeding step was repeated, the 100 μl of aspirated medium being saved for screening by enzyme-linked immunosorbent (ELISA) assay (see below). The next day, the cell lines were expanded in 2 ml Linbro wells, and feeder layers were no longer required. At this time, the supernatant from phytohemagglutinin (PHA)-stimulated tonsillar cells (Nowell *et al.*, 1981), at a final concentration of 10% was added. A second screening of supernatant one week later confirmed the specificity and isotype of the antibodies produced. Cell lines were then sub-cloned using the limited dilution culture conditions outlined above (dilutions at 10 and 1 cell/well). Cultures were expanded to a 10 ml stage, screened and the excess cells frozen. Expansion continued using 1 in 5 dilutions, with a starting cell density of 10^5/ml. Antibody containing supernatants were centrifuged at 1,000 *g* to remove any contaminating cells, and stored frozen prior to purification.

Antibody purification. Referring to Fig. 18, segment 3, human monoclonal IgM antibodies were precipitated from culture supernatants (100-250 ml) by the addition of cold (4°) saturated ammonium sulfate to a volume ratio of 1 and stirring overnight at 4°. Precipitated antibody was collected by centrifugation at 24,000 g for 2 hr in a Beckman JA-14 rotor. After centrifugation the antibody was resuspended in 5 ml of distilled water and dialyzed for 24 hrs against S-300 buffer (0.5 M NaCl, 50 mM Tris-HCl, pH 8.0, 1mM EDTA). Antibodies were subjected to gel exclusion chromatography on Sephacryl S-300 and the eluant was monitored by measuring its absorption at 280 nm. Fractions of 4 ml were collected and those corresponding to the immunoglobulin M pooled. Purified antibodies were stored at -20°.

The purification of human monoclonal IgG antibody from cell supernatants or from ammonium sulfate precipitates was achieved by chromatography on 1cm pasteur pipet columns of Sepharose-linked goat anti-human IgG (Sigma). The columns were washed with PBS, and specifically bound IgG's were then eluted by 0.1M glycine-HCl, pH 2.5. Elution was monitored at 280 nm and the peak corresponding to immunoglobulin G collected. The eluant was neutralized with 3 M Tris, pH 8.5, and stored at -20°.

ANTIBODY ASSAYS

A. **Specificity and isotype.** Antibodies raised to HCMV were detected and characterized by an ELISA assay. Purified HCMV or anti-human IgG or IgM at 5 μg/ml was dispensed in 100 μl aliquots onto the ELISA plate and allowed to adsorb overnight at 4°. The enzyme-linked antibodies used were alkaline phosphatase conjugated goat anti-human IgM and IgG (Miles Scientific, Rexdale, Ont.), and the substrate used was nitrophenyl phosphate (Sigma). The reactions were quantitated by measuring absorbance at 410 nm on a Titertek Multiskan Microplate reader.

B. IgG Subclass Identification. The HCMV subclass assays were performed essentially as described by Linde *et al.* (1983). Microplates (Immulon 1, Dynatech Laboratories, Alexandria, VA) were coated with viral antigen 10 μg/ml and incubated at 4° overnight. Serial dilutions of the human monoclonal antibodies were then added to the plates and incubation was allowed to proceed for 90 min at 37°. After washing, 100 μl of ascites fluid containing monoclonal antibodies against IgG 1, 2, 3 or 4 were added. These monoclonals were obtained from Miles Scientific Ltd. (Naperville, IL) and were used at the following dilutions: IgG1 clone SG16 (1:1000), IgG2 clone GOM-1 (1:1000), IgG3 clone SJ-33 (1:50), IgG4 clone SK-44 (1:3000). As a control, the monoclonal antibodies were also added to antigen-coated wells which had not been exposed to human monoclonal antibody. Alkaline phosphatase-labeled sheep anti-mouse IgG (Miles) diluted 1:500 was used in subclass assays, with the nitrophenyl phosphate substrate. The background reactivity of the monoclonal antibodies to human IgG 1-4 was established using HCMV negative serum diluted 1:16.

C. IgG Light Chain Characterization. The nature of the IgG light chain was also determined by ELISA. Duplicated Dynatech Immulon 2 plates were coated overnight with rabbit anti-human Ig antibody (αIgM + G + A; Cappel Laboratories, West Chester, PA) at a concentration of 10 μg/ml. Following incubation with dilutions with affinity-purified human monoclonal antibody, to the wells of one plate was added 1:300 and 1:900 dilutions of mouse anti-kappa and to the other plate similar dilutions of mouse anti-lambda (ακ, αλ; Bethesda Research Laboratories, Bethesda, MD). Bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse antibody (Boehringer Mannheim, Dorval, Que.) at a 1:1000 dilution, using the nitrophenyl phosphate substrate.

Immunofluorescence Assays. Monolayer cell cultures were grown on glass coverslips and infected with virus once the cells neared confluency. Culturing was continued with

the periodic harvesting of coverslips representative of the immediate early, early and late stages of HCMV infection. For viral cross-reactivity studies, culturing was continued until such a time that cytopathic effects were visible in about 30% of the cells. Coverslips were fixed in methanol, dried and stored at -20° until assayed. They were then incubated for 1 hour at room temperature with a 1 in 10 dilution of monoclonal culture supernatant. After repeated washings with PBS the cells were covered with fluorescein isothiocyanate-labeled antiserum against human IgG (Hyland Diagnostics, Deerfield, IL) for 1 hr. After washing, the cells were mounted in a mixture of 1 part PBS and 9 parts glycerol. Appropriate positive and negative controls were included for each test, and photography was carried out on a Leitz Ortholux II fluorescent microscope using Kodachrome 400ASA film.

Dot Blotting. Dot-blot analysis was carried out prior to Western blotting in order to establish optimal antigen renaturation/reaction conditions. Antigen in both native and denatured (boiled for 3 min in 63 mM TRIS buffer pH 6.8 containing 2% SDS and 5% β-mercaptoethanol) forms, at a concentration of 10 μg/ml, was dispensed in 100 μl aliquots into the wells of a minifold Dot-blot apparatus, (Schleicher & Schuell, Keene, NH) lined with 0.45 μ pore-size nitrocellulose filters. Antigen binding took place overnight at 4°. Renaturation of the denatured antigen was then carried out by incubation with 8M urea. Wells were washed 3 times with PBS prior to blocking any remaining protein binding sites with 1% ovalbumin (OA; Sigma). The following steps were all carried out at 37°. Into the appropriate dot blot well was dispensed 100 μl of affinity purified human monoclonal antibody, to which had been added OA to a final concentration of 1%. Antigen-antibody interaction was allowed to proceed for 90 min prior to washing. Immunodetection was with the BRL Biotin-Streptavidin system (Bethesda Research Laboratories) with β-galactosidase as the enzyme conjugate. The manufacturers' instructions were followed with the exception of the substitution of 1% OA for bovine serum albumin as a carrier, and Tween 20 was not included in the wash

buffers until second antibody binding was complete. The β-galactosidase substrate Bio-gal was employed in an "iron" (ferric) cyanide system to visualize antibody binding.

Protein Blotting. The discontinuous system of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Prestained marker proteins (Bethesda Research Laboratories) were added alongside sample lanes and electrophoresis was carried out at 6mA/gel for 16 hrs in a Protean I apparatus (Bio-rad Laboratories, Richmond, CA.). Gel replicas were prepared by electrophoretic transfer to nitrocellulose sheets (0.45μm pore size, Schleicher and Schuell) using a Trans-Blot cell (Bio-rad). The electrophoretic transfer method of Towbin *et al.* (1979) was used with the following modifications. The transfer buffer consisted of the Laemmli electrophoresis buffer without SDS. Electrophoretic transfer was carried out by blotting at a potential difference of 20 volts for 60 min, followed by 200 volts for a further 90 minutes. After transfer, the blots on nitrocellulose were treated with 8M urea for 1 hr in order to remove SDS and subsequently washed with PBS (3 changes of buffer) in order to remove the urea and to allow the renaturation of the active site. The nitrocellulose was blotted dry between two pieces of Whatman 3mm filter paper and the antigen containing lanes excised. Remaining protein binding sites were blocked with 1% OA prior to incubation with 5 ml of the purified antibody. Immunodetection then followed the same protocol as the dot-blotting. Wash steps were of 30 min duration with at least four changes of buffer.

Virus Neutralization. Virus neutralizing antibody was detected by the microneutralization assay of Rasmussen *et al.* (1984). Briefly, antibody together with dilutions of HCMV, in both the presence and absence of 2% rabbit serum (as a source of complement), were incubated for 45 min at 37°. Then 0.2 ml of virus-antibody mixture was applied to monolayers of HEL cells, seeded the day before at a concentration

of 2.5 x 10^4 cells per well in 96-well tissue culture microtiter plates. Medium was changed 1 day later to BME containing 2% FCS. Three days post infection, each well was assayed microscopically for viral cytopathic effect as compared to control wells with virus alone. Only antibodies that totally inhibited the development of viral cytopathic effects were considered to have neutralized the virus.

RESULTS

Antibody Generation. Initial panning studies using HCMV nucleocapsids as the selecting antigen yielded three lines of IgM secreting cells. These lines, however, proved to be unstable in the long term, with antibody production decreasing after 8-12 weeks of continuous culture. Since our objective was to establish cell lines which were representative of the human long-term immune response to specific antigens, subsequent transformation protocols included two additional steps. The first was to supplement the growth medium with supernatant taken from phytohemagglutinin (PHA) stimulated tonsil cells (Nowell *et al.*, 1981). Such a supernatant (which contains B-cell growth and differentiating factors) would, it was hoped, give rise to the differentiation of IgM secreting lines into IgG producers. However, the inclusion of 10% PHA supernatant gave no enhancement of IgG production and produced variable results in maintaining antibody secretion. Nevertheless, the inclusion of PHA supernatant into the resuscitation media for frozen cell lines was vital, since unsupplemented cell-lines recovered only slowly after thawing, whereas those incubated with PHA supernatant recovered rapidly and continued growing for long periods of time.

The second additional step was the sequential decrease in FCS concentration from 20% to 5%. This procedure was based upon an earlier observation that consistently higher ELISA readings were obtained from cell-lines grown in 5% FCS than in 20% FCS, and that these readings represented increased antibody secretion. By examining the numbers of antibody-producing cell lines generated in 5% FCS compared to those in 20% FCS, we noted an increase in both IgM and IgG secreting lines. All following transformations using HCMV antigens therefore included the FCS reduction step.

The transformation of lymphocytes from three donors, using both intact HCMV and HCMV nucleocapsids as panning antigens, yielded six stable IgG producing cell lines; the IgM lines were discarded at the first screening. All of the stable IgG lines were found to have been derived from a single donor, and in each case intact HCMV

was the selecting antigen.

The stability of the IgG monoclonal antibody lines proved to be significantly better than that exhibited by the IgM producer lines. The six IgG secreting lines were cultured for 12 weeks in order to build up antibody stocks for characterization. During this period no decrease in antibody production or overall cellular proliferation was detected. Our better producing lines have now been cultured continuously over a 6 month period, throughout which antibody production was maintained.

IgG Subclasses and Light Chains. The subclass response of the six human monoclonal antibody producing cell lines was found to be limited to IgG1. IgG subclass responses of IgG1 alone (Vandvik *et al.*, 1976), IgG 3 alone (Beck, 1981) or IgG1 with IgG3 (Gilljam *et al.*, 1985) have been described in connection with human viral infections. It has been reported by Linde *et al.* (1983) that in primary HCMV infection, IgG3 was detectable first in serum, but that IgG1 levels increased and persisted for a longer period of time. During a reactivated infection IgG1 and IgG3 were present during both the acute and convalescent periods. Since IgG1 constitutes 60.9% of the total IgG and IgG3 only 5.3% in sera from healthy individuals (Morell *et al.*, 1972), the absence of IgG3 from our transformed cell lines is perhaps due simply to a subclass distribution effect. Since HCMV serology was not performed on the lymphocyte donors, the question as to whether or not the lymphocytes transformed represent cells that had prior exposure to HCMV must remain unanswered.

The results of the light chain characterization are shown in Table 6. Our findings are in general agreement with the normal human kappa/lambda ratio of 70/30 reported by Nisonoff *et al.* (1975), and confirm the observation of Stein and Sigal (1984) that random Ig producing clones derived by limiting dilution culture show no preference for either light chain.

Immunofluorescence Assays. Indirect fluorescence assays were carried out on HEL fibroblasts infected with HCMV strain AD 169 or strain Davis (ATCC VR-807), as well as with virus isolated from six clinical cases of HCMV. The latter were generously provided in the form of primary infected cell cultures by Mr. R. Devine (Microbiology Unit, Alberta Provincial Laboratory, Edmonton). Immune serum from an HCMV seropositive patient was used as a positive control. In each case the pattern of fluorescence obtained with the human monoclonal antibodies was similar, and clearly demonstrated the development of perinuclear cytoplasmic inclusions at 40-45 hr and an alteration in the shape of the nucleus and the appearance of nuclear inclusions at times later than 72 hours post-infection (Fig. 19C). This pattern differed somewhat from that obtained with the positive control serum, the latter exhibiting less well-defined concentrations of fluorescence and a higher overall intensity produced by the binding of a polyclonal mixture of antibodies to the HCMV antigens (Fig. 19a).

The absence of cross-reactivity of the human monoclonal IgG's with other viral antigens was also shown by indirect fluorescence assays. No interaction was found with cells infected with herpes simplex type 1 (Strain Kos), herpes simplex type 2 (Strain 333), Epstein-Barr virus (viral capsid antigen) or measles (Strain Edmonston:ATCC VR 24).

Dot Blotting/Western Blot Analysis. Optimal renaturation conditions for HCMV antigens denatured by heating in the presence of SDS and β-mercaptoethanol were obtained by incubating with 8M urea for 1 hr at room temperature; less antigen-antibody interaction was observed when 4M urea or 0.1% NP-40 were used as renaturants. Erickson *et al.* (1982) had found that 8M urea did not affect the binding of antigen to nitrocellulose and our results confirm this. Indeed, as Fig. 20 shows, with the exception of monoclonal line A, antigen-antibody interactions were better with the renatured antigen. In each case, the dot-blot analysis showed that the monoclonal antibodies reacted with nucleocapsids as well as with whole virion antigens. In relative

FIGURE 19

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY OF

HCMV INFECTED HFL CELLS

Panel A shows infected cells following incubation with HCMV immune serum; notice the cytomegalic cell. Panel B shows uninfected HFL cells incubated with anti-HCMV human monoclonal IgG antibody from cell line A. Panel C shows HCMV-infected monolayers incubated with the same human monoclonal antibody. The arrow designated 1 in this panel indicates an intense fluorescence associated with a perinuclear cytoplasmic inclusion and arrow 2 points to a nuclear viral inclusion (magnification 420x).

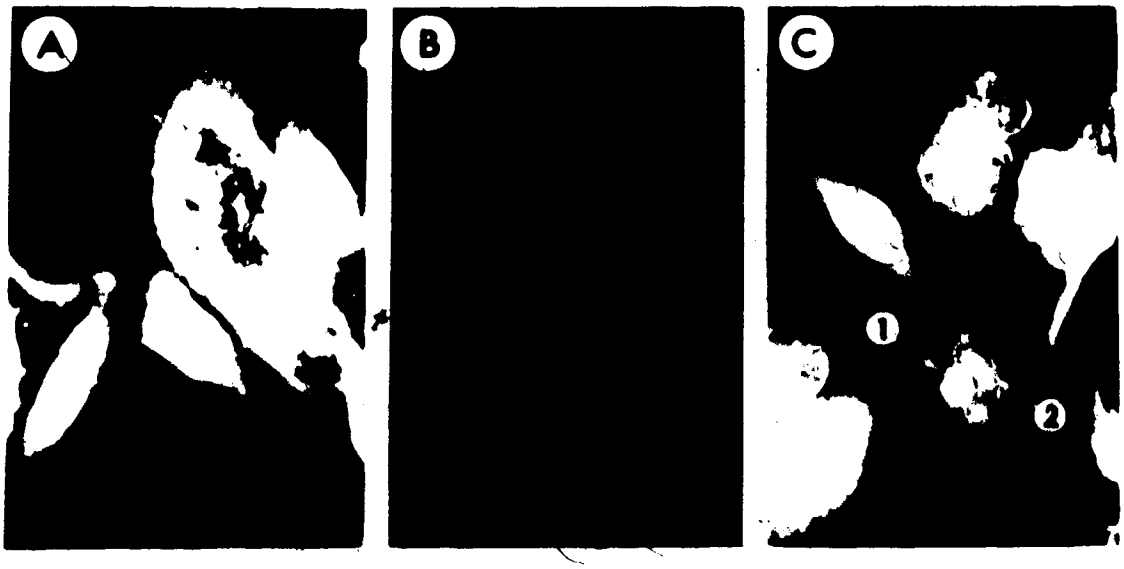
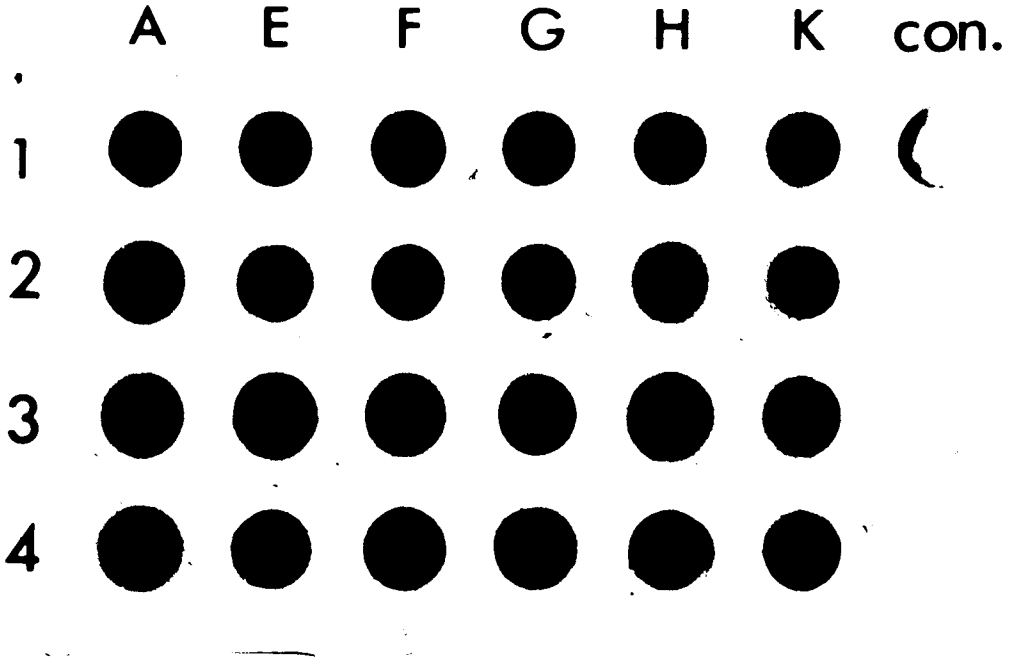

A
B
C
1
2

FIGURE 20

NITROCELLULOSE DOT BLOT ANALYSIS OF HUMAN MONOCLONAL ANTIBODIES A K USING INTACT AND DENATURED RENATURED ANTIGENS

The blots in row 1 contained intact HCMV nucleocapsids and row 2, denatured-renatured nucleocapsids. Rows 3 and 4 blots contained native and renatured whole virus, respectively. Following interaction with the appropriate monoclonal antibody, the blots were probed with biotinylated goat anti human IgG and streptavidin conjugated to β-galactosidase. Visualization with bluo gal showed widespread intense coloration associated with strong antigen-antibody interaction and a more centralized coloration with weaker interaction. Control wells containing viral antigens and control antibody showed some peripheral staining but lacked the intensity of the positive results.


A
E
F
G
H
K
con.
1
2
3
4

terms, antibodies A and F reacted strongly with all of the antigens; antibodies E, G, H and K reacted less strongly with native virions and undenatured nucleocapsids.

Western blot analysis (illustrated in Fig. 21) divided the IgG antibodies into two groups. Antibody A showed specificity towards virion proteins of 95 kD and 66 kD with very faint staining of a protein ~160 kD. Against nucleocapsid antigens the only interaction was with a protein of 95 kD (Fig. 21, middle panel). This pattern was also observed with antibodies E, F, G and H. Antibody K showed an interaction with a virion protein of 77 kD (Fig. 21, right-hand panel), but no easily discernable interactions with nucleocapsid proteins. Occasionally a faint interaction was observed between Antibody K and a nucleocapsid protein of mass 130 kD. This observation is in contrast to the observations that Antibody K reacts well with both viral and nucleocapsid antigens in dot-blot and immunofluorescence experiments. A possible explanation for this discrepancy could be the concentration effect, since the antigenic proteins in western blots were dispersed over a greater area than in the dot-blots.

The proteins reactive with antibodies A,E,F,G and H coincide with HCMV glycoproteins (Figure 16) and this is an explanation for multiple protein specificity. The immunoreactive proteins may represent precursors/products of glycosylation reactions similar to those described by Pereira *et al.* (1984).

Probing western blots with ^{125}I-goat anti-human F(ab')$_2$ (Amersham) established that the antibodies of IgM isotype which we had isolated have affinities for 2 nucleocapsid proteins. As shown in Fig. 22, IgM antibody 1 interacted with a 12 kD protein whilst antibody 2 reacted with a 18 kD protein.

Neutralization Studies. Four of the monoclonal cell-lines were found to neutralize HCMV in the absence of complement; the remaining two lines failed to completely neutralize virus even in the presence of 2% rabbit serum, as a source of complement, despite an overall reduction in viral cytopathic effect. Antibodies produced by cell line F were found to neutralize 3.75 x 10^5 pfu of virus (m.o.i. = 10) at a concentration of

FIGURE 21

WESTERN BLOTS FOR HUMAN ANTI-HCMV IgG LINES A AND K

Viral (V) and nucleocapsid (N) proteins were electrophoresed on 10% Laemmli gels, electrophoretically transferred to nitrocellulose and probed with antibody. Antibody-antigenic protein interaction was visualized with biotinylated goat anti-human IgG and streptavidin-β-galactosidase conjugate. Positive results produced blue bands. The arrow-heads denote proteins interacting with antibodies produced by line A; the arrow indicates that interacting with antibodies from line K. The control panel shows Silver stained and Coomassie blue counterstained duplicate electrophoretic gels containing viral (V) and nucleocapsid (N) proteins. The molecular weight marker proteins (M) shown on the left were from a high-molecular-weight standard kit supplied by Bethesda Research Laboratories.

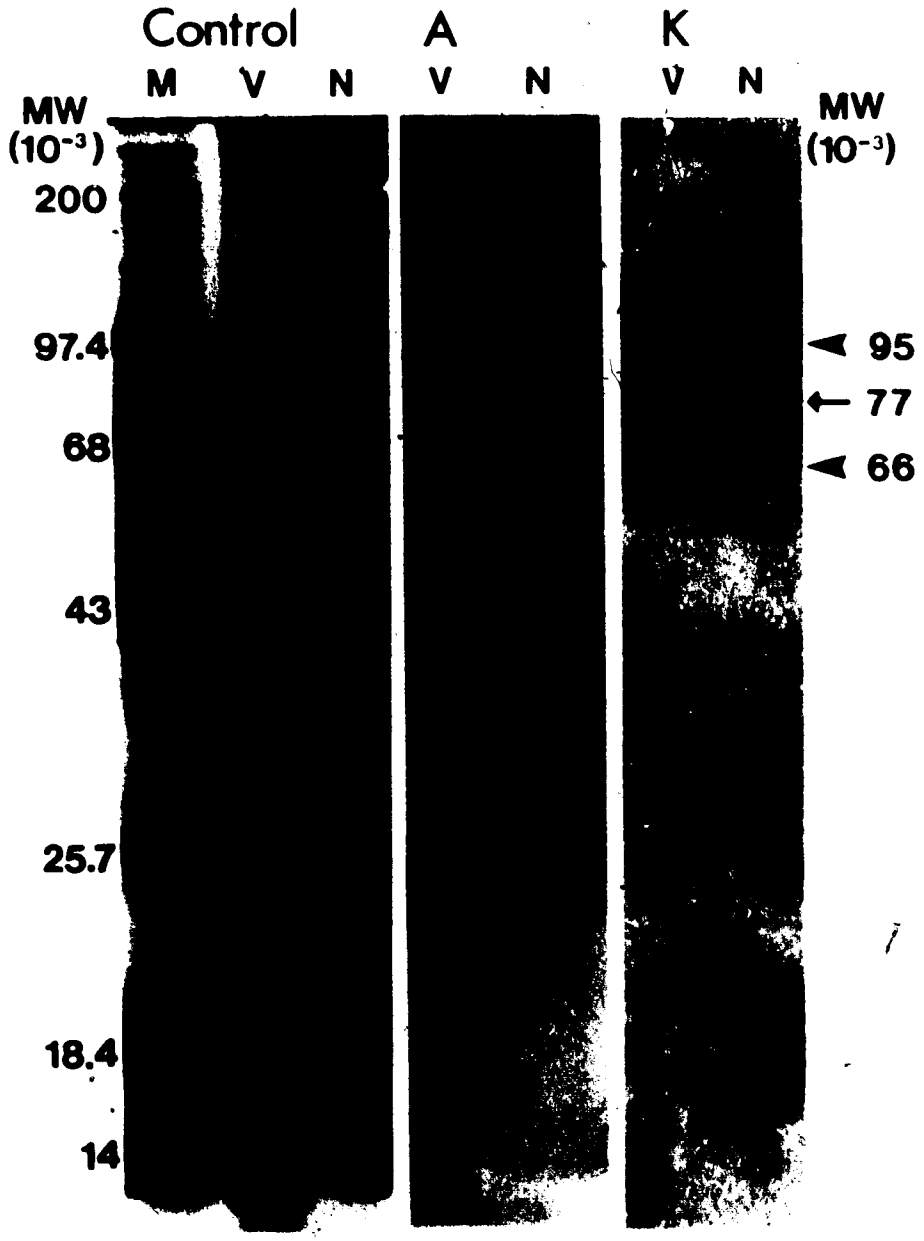
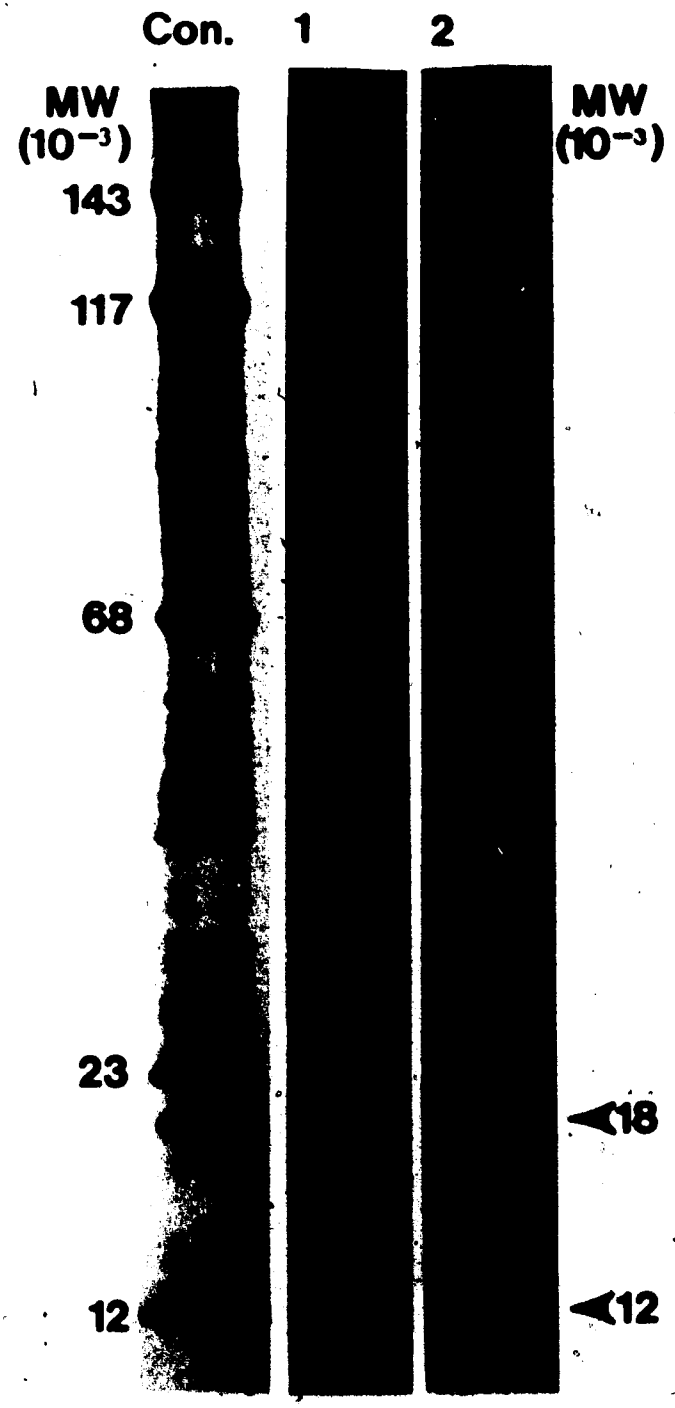

Control
A
K
M
V
N
V
N
V
N
MW
(10⁻³)
200
97.4
68
43
25.7
18.4
14
MW
(10⁻³)
95
77
66

FIGURE 22

AUTORADIOGRAPHS OF WESTERN BLOTS FOR HUMAN ANTI-HCMV

IgM LINES 1 AND 2

Blots were prepared following SDS-PAGE by the electrophoretic transfer of nucleocapsid proteins from 10% Laemmli gels to nitrocellulose. Following incubation with monoclonal antibody, the blots were probed with ^{125}I-goat anti-human Ig(Fab')$_2$ fragments. The control panel shows an autoradiograph of [^{3}H]leucine-labeled HCMV nucleocapsids subjected to SDS-PAGE on the same original gel.


Con.
1
2
MW
(10⁻³)
143
117
68
23
12
MW
(10⁻³)
18
12

10 μg/ml. Line A antibodies did not neutralize even at 25 times this concentration of IgG.

SUMMARY

Table 6 lists the results for the characterization of the IgG monoclonal antibodies produced by our transformed lymphocytes. Comparing the six antibodies in terms of HCMV proteins recognized, neutralizing activity and light chain type, four groups can be distinguished: antibodies F, G and H recognize proteins of molecular mass 95 kD and 66 kD, are neutralizing and have kappa light chains; antibody E has the same characteristics except that its light chains are of the lambda class; antibody A resembles antibody E except that it is unable to neutralize virus infectivity; and antibody K is non-neutralizing, has kappa light chains, and is unique in recognizing a 77 kD HCMV protein. Since these four different groups of IgG secreting cell lines were all derived from a single donor, the results are indicative of at least part of the human immune response to challenge with HCMV.

DISCUSSION

This chapter describes the selection and characterization of human monoclonal antibodies to human cytomegalovirus, with particular emphasis on the production of immunoglobulin G. Crawford *et al.* (1983a,b), for the purposes of generating human IgG monoclonal antibodies towards Rhesus D antigen and the nucleoprotein of influenza virus, established the immune status of lymphocyte donors through deliberate prior immunization. Having no details on the immune status of our donors, which were selected at random from a lymphocyte library generated at the North London Blood Transfusion Centre, but estimating approximately 50% of the adult population of London to have been exposed to HCMV (Stern & Elek; 1965), we adopted the strategy of panning on HCMV antigens with the lymphocytes from 3 donors. The six IgG producing lines characterized here were all derived from a single donor using HCMV as the

TABLE 6

Summary of the characteristics of human monoclonal cell lines

Antibody designation	IgG Subclass	Light Chain	Molecular mass of antigenic protein (kD)	Neutralising activity
A	IgG_1	λ	66, 95	No
E	IgG_1	λ	66, 95	Yes
F	IgG_1	κ	66, 95	Yes
G	IgG_1	κ	66, 95	Yes
H	IgG_1	κ	66, 95	Yes
K	IgG_1	κ	77	No

panning (selecting) antigen; panning with the HCMV nucleocapsid antigen gave rise only to IgM-secreting cell lines. It is therefore most likely that our IgG's are the fortuitous result of having selected blood from a naturally immune donor primed against native HCMV.

Characterization of the IgG antibodies would appear to support this theory: first, the neutralizing antibodies recognize major antigenic HCMV proteins; second, the IgG1 subclass of antibodies are recognized as offering long-term immunity against viral challenge. The IgM lines would appear to have been produced in a typical primary antigenic response. This would apply to non-immune donors exposed to the virus for the first time, and would likewise apply to the HCMV nucleocapsid, an antigen which would not normally be presented to the immune system.

Electrophoretic analysis of polypeptides immune precipitated from HCMV infected cell extracts by immune human sera (Pereira *et al.*, 1982) and sera from children with congenital and perinatal HCMV infections (Pereira *et al.*, 1983) indicated polypeptides with M_r's of 140 and 66 kD are primary immunogens. The proteins of M_r 140 and 66 kD comigrated with glycoproteins and contained neutralizing sites (Stagno *et al.*, 1984). Smaller amounts of protein M_r 74 kD was also precipitated. Therefore, the production of our human monoclonal antibodies to these proteins would again suggest that we are activating memory B lymphocytes into the production of IgG antibody.

The production of neutralizing antibodies is of major interest because of the potential therapeutic role of such antibodies in conferring passive immunity to HCMV. Long-lived cell lines such as those that we have described could be continuously cultured to produce large amounts of antibody. It has been suggested that the use of such antibodies could involve a risk of oncogenesis due to the presence of Epstein-Barr viral particles or genomes in the preparations. However, antibody purification by microfiltration (0.1 μm filters), DNase treatment, and affinity purification on anti-human IgG columns would yield a product that is safe to use. An obvious use for anti-HCMV

antibodies would be in the treatment of immunosuppressed patients at risk from HCMV infection following transplant surgery.

Since the completion of our work on human monoclonal antibodies directed against HCMV there have been a number of other reports describing human monoclonal antibodies directed against HCMV (Masuho *et al.*, 1987 and Matsumoto *et al.*, 1986). Two groups of monoclonal antibodies were produced, one group recognizing HCMV proteins M_r 130/55 kD that also had neutralizing activity, the other recognized only a 64 kD protein in immunoprecipitation and failed to neutralize virus. Serum antibodies from this group's lymphocyte donor precipitated large amounts of the 64 kD protein and smaller amounts of 130/55 kD proteins.

In comparing the specificity of antibodies generated by human monoclonal cell lines to those of mouse monoclonal cell lines when presented with nucleocapsid antigen, we find marked differences. Whereas the human antibodies directed response to proteins M_r 12 and 18 kD, the mouse response was towards the 64 kD protein. It is therefore important not to extrapolate data obtained from one species to another.

CHAPTER IV

HERPES SIMPLEX VIRUS: THE USE OF SYNTHETIC PEPTIDES TO MAP THE ANTIGENIC DETERMINANTS OF GLYCOPROTEIN D OF HERPES SIMPLEX VIRUS

INTRODUCTION

Herpes simplex viruses (HSV's) are among the most common infectious agents of man. There are two distinct serotypes (HSV-1 and HSV-2) and they usually have different modes of transmission. HSV-1 is transmitted primarily by non-genital routes whereas HSV-2 is most often transmitted venereally or from a mother's genital infection to the newborn.

The clinical course of an HSV infection can vary widely in severity and duration. The primary infection may be inapparent, partly because the primary sites of infection, in the mouth or cervix, are not readily visible, or it may have a clinically observable course. Most HSV type 1 infections are asymptomatic and many recurrent oral infections are also subclinical (Nahmias and Josey, 1982). The infection may become latent, and the latency is independent of the previous form of the disease. A recurrent infection may follow one of several stimuli (for example, upper respiratory tract infection, overexposure to sunlight or physical or emotional stress), or it may appear simultaneously with other conditions such as fever, viral or bacterial diseases (measles, varicella or tuberculosis; Nahmias *et al.*, 1981), menstruation or general trauma.

A primary infection in an individual without preexisting antibodies to HSV 1 or 2 is usually more severe than secondary (recurrent) infections, and is often accompanied by fever, extensive lesions localized to the skin, mouth or eyes including not only keratinitis but also chorioretinitis, and lymphadenopathy of the groin and pelvic region. Primary infections are normally of longer duration than secondary infections. The clinical manifestations of either virus may also be more severe in certain types of hosts, for example, a newborn infant or immunocompromised individual, and with involvement

of certain sites, for example, the central nervous system. In the absence of antiviral therapy, the mortality ensuing from HSV encephalitis is 70% and over one-half of the survivors are left with severe neurological disorder (Whitley *et al.*, 1977).

HSV Latency. From the site of inoculation on epithelial surfaces, the virus enters nerve terminals and travels to ganglia (Cook and Stevens, 1973; Openshaw *et al.*, 1981; Wildy *et al.*, 1982). An acute, productive infection takes place in the ganglion cells, and high concentrations of viral antigen can be demonstrated in these cells by immunofluorescence. At approximately two weeks after infection, the infection passes from an acute into a latent stage (Cook and Stevens, 1973). Viral antigens can no longer be demonstrated by immunofluorescence; however, by explantation of ganglia cells and culturing *in vitro*, infectious virus can be demonstrated. It has been proposed (Roizman, 1965) that during latency the viral genome is in a non-replicating, non-expressed state. However, HSV mRNA has been detected in ganglia cells by *in situ* hybridization (Schwartz *et al.*, 1978), and the impairment of transcription may only be partial. Reactivation of the virus occurs when this impairment is removed. The actual mechanism of the reactivation process is still unknown. At this time virus replication resumes and virus spreads from the ganglia back along the nerves to the sensory cells, giving rise to the clinical symptoms of HSV infection at or near the site of primary infection.

Immunity to HSV. The normal host has a series of defense mechanisms that participate in sequestering and clearing invading viral pathogens (Allison, 1974). The defense systems involved fall into two major categories: natural resistance and adaptive immunity.

Natural resistance mechanisms probably play a decisive role during early stages of primary HSV infections (Lopez, 1985). Unlike antibody and cell-mediated immunity, these mechanisms do not require prior exposure to viral antigens to be operative and a significant response can be detected as early as 2-4 hr after infection. Adaptive immune

mechanisms require resensitization and are not active until several days after the primary virus infection. The viral cellular proteins synthesized during a primary as well as during a recurrent infection interact with the immune system and give rise to the formation of both circulating HSV specific antibodies and activated lymphocytes that have the potential to destroy virus infected cells.

During an infectious cycle, HSV 1 and HSV 2 specify the synthesis of more than 50 proteins. Five HSV glycoproteins have been identified and designated gB, gC, gD, gE and gG. (It should be noted that gC of HSV 2 was formerly named gE and that gG is a recently discovered glycoprotein in HSV 2; Roizman *et al.*, 1984). These viral glycoproteins have been shown to be inserted into the plasma membrane of infected cells and thence into the virus envelope (Jennings, 1987).

Several of the HSV proteins are strong immunogens, and especially the glycoproteins that are exposed on the surface of infected cells and virions have been studied extensively with respect to their ability to induce the production of both specific antibodies and activated cytolytic lymphocytes (Kapoor *et al.*, 1982). Antibodies to the glycoproteins gB and gD were found to be dominant when 100 human sera were analyzed by crossed immunoelectrophoresis (Vestergaard, 1979). Glycoprotein D is a major protein component of the envelope of the mature virion and plays an important role in initiating the infectious process (Spear, 1985). The two HSV serotypes also show a high degree of gD sequence homology. Glycoprotein D has therefore been considered as a good candidate as a subunit vaccine against HSV infections.

Herpes Simplex Virus Vaccines. Lipschutz first demonstrated the feasibility of immunization against herpes infection in rabbits in 1921. Since then there have been many studies that have attempted to devise safe and effective preparations for use in man. These have included live virus (Blank and Haines, 1973), attenuated virus (Asher *et al.*, 1978), formulations of whole killed virus (Schmersahl and Rudiger, 1975), and preparations based on purified sub-viral membrane fractions (Skinner, 1978; Skinner, 1980).

However, the risks of oncogenicity (Parks and Rapp, 1978) from the HSV DNA and the risk of latency arising from the use of attenuated strains preclude the use of live or modified strains for human vaccination (Hall and Kattak, 1986). With the advent of molecular genetics two new approaches have emerged which may overcome the earlier difficulties or ethical objections. Recombinant vaccines of herpes virus nucleotide sequences expressed in a vaccinia virus infection, for example glycoprotein D (Cremer *et al*., 1985; Paoletti *et al*., 1984), have been reported to protect mice from lethal infection with HSV 1. Although cloning represents a major advance in vaccine development, an equally valid approach would be to synthesize peptide epitopes with potent and relevant immunological activity (Shinnick *et al*., 1983; Brown, 1984).

It has been shown that peptides of synthetic origin can elicit the production of antisera capable of neutralizing viruses such as polio (Emini *et al*., 1983) or influenza (Muller *et al*., 1982), bacteria such as *Streptococcus* (Beachey, 1985) or *Vibrio* (Jacob *et al*., 1983) and the sporozoites of *Plasmodium* (Egan *et al*., 1987; Harrington *et al*., 1987). The efficacy of employing a synthetic peptide as an anti-virus vaccine was first demonstrated by Bittle *et al* (1982). They synthesized peptides corresponding to various sequences of the capsid protein VP-1 of foot-and-mouth disease virus and found that one of them corresponding to residues 141-160, produced high levels of virus neutralizing antibody when it was conjugated to the protein carrier, keyhole limpet hemocyanin (KLH), and inoculated into cattle, rabbits or guinea-pigs. Furthermore, animals which had received a single inoculation were protected against subsequent challenge with virulent virus. A second peptide, corresponding to residues 200-213 of VP1 was also shown to elicit the production of neutralizing antibodies in rabbits, and more recently Di Marchi *et al*. (1986) synthesized the composite peptide 141-158, 200-213. This composite peptide, administered without conjugation to carrier protein, was shown to "elicit high levels of neutralizing antibody" and to protect cattle against "intradermolingual challenge by inoculation with infectious virus". Larger scale field

trials of this vaccine are currently underway.

The development of peptide vaccines for protection against foot and mouth disease was stimulated by the dramatic illustration of one of the problems with conventional "killed-virus" vaccines, namely their potential to cause disease. A serious outbreak of foot and mouth disease in Brittany, the Channel Islands and the south of England in 1981 was shown by careful molecular analysis to have been caused by an incompletely inactivated virus vaccine (King *et al.*, 1981).

Our interest in the preparation of a synthetic vaccine for HSV arose in part from the development of a predictive algorithm called "Surfaceplot" by Parker *et al.* (1986). Surfaceplot predicts from the amino acid sequence of a protein which residues have a high probability of being exposed on the molecular surface. Since it is now generally regarded that the entire surface of a protein is antigenic (Benjamin *et al.*, 1984) predicted surface sites are potential antigenic sites. Glycoprotein D of HSV is one of the most studied antigens of any herpesvirus and may represent a useful candidate for provision of protection against both HSV-1 and HSV-2 infections (Eisenberg *et al.*, 1980). Glycoprotein D therefore presented itself as a prime candidate for Surfaceplot analysis and antigenic site mapping using polyclonal antisera raised to synthetic peptides of the predicted surface-sites. Sites producing a strong virus-neutralizing response are potentially useful as synthetic vaccines.

The application of Surfaceplot to the amino acid sequence of glycoprotein D of HSV-1 revealed 15 linear segments (5 or more residues) of the polypeptide which have a high probability of surface exposure. Surfaceplot also identified a number of segments of glycoprotein D with a very low probability of surface exposure; two of these were selected for control experiments. Fourteen peptides corresponding to the predicted surface and interior segments were synthesized, photochemically linked to protein carriers and injected into rabbits. The polyclonal sera produced in response to these peptides were then screened by ELISA for reactivity against peptide-conjugates, purified glycoprotein D

and HSV-1 virions.

The results communicated in this chapter show that all of the peptides selected produced positive immune responses (ELISA) in rabbits, and that all of the rabbit antisera reacted with isolated glycoprotein D. Eight of the antisera were found to react with intact herpes virions, confirming the presence of these sequences on the surface of glycoprotein D *in situ*. In plaque-reduction (neutralization) tests, five of the antisera inhibited virus infection. The greatest virus neutralization capacity was found to be associated with antisera raised to the peptide-conjugate of the N-terminal amino acid residues 2-21. Strong neutralization was also produced by antisera to the sequence 267-276.

MATERIALS AND METHODS

Surface Profile Prediction. Synthetic peptides were chosen from surface regions predicted from the algorithm of Parker *et al.* (1986). This algorithm (Surfaceplot) combines the HPLC hydrophilicity parameters of Guo *et al.* (1986), the accessibility parameters of Janin (1979) and the flexibility parameters of Karplus and Schultz (1985). The HPLC parameters were derived from the retention times in reversed-phase chromatography of model synthetic peptides, Ac-Gly-X-X-(Leu)$_2$-(Lys)$_2$-amide, where X was substituted with each of the twenty amino acids found in proteins. The accessibility parameters derived from X-ray crystallographic data from twenty-two proteins represent the partition coefficient of each amino acid residue between the surface and the inside volume of the protein. The flexibility parameters were determined from the X-ray crystallographic data of the average temperature values of all c_α-atoms of each amino acid in thirty-one proteins. It has been shown that a combination of these three parameters improved the prediction of surface regions compared to using any single parameter alone (Parker *et al.*, 1986). The Surfaceplot program is available from Synthetic Peptides Incorporated, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2H7.

Synthesis of Peptides. Peptide synthesis and purification was carried out by Dr. J.M.R. Parker and Miss N.C.J. Strynadka in the Biochemistry Department, University of Alberta. All chemicals and solvents used were reagent grade. Co-poly(styrene 2% divinylbenzene) benzhydryl amine resin (hydrochloride salt, substitution = 0.8 mmole/gm of resin) was purchased from Protein Research Foundation, Osaka, Japan. Dichloromethane was distilled from calcium carbonate. Trifluoroacetic acid was purchased from Halocarbon, Hackensack, N.J., p-benzoyl-benzoic acid from Aldrich Chem. Co., Milwaukee, WI., and N^{α}-t-Boc-L-lysine from Sigma Chemical Co., St. Louis, MO.. All other protected amino acids were purchased from Institute Armand Frappier, Laval,

Quebec.

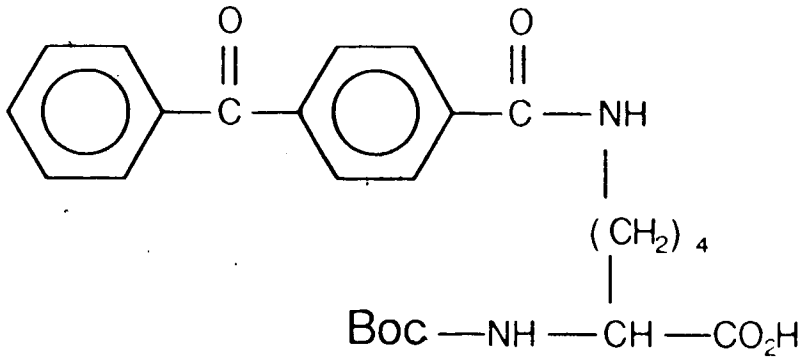
Peptides were synthesized on a Beckman peptide synthesizer Model 990 using the general procedure outlined by Erickson and Merrifield (1976) for solid-phase synthesis, with the modifications described by Parker and Hodges (1985). Amino groups were protected at the α-amino position with a t-butyloxycarbonyl (Boc) group, and the following side chain blocking groups were used: Lys(2-chlorobenzoxycarbonyl), Thr (benzyl), Glu (O-benzyl), Asp(O-benzyl), Arg (tosyl), Cys (methoxybenzyl) and Tyr (2,6-dichlorobenzyl). A radioactive label Boc[1-^{14}C] glycine, prepared as described previously (Worobec *et al.*, 1983), was introduced at an appropriate glycine in the sequence. Since the peptide corresponding to residues 12-21 of glycoprotein D was to be used to investigate different peptide-protein conjugation procedures other than the photochemical coupling method described below, two analogs were synthesized, one with a cysteine added to the N-terminus and a second with a tyrosine added to the N-terminus.

The photochemical coupling reagents N^{ϵ}-(p-benzoylbenzoyl)-N^{α}-t-butyloxycarbonyl-lysine, Boc-Lys(BB) (Parker and Hodges, 1985), and p-benzoylbenzoic acid, BBA (Figure 23), were coupled to the N-terminal α-amino group of the peptide-resin or Boc-Lys(BB) was coupled as the first C-terminal amino acid during synthesis. To obviate the limited solubility of these reagents in dichloromethane the following procedure was found to give high reproducible coupling yields. Boc-Lys(BB) or BBA (1600 μmoles/800 μmole peptide resin) was dissolved in dimethylformamide (200 - 500 μl) diluted with dichloromethane (4.5 ml) and cooled to 4°C. Dicyclohexylcarbodiimide (DCC, 800 μmoles) was added and the reaction was left at 4°C for 30 min before being filtered and added to the deprotected and neutralized peptide resin. After stirring for 30 min, an additional portion of DCC (800 μmoles) in dichloromethane (1 ml) was added and the reaction was stirred for one hour. All peptides were synthesized as C-terminal amides in order to eliminate the presence of charged residues

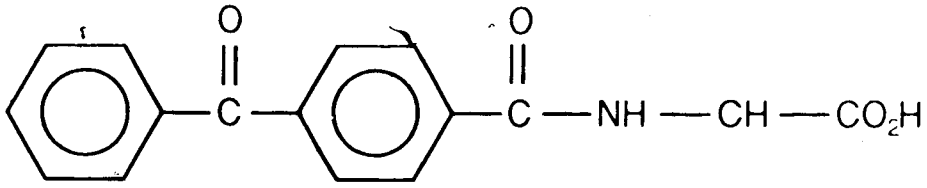
FIGURE 23

STRUCTURE OF THE PHOTOAFFINITY PROBES

The structures of the photoprobes p-Benzyoylbenzoyl glycine (BBGY) and N^{ϵ}-(p-benzoylbenzoyl)-N^{α}-t-butyloxycarbonyl-lysine (BB Lys) are shown. The BB Lys probe is protected at the N^{α} terminal with a t-butyloxycarbonyl (BOC) group.



BB Lys



BB Gly

at the C-terminus. When Lys(BB) was synthesized at the C-terminus, the N-terminal residue of the completed peptide was deprotected, neutralized and acetylated with acetic anhydride/pyridine/toluene (1:2:2,v:v:v). Acetylation was performed to eliminate the charge on the amino group on the terminal lysine residue.

Peptides were cleaved from the resin support using hydrofluoric acid (HF):10% anisole (v:v) at 4° for 45 min (Hodges and Merrifield, 1975a). The solvents were removed under reduced pressure at 4°, the resin was washed with ether and the peptide was extracted with TFA (4 x 5 ml). The combined TFA washes were evaporated, and the peptide redissolved in water and lyophilized.

Purification of peptides was accomplished by reversed-phase high performance liquid chromatography (HPLC). Samples in 0.1% trifluoroacetic acid in water were applied to a Whatman Partisil CCS/C8 column (Whatman, Clifton, NJ, 250 x 4.6 mm I.D.) or a Synchropak RP-P C18 reversed-phase column (250 x 10 mm I.D.). Elution was with a gradient of 0.05% trifluoroacetic acid in acetonitrile. The absorbance was recorded at two wavelengths, 210 nm for the peptide bond and 260-270 nm for the photoprobe (BB or BBA).

Amino acid analysis were performed on a Durrum Model D-500 high pressure automatic analyzer after hydrolysis of samples with 6 N HCl:0.1% phenol in sealed, evacuated tubes at 110° for 24 h. When peptide-resin hydrolysates were required the peptide-resins were hydrolyzed as described by Hodges and Merrifield, 1975b. The mean of the molar ratios of all accurately measurable amino acids in the acid hydrolysate was used to calculate the concentration of the peptide.

Conjugation to Carrier Proteins. Concentrated solutions of carrier proteins were prepared in the following manner. To 2 g of bovine serum albumin (BSA) (Fraction V; Sigma) was added 1 ml of 0.1 M phosphate buffer, (pH 6.8), and the gummy mixture allowed to stand overnight before being further diluted to 4 ml (1 mg/2 μl). Similarly, 500 mg of keyhole limpet hemocyanin (KLH) (Sigma) was mixed with 1 ml of 0.1 M

$NaHCO_3$ and allowed to stand overnight before being further diluted to 5 ml (1 mg/10 μl).

Photochemical coupling of peptide to carrier was accomplished by dissolving or suspending lyophilized peptide (1 to 2 mg) in a minimum volume of phosphate buffer (10-30 μl of 0.1 M KH_2PO_4, pH 6.8) and adding ~ 10 mg of BSA or KLH from the stock solutions, (20-100 μl). The photolysis was carried out for 45 min in a Rayonet RPR 208 preparative apparatus (Southern New England Ultraviolet Co., Middletown, CT) equipped with 350 nm lamps. The reactor was placed in a cold room and a constant air temperature (8-10°) surrounding the sample was maintained by an electric fan mounted in the bottom of the reactor. Following photolysis the conjugates were dissolved in 4 ml 8 M urea, dialyzed (Spectrapor tubing, 6000-8000 MW cutoff, pretreated by boiling and extensive washing in distilled water) against 0.1 M NH_4HCO_3 (once) and 0.025 M NH_4HCO_3 (twice), and lyophilized. Peptide conjugation to proteins via the peptide N-terminal cysteine or tyrosine was carried out according to the methods of Liu *et al.* (1979) and Bassiri (1980), respectively. Peptide:protein ratios were ascertained from the specific activity of the radioactively-labeled peptide, or by amino acid analysis, and ranged from 5 to 15 moles of peptide per mole of carrier protein.

Production of Antisera. Antisera to synthetic peptide-KLH conjugates were obtained from 6 week old male New Zealand white rabbits. On day 1, 40 μg of peptide-KLH conjugate (in sterile 0.9% NaCl, 500 μl) was thoroughly mixed with an equal volume of Freund's complete adjuvent (Difco Laboratories, Detroit, MI.) and injected (200 μl per injection) into the left and right subscapular regions and gluteal muscle. The same procedure was repeated at day 14 except that 200 μg of peptide (in sterile 0.9% NaCl, 500 μl) in incomplete Freund's adjuvant (500 μl) was used as before (200 μl per injection). The rabbits were bled from the ear vein at day 28 and reinnoculated as described for day 14. This bleeding/innoculation protocol was carried out for a total of 6 weeks.

Cells and Virus. Human fetal lung cells (Flow 2000 line; Flow Laboratories, McLean VA.) were cultured in 175 cm^2 plastic flasks (Corning Glass, Corning, N.Y.), containing Eagle's basal medium (BME; Flow Labs) supplemented with 10% (v:v) fetal calf serum (Gibco, Grand Island, N.Y.). Herpes simplex virus type 1 (strain Kos) was used to infect confluent monolayers at an input multiplicity of infection of between 1 and 5. Infection was allowed to proceed for 18 hr. The infected cells were scraped from the plastic surface, resuspended in phosphate buffered saline (PBS) (Dulbecco & Vogt, 1954) and homogenized (Dounce homogenizer, close-fitting pestle). Following centrifugation (400 *g*, 10 min), the pelleted material was resuspended, re-homogenized and recentrifuged (1800 *g*, 10 min). The two supernatants were combined and centrifuged at 100,000 g for 1 hr (Beckman Ti60 rotor). The virion-containing pellet was resuspended in a minimal volume of PBS, layered onto a discontinuous gradient of sucrose (20%, 40%, 60% w/w) and centrifuged (Beckman SW 27.1 rotor) at 100,000 *g* for 1 hr. The opalescent virion band (at ~45% sucrose) was removed from the side of the tube with a hypodermic syringe. The presence of intact virions was confirmed by electron microscopy.

Purification of Glycoprotein D. N-octyl glucopyranoside (Sigma) was added to the supernatants obtained from homogenized HSV-1 infected cells to a final concentration of 1% (w:v). Potentially infectious DNA was inactivated by incubating with 1 mg DNase I/ml (Sigma) for 30 min at 37°. This lysate was then dialyzed overnight (4°) against PBS to remove the detergent. An affinity column of Concanavalin A-Sepharose (Pharmacia, Dorval, Que.) was prepared and equilibrated with 50 mM Tris-HCl, (pH 7.2), containing 0.5 mM $MgCl_2$ and 250 mM $MnCl_2$. The dialyzed HSV-1 lysate was then applied to the column washed with 10 volumes of buffer and the bound glycoproteins subsequently eluted with 0.2 M mannose (Aldrich). The eluant was dialyzed overnight at 4°C against water, then lyophilized. The resulting powder was dissolved in 1 ml of water and subjected to isocratic HPLC on a Superose 12 gel

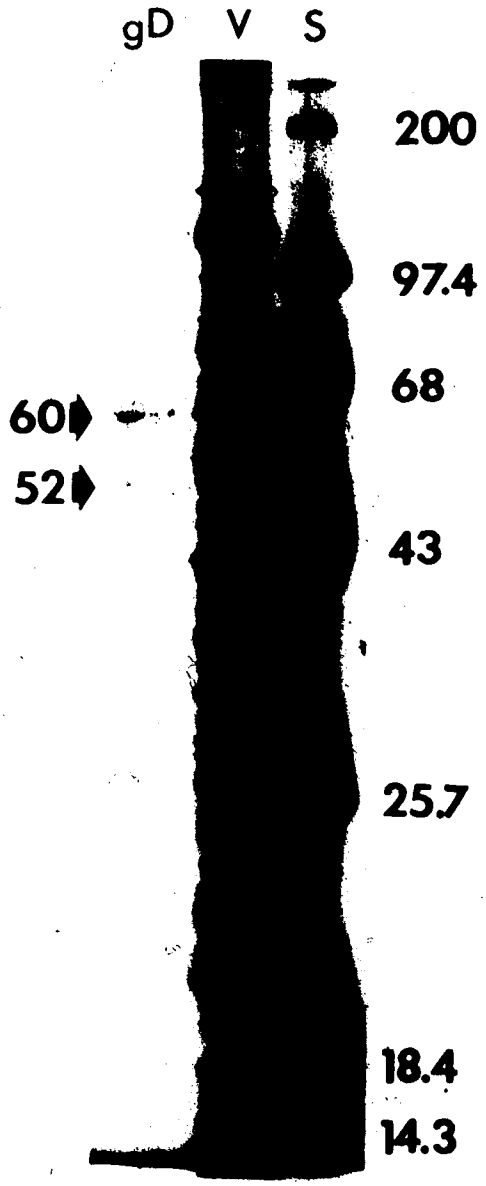
filtration column (molecular weight range 10^3 - 10^5 daltons; Pharmacia) using 0.1 M phosphate buffer (pH 7.0) 50 mM NaCl as eluting buffer. Protein elution was monitored by absorption at 280 nm, and glycoprotein D was found to be eluted as a single peak after appoximately 30 min. The purity of this product was confirmed by SDS-PAGE. Samples of the 30 min peak were electrophoresed on 10% polyacrylamide gels (Laemmli, 1970). The gels were fixed in methanol:acetic acid (40:10; v/v) and stained for protein using a protein-silver stain kit (Biorad Laboratories, Richmond, CA.). Two bands of apparent molecular masses 60 kD and 52 kD were detected. These are shown in Fig. 24, and correspond to the apparent molecular masses reported for mature glycoprotein D (59-65 kD) and pre-glycoprotein D (52 kD) (Spear, 1976; Eisenberg *et al.*, 1979).

Enzyme-Linked Immunosorbent Assays (ELISA). Antisera titers were determined using a modification of the ELISA protocol described by Voller *et al.* (1974). Microtitre plates (Immunolon II; Dynatech, Alexandria, VA.) were coated with 100 μl of peptide-BSA conjugate (50 μg/ml) in 0.5 M Na_2CO_3 (pH 9.6) for 16 hrs at 4°C in a moist chamber. This represented a saturating concentration of protein carrier. Peptide-BSA conjugates were used in the ELISA wells when KLH peptide-conjugates were used for immunization in order to measure antipeptide antibody titre and to exclude anticarrier antibody titre. The antigen coated wells were washed (3 x) with PBS containing 0.05% (v:v) Tween 20 (PBS-T). After washing, the plates were incubated for 2 hr at room temperature with 100 μl/well of the appropriate dilution of antisera in PBS-T containing 0.5% (w:v) BSA. Unbound or excess Ab was removed by washing with PBS-T (3 x) and the plate was incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (1:1000 dilution; Boehringer-Mannheim Corp., Dorval, Quebec). After washing with PBS-T (3 x), p-nitrophenyl phosphate (Sigma 104 substrate) in 10% (v:v) diethanolamine was added. Thirty to 60 min later the absorbance at 410 nm was measured on a Titertek Multiscan plate reader (Flow Laboratories, Mississauga, Ontario).

FIGURE 24

SDS-PAGE OF GLYCOPROTEIN D AND HSV-1 VIRIONS

Samples of glycoprotein D extracted with detergent from HSV-1 infected cells and purified by affinity chromatography on Concanavalin A-Sepharose and isocratic HPLC, together with virions obtained by mechanical disruption of infected cells and purification on sucrose gradients, were boiled and electrophoresed under reducing conditions in 10% polyacrylamide gels containing 0.1% SDS. Following electrophoresis the protein bands were visualized by silver staining. The lane marked S contained a standard mixture of proteins of known molecular weight. The glycoprotein D isolate was found to contain protein bands equivalent to polypeptides of apparent molecular masses 60 kD and 52 kD. The virion preparation was found to contain in excess of 50 polypeptides, ranging in apparent molecular mass from 14 kD to > 200 kD.


gD
V
S
200
97.4
68
60
52
43
25.7
18.4
14.3

Titres from ELISA assays are expressed as the negative logarithm of the reciprocal of the highest dilution of serum which still gave a positive response. In ELISA assays for glycoprotein D and virion reactivity, the same protocol was followed except for coating the plates with 100 μl (10 μg/ml) of gD or HSV-1 preparations. Acute and immune sera from individuals with HSV-1 infections were generously provided by Mr. R. Devine of the Alberta Provincial Laboratory, Edmonton, Alberta.

Neutralization Tests. Prior to analysis in plaque reduction assays, the immunoglobulin component of each antiserum was separated from complement and other serum proteins by affinity chromatography on DEAE-Affigel Blue columns (Biorad). This was done in order to estimate the contribution that complement makes to virus neutralization under our experimental conditions. Dilutions of antibody with and without the addition of 5% guinea-pig complement were incubated for 1 hr at room temperature with 100 and 200 plaque forming units of virus. These mixtures were then assayed for unneutralized virus using the plaque technique of Wentworth and French (1969). Briefly, monolayers of fibroblast cells were prepared by seeding 0.75×10^6 cells in each well of 6-well tissue culture plates (Costar, Cambridge, MA). After overnight incubation, the growth medium was removed and each plate inoculated with 0.4 ml of the neutralization mixture. Inocula were allowed to adsorb for 30 min at room temperature after which time 4 ml of overlay medium (MEM containing 0.1% $NaHCO_3$ and 0.3% agar) was added. When the overlay had solidified, the plates were incubated at 37° in a 5% CO_2 atmosphere for 72 hrs. The monolayers were fixed using 4% neutral buffered formalin prior to the removal of the overlay, and subsequently stained with 1% crystal violet. Clear plaques of dead cells were enumerated.

Immunofluorescence. Confluent monolayers of Flow 2000 cells on glass coverslips were infected with HSV-1 or HSV-2 at an input multiplicity of between 1 and 5 pfu/cell. Culturing was for 24 hr after infection, at which time the cells were washed twice with

PBS and fixed in methanol. After drying, the coverslips were stored at -20° until assayed. The coverslips were then incubated for 1 hr at room temperature with 10% normal goat serum in PBS to block HSV induced Fc receptors, washed with PBS, and covered with a 1:500 dilution of the peptide specific polyclonal rabbit sera. After 1 hr the coverslips were washed (3 x) with PBS and then incubated for 1 hr with fluorescein isothiocyanate labeled (FITC) goat anti rabbit IgG (1:32 dilution; Sigma). Following a subsequent wash the cells were mounted in a mixture of 1 part PBS and 9 parts glycerol. Negative controls of pre immune rabbit sera were included for each test, and photography was carried out with a Leitz Orthoplan II fluorescent microscope using Kodacolor VR 400 film.

RESULTS

Selection of Potential Linear Antigenic Sites. Dr. Parker, using the surface profile algorithm, Surfaceplot, has shown for several proteins that there is a good correlation of its predictions with surface sites determined from immunological and X-ray structural data (Parker *et al.*, 1986). In this instance, Surfaceplot was applied to glycoprotein D of HSV-1, using the amino acid sequence reported by Watson *et al.* (1982). The computer-generated surface profile of glycoprotein D is shown in Figure 25 and the predicted surface residues are listed in Table 7. A predicted large buried region (amino acid residues 314-339) corresponds to the proposed membrane-spanning area, and two large surface areas (340-351 and 356-365) correspond to the proposed hydrophilic C-terminal cytoplasmic tail. In the external N-terminal region, Surfaceplot predicts five major (sequences of 10 or more residues) linear surface regions (12-21, 83-93, 130-140, 244-275 and 299-313) and eight minor (sequences of 5 or more residues) linear surface regions (28-33, 44-51, 73-78, 116-120, 144-148, 184-189, 206-213, 224-229). By comparison, analysis using Hopp and Woods (1981) hydrophilicity parameters reveals only four antigenic sites in the external region of glycoprotein D. As shown in Figure 25, the sequences predicted using Hopp and Woods parameters exist as smaller subsets of those predicted using Surfaceplot. We have synthesized peptides corresponding to all major predicted surface sequences which were 10 or more residues in length (Table 8). Peptides 52-61 and 150-159 were synthesized as negative controls since these regions were predicted not to be on the surface. The remaining sequences were chosen since they were minor predicted surface regions (181-190, 206-215, 288-297) or they contained one or two single predicted surface residues (63-72, 121-130, 314-323). Sequence 314-323 is within the twenty-five amino acid sequence (314-339) that was predicted by Watson *et al.* (1982) to be the transmembrane region.

TABLE 7.

Surfaceplot predicted surface sites and antigenic sites predicted using Hopp and Woods (1981) parameters

SURFACE SITE	SURFACEPLOT RESIDUES	HOPP & WOODS RESIDUES
1	4 - 4	
2	12 - 21*	11 - 13, 15 - 21*
3	28 - 33*	32 - 34
4	44 - 51*	-
5	66 - 66	-
6	73 - 78*	
7	83 - 93*	84 - 91*
8	104 - 104	-
9	114 - 114	-
10	116 - 120*	119 - 120
11	122 - 122	-
12	124 - 124	-
13	130 - 140*	-
14	142 - 142	-
15	144 - 148*	145 - 147
16	160 - 162	-
17	172 - 172	-
18	174 - 175	-
19	184 - 189*	
20	198 - 199	-
21	206 - 213*	-
22	224 - 229*	227 - 2
23	244 - 275*	256 - 2 268 - 2 *, 277 8
24	281 - 283	-
25	289 - 292	-
26	299 - 313*	-
27	322 - 322	-
28	324 - 324	-
29	340 - 351*	341 - 351*
30	354 - 354	-
31	356 - 365*	-

Asterisks denote sequences of five or more residues

FIGURE 25

GRAPHIC REPRESENTATION OF HYDROPHILICITY AND SURFACEPLOT VALUES FOR GLYCOPROTEIN D OF HERPES SIMPLEX VIRUS

Panel A shows a Hopp and Woods (1981) hydrophilicity plot while panel B shows a Surfaceplot (Parker *et al.*, 1986) plot. Residues are numbered from the N- to the C-terminal. The surface value ordinate has been arbitrarily re-scaled that the O value corresponds to a cut-off of 25% above the mean of hydrophilicity/hydrophobicity values (Panel A) or the mean of exterior/interior values (Panel B). A surface value of 100 corresponds to the height of the highest peak in either case. The scale is therefore a relative one. Shaded peaks represent predicted surface regions of 5 or more contiguous residues.

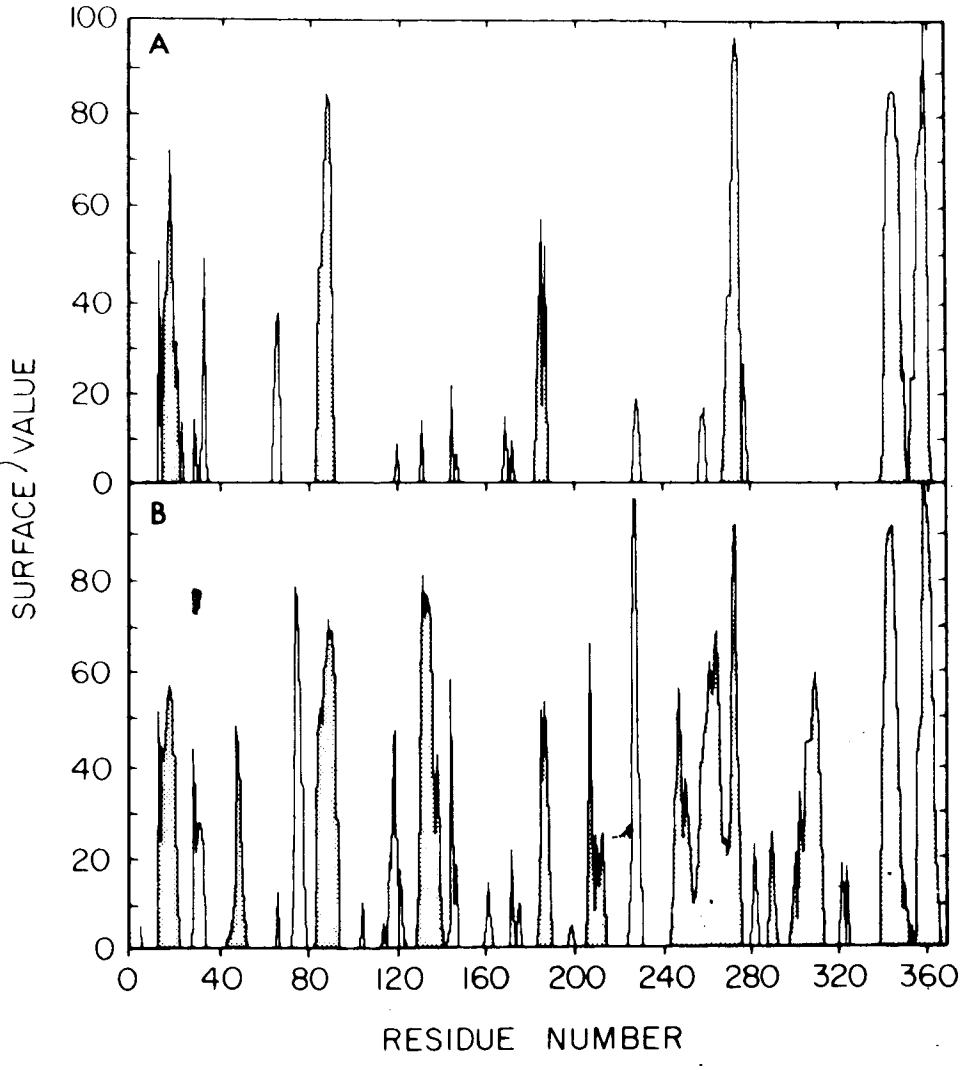

A
B
100
80
60
40
20
0
SURFACE VALUE
0
40
80
120
160
200
240
280
320
360
RESIDUE NUMBER

TABLE 8.

Sequences of HSV-1 glycoprotein D peptides synthesized.

SEQUENCE NUMBER	PEPTIDE SEQUENCE[a]
2-21	BB-G-G-(Y-A-L-A-D-A-S-L-K-M-A-D-P-N-R-F-R-G-K-D)-G-G-amide
12-21	BB-G-G-(A-D-P-N-R-F-R-G-K-D)-G-G-amide
52-61	BB-G-G-(S-L-P-I-T-V-Y-Y-A-V)-G-G-amide
63-72	BB-G-G-(E-R-A-C-R-S-V-L-L-N)-G-G-amide
83-92	BB-G-(G-A-S-E-D-V-R-K-Q-P)-G-G-amide
121-130	BB-G-G-(N-L-S-L-G-A-C-P-I-N)-G-G-amide
130-139	BB-G-G-(R-T-Q-P-A-W-N-Y-Y-D)-G-G-amide
150-159	BB-G-(G-F-L-M-H-A-P-A-F-E)-G-G-amide
181-190	BB-G-G-(L-E-H-R-A-K-G-S-C-K)-G-G-amide
206-215	BB-G-G-(Q-A-Y-Q-Q-G-V-T-V-D)-G-G-amide
267-276	BB-G-G-(E-L-A-P-E-D-P-E-D-S)-G-G-amide
288-297	BB-G-G-(P-Q-I-P-P-N-W-H-I-P)-G-G-amide
303-312	BB-G-G-(A-T-P-Y-H-P-P-A-T-P)-G-G-amide
314-323	BB-G-G-(N-M-G-L-I-A-G-A-V-G)-G-G-amide

[a] Peptides were synthesized based upon the HSV-1 glycoprotein D sequence (Watson *et al.*, 1982).

Affect of Linkers and Coupling Position on Antisera Production. Five methods of conjugation of peptide to carrier protein were investigated (Table 9). All five of these conjugates elicited a strong antibody response in rabbits as tested in ELISA against peptide-conjugate antigen (Figure 26). As controls, there was no ELISA reaction when the plates were coated with the coupling reagents BB-Lys or BB-Gly. Peptide-conjugates with photoprobe attached to the ϵ-amino group of the N-terminal lysine residue (BB-Lys 12-21) or photoprobe attached to the ϵ-amino group of the C-terminal lysine residue (12-21 BB-Lys) showed significantly reduced reactivity in ELISA assays with isolated glycoprotein D or with intact virions, in comparison to the reactivity produced by a peptide-conjugate with the photoprobe attached to the N-terminal α-amino group of glycine (BB-Gly 12-21). Since the method of coupling, the photoprobe and the sequence of peptide are identical, these results suggest that the position of the photoprobe provides a different anti-peptide antibody response even though the titres against peptide-conjugates were similar. The chemically linked peptides, Cys 12-21 and Tyr 12-21, also showed a positive immune response in ELISA assays against isolated glycoprotein D and intact virions. The titres produced by peptides chemically linked to carriers were similar to those of the photoprobe linked peptide, BB-Gly 12-21. Given these results and the ease of coupling with the photochemical reagent, all subsequent experiments were done with BB-Gly peptide-conjugates.

Antibody Titre. The ELISA titres (expressed as the negative logarithm of the reciprocal of the highest dilution of serum which gave a positive response) of antisera raised against BB-Gly peptide-KLH conjugates and tested against BB-Gly peptide-BSA conjugates were found to be approximately 4-5. All antisera were also found to react with isolated glycoprotein D, with a mean titre of 3. The positive reactivity of control peptide antisera (52-61, 150-159) with isolated glycoprotein D (ELISA titers of <1.7; *i.e.* 1:50 dilution) indicated that isolated glycoprotein D was no longer in its native conformation.

TABLE 9.

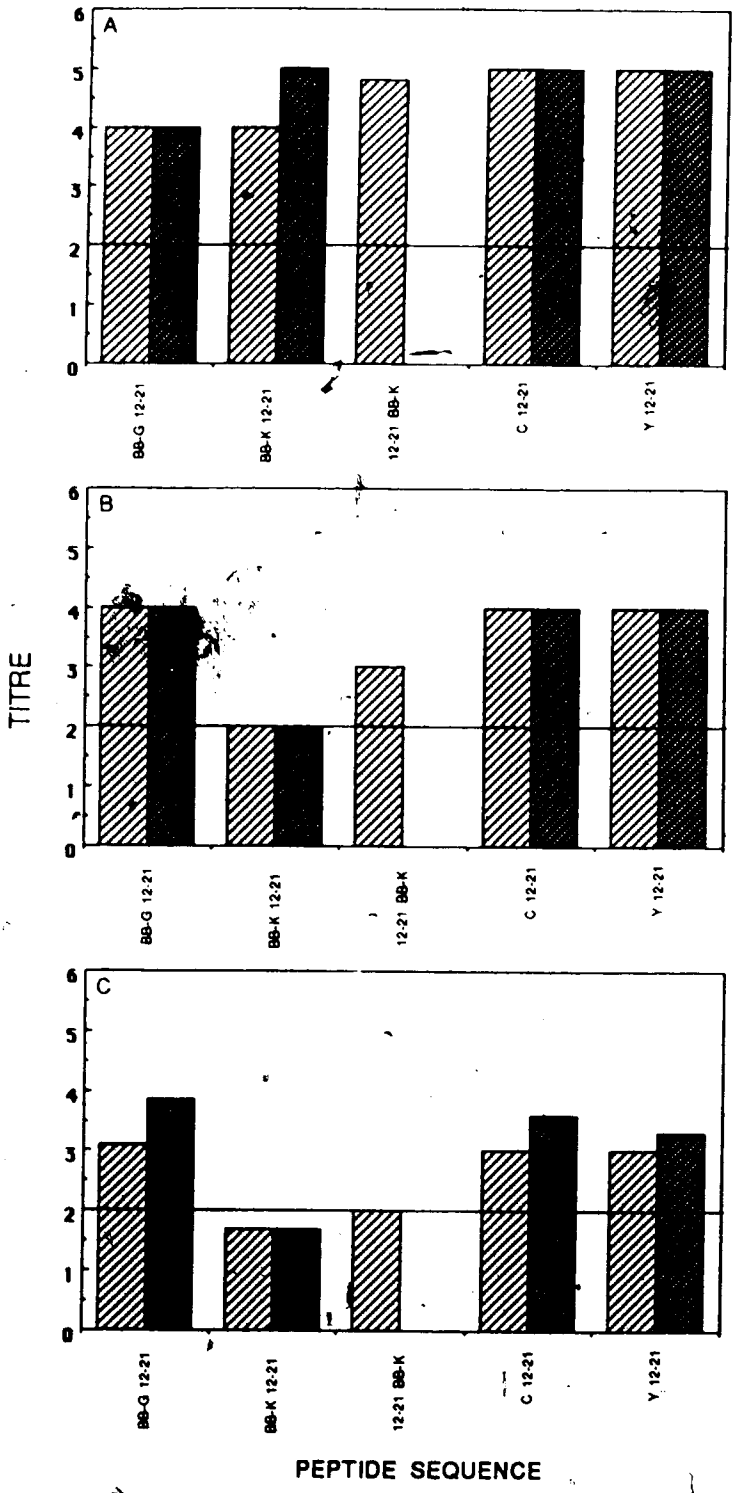
Sequences of peptides synthesized to investigate the effect of linker position and type of linker on the immune response to synthetic peptide conjugates.

SEQUENCE	PEPTIDE SEQUENCE
BB-G 12-21	BB-G-G-(A-D-P-N-R-F-R-G-K-D)-G-G-amide
BB-K 12-21	NAc-K(BB)-G-(A-D-P-N-R-F-R-G-K-D)-G-G-amide
12-21 BB-K	NAc-(A-D-P-N-R-F-R-G-K-D)-G-K(BB)-amide
C 12-21	C-(A-D-P-N-R-F-R-G-K-D)-G-G-amide
Y 12-21	Y-(A-D-P-N-R-F-R-G-K-D)-G-G-amide

FIGURE 26

ACTIVITY IN ELISA OF RABBIT ANTISERA PRODUCED USING PEPTIDES CONJUGATED TO KLH USING DIFFERENT LINKERS

Antisera were tested against: peptides conjugated to BSA, isolated glycoprotein D and HSV virions. The shaded bars indicate the results from two different rabbits immunized in each case (the second rabbit immunized with BB-Lys (BB-K) conjugated via the C-terminus unfortunately died). Titers are expressed as the negative logarithm of the reciprocal of the highest dilution of serum which still gave a positive response. Sera with significant activity was judged to be that which reacted at a dilution of more than 1:100 (titer > 2). C and Y refer to the chemically-linked (cysteine, tyrosine) peptides.


A
B
C
TITRE
0
1
2
3
4
5
6
BB-G 12-21
BB-K 12-21
12-21 BB-K
C 12-21
Y 12-21
PEPTIDE SEQUENCE

Antibody Reactivity with HSV-1 Virions. Interaction of the peptide antisera with purified virions is shown in Figure 27. Eight of the peptide antisera reacted with titres > 2 (*i.e.* 1:100 dilution) with this antigen. It is interesting that antibodies raised to the peptides which showed low titres to the whole virion did react well with isolated gD. This particular result indicates that either a conformational change altering antigenicity occurs during incorporation of glycoprotein D into the virion envelope, or that close packing masks an antigenic site. Antisera raised to the sequence 314-323 also shows reactivity with the whole virion. It has been proposed (Watson, 1982) that this sequence is likely to be part of the transmembrane region. The reactivity of the antisera raised to peptide 314-323 (which was predicted by Surfaceplot to have surface residues) would place the start of transmembrane region closer to the C-terminus. Antisera raised to the control sequences (52-61, 150-159) did not react with the intact HSV-1 virion.

Neutralization Tests Antisera which reacted with intact virions in ELISA was screened for virus-neutralization capacity using a plaque-reduction test. Antibodies to five of the eight surface regions were found to elicit a neutralizing response (Table 10). The strongest neutralization was associated with the antisera to N-terminal sequence 2-21 in the presence of guinea pig complement. Without complement the neutralization associated with these antisera was found to be much weaker (1:25 dilution compared to 1:200 for 50% plaque reduction). A fragment of sequence 2-21, namely residues 12-21, was found to neutralize virus equally with or without complement. Since the extent of this neutralization was equal to that of antisera 2-21 in the absence of complement, one may conclude that sequence 2-21 contains two epitopes; one in the sequence 12-21 and the other, which requires complement for neutralization, elsewhere within residues 2-21.

The rabbit antisera raised to predicted surface regions 267-276 and 288-297 were also found to contain neutralizing antibodies. It is interesting to note that in the plaque reduction assay, the antisera to 267-276 exhibited the strongest complement-independent response.

FIGURE 27

REACTIVITY IN ELISA OF RABBIT PEPTIDE ANTISERA WITH HSV-VIRIONS

(*i.e.* INTACT GLYCOPROTEIN D)

The asterisks indicate sequences which were selected and synthesized as negative controls. Sera with significant activity was judged to be that which reacted at dilutions of more than 1:100 (titer $>$ 2.0).

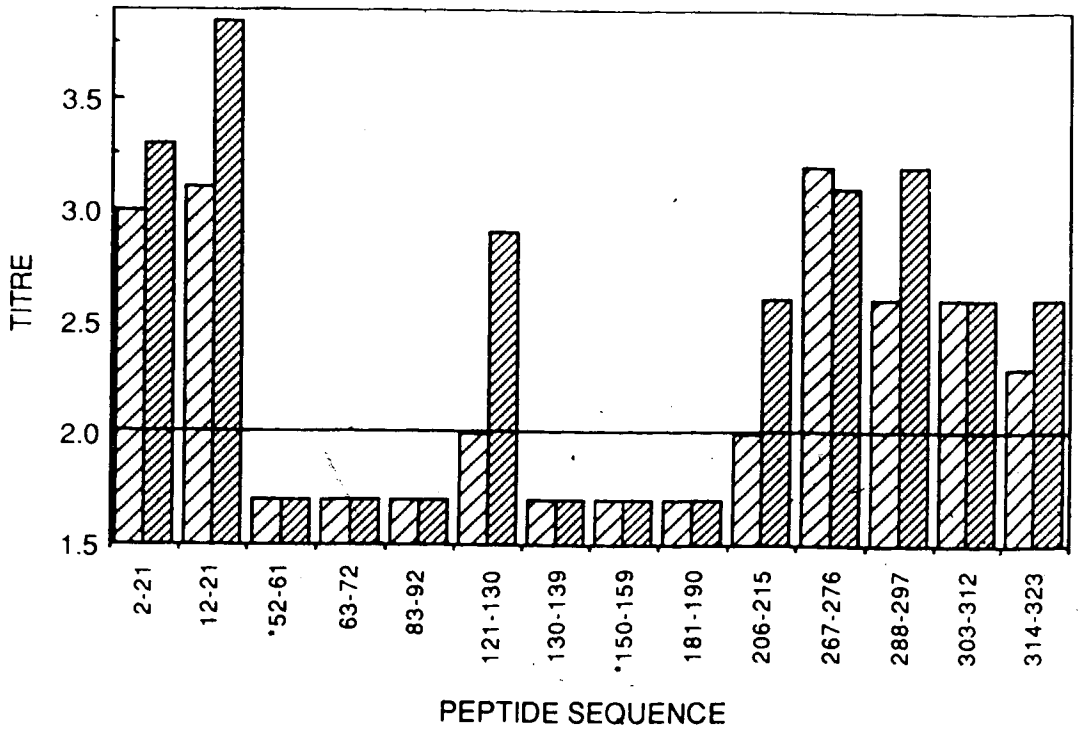

TITRE
3.5
3.0
2.5
2.0
1.5
2-21
12-21
*52-61
63-72
83-92
121-130
130-139
*150-159
181-190
206-215
267-276
288-297
303-312
314-323
PEPTIDE SEQUENCE

TABLE 10.

Results of neutralization assays

Antibody to Sequence	Neutralization	50% Plaque-reduction (Dilution)[a]
2 - 21	+	1:200[b]
12 - 21	+	1:25
121 - 130	x	-
206 - 215	x	-
267 - 276	+	1:50
288 - 297	+	1:25
303 - 312	x	-
314 - 323	+	1:25

a Control sera from pre-immune rabbits did not cause any plaque reduction at a dilution of 1:25

b Neutralization capacity was enhanced by the presence of 5% guinea-pig complement. Dilution without complement was 1:25.

It was surprising to find neutralization associated with the antisera to peptide 314-323. As we have stated previously, our results suggest that this sequence lies adjacent to the transmembrane region of glycoprotein D.

Reactivity of Human Sera with Synthetic Peptide Antigens. The recognition of the synthetic peptide antigens by sera obtained from a small number of individuals with acute or convalescent HSV-1 infections was assessed using ELISA assays. The results of these assays are shown in Figs. 28 and 29 for acute and convalescent sera respectively. For acute sera, although the titre against viral antigen was found to be significant, no strong response to any one peptide was observed. It is known that in the primary stages of herpesvirus infection, it is primarily the cytotoxic T cells and cell mediated immune responses (Nash *et al.*, 1985) which combat infection. The lack of development of humoral response to linear antigenic determinants was not, therefore, unexpected.

The response of immune sera in some cases showed a lack of specificity for surface sites, for example, some sera reacted with the predicted internal sequences 52-61 and 150-159. This reactivity was probably produced by a fortuitous cross reaction of non-HSV human antibodies with these sequences. The N-terminal sequence (2-21) as well as the sequence 267-276 were recognized by a number of sera. This may be indicative of the immunological importance of these sites, and these are potential candidates for a synthetic vaccine. De Freitas *et al.* (1985) have reported that a synthetic peptide corresponding to residues 8-23 activated human peripheral blood T-cells. A vaccine containing this sequence might be expected to elicit both a strong humoral immune response and to prime cell-mediated immunity to herpes simplex virus. These studies represent a preliminary analysis of the interaction of human sera with synthetic peptide antigens. Future studies will be expanded to incorporate the screening of larger numbers of sera and will include the analysis of paired sera. Paired sera represent samples from a single donor taken during the acute and convalescent stages of infection. Analysis of such sera will monitor the changes in immune response during

FIGURE 28

REACTIVITY IN ELISA OF HUMAN SERA WITH PEPTIDE BSA CONJUGATES

CONJUGATES AND HSV 1 VIRIONS

Sera was obtained from three individuals in the acute stage of HSV 1 infection

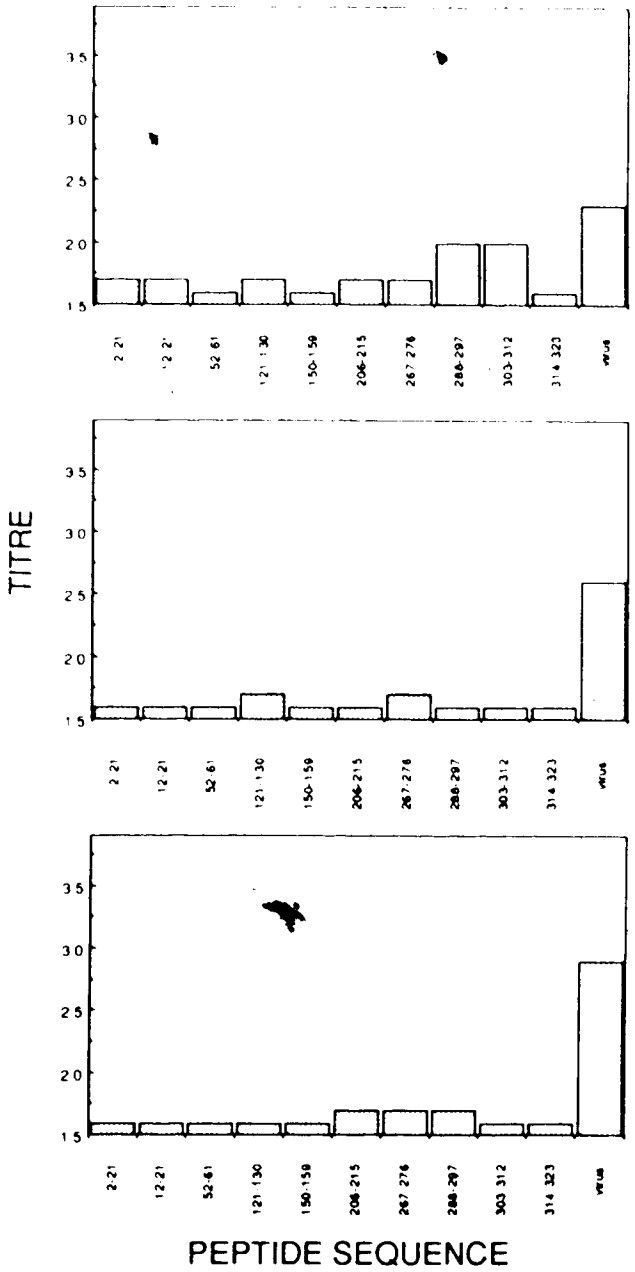
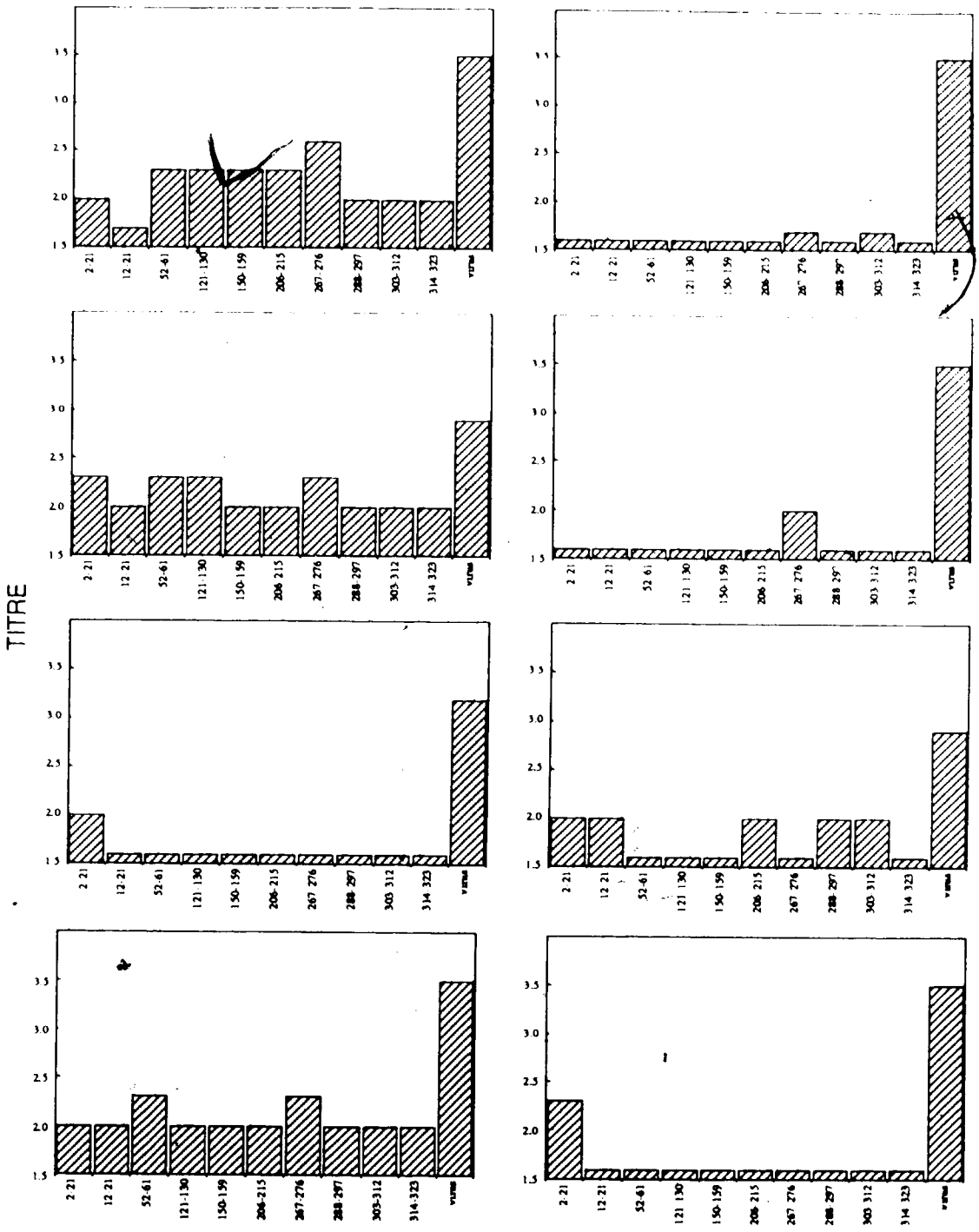

TITRE
35
30
25
20
15
2-21
12-21
52-61
121-130
150-159
206-215
267-276
288-297
303-312
314-323
virus
PEPTIDE SEQUENCE

FIGURE 29

REACTIVITY IN ELISA OF HUMAN SERA WITH PEPTIDE-BSA CONJUGATES AND HSV-1 VIRIONS

Sera was obtained from eight individuals in the convalescent stage of HSV-1 infection (none of these correspond to the sera used for the tests shown in Figure 28).


TITRE
PEPTIDE SEQUENCE

recovery from an HSV infection.

HSV Type-Specific Immune Response. The interaction of antisera raised to type specific sequences with cells infected with HSV-1 or HSV-2 was detected by indirect fluorometric assay. Three type specific sequences from HSV-1 (those in which there are a number of non-conservative amino-acid changes between corresponding peptides for glycoprotein D of HSV-1 and HSV-2) have been synthesized (sequences 38-47, 83-92 and 199-208; Table 11). These type specific peptides also contained residues predicted to be at or near the protein surface. Antisera to these sequences showed bright cytoplasmic fluorescence when assayed against HSV-1 infected cells (Figure 30, panel A). In fluormetric analysis of cells infected with HSV-2 (strain 333), antisera to peptide 83-92 produced a pattern of fluorescence identical to that obtained with HSV-1 infected cells; *i.e.* bright pools of cytoplasmic fluorescence (Fig. 30, panel C). Antisera to peptide 38-47 produced plasma membrane fluorescence (Fig. 30, panel B), similar to that which we have previously observed with non-specific or cross-reactive antibody binding to membrane components. The antisera to peptide 199-208 was non-reactive. Controls of antisera raised to the type-common peptide sequences showed identical patterns of fluorescence with both types of herpes simplex virus infection. Negative controls of pre-immunization rabbit sera gave low overall fluorescence with small centers of apparently non-specific binding. The immunofluorescence results suggest that the single amino acid change in peptide 83-92 does not significantly affect antibody binding, whereas the differences in peptides 38-47 and 199-208, which constitute major sequence changes, profoundly affect antibody binding. Antisera to peptide 199-208 could conceivably be useful as a type-specific diagnostic reagent, permitting one to differentiate between HSV-1 and HSV-2 infections.

TABLE 11.

Sequences of synthetic peptides based on type-specific regions of glycoprotein D of herpes simplex virus.

SEQUENCE NUMBER	PEPTIDE SEQUENCE
HSV1-gD 38-47	BB-G-G-(Y-H-T-Q-A-G-L-P-D-P)G-G-amide
	* * * *
HSV2-gD 38-47	Y-H-I-Q-P-S-L-E-D-P
HSV1-gD 83-92	BB-G-(G-A-S-E-D-V-R-K-Q-P)G-G-amide
	*
HSV2-gD 83-92	G-A-S-E-D-V-R-K-H-T
HSV1-gD 199-208	BB-G-G-(P-S-A-C-L-S-P-Q-A-Y)G-G-amide
	* * * *
HSV2-gD 199-208	P-A-A-C-L-T-S-K-A-Y

Asterisks denote non-conservative amino-acid changes between corresponding peptides for the glycoproteins D of HSV-1 and HSV-2.

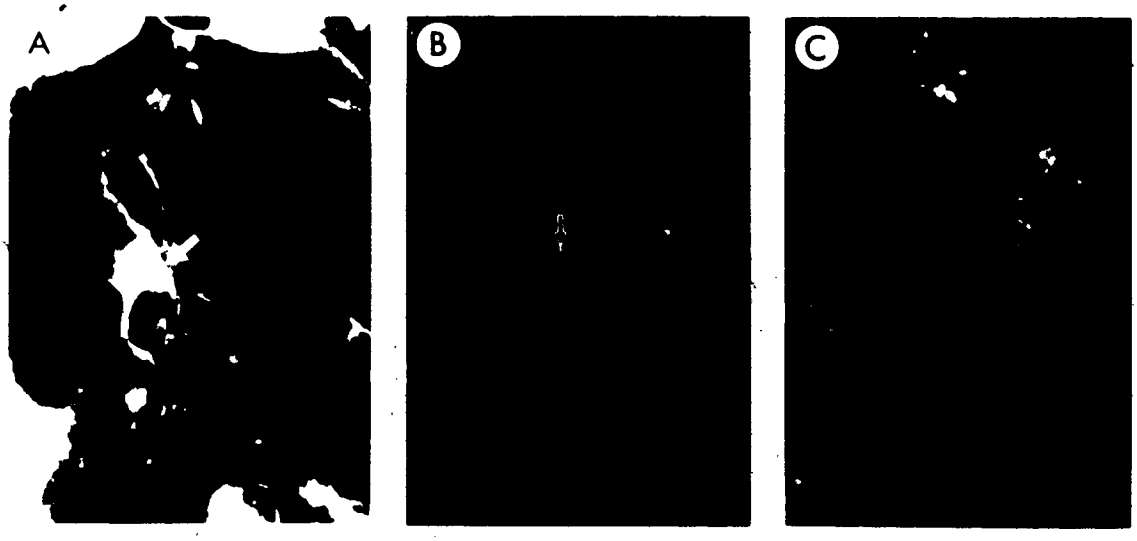
HSV-1 peptides were synthesized based upon the HSV-1 glycoprotein D sequence of Watson *et al.*,1982.

HSV-2 peptides were from the HSV-2 glycoprotein D sequence of Watson *et al.*,1983.

FIGURE 30

IMMUNOFLUORESCENCE OF TYPE-SPECIFIC PEPTIDE ANTISERA WITH HSV-1 AND HSV-2 INFECTED CELLS

Panel A shows the fluorescence pattern observed when HSV-1 infected cells are incubated with all three type-specific antisera. Note the pool of cytoplasmic fluorescence (arrow). Panel B shows the weak plasma membrane associated fluorescence observed when antisera to peptide sequence 38-47 is incubated with HSV-2 infected cells. Panel C shows the fluorescence observed when HSV-2 infected cells are incubated with antisera to peptide sequence 199-208. The photographic exposure for panels B and C was 5x longer than for panel A (Magnification: 400x)


A
B
C

DISCUSSION

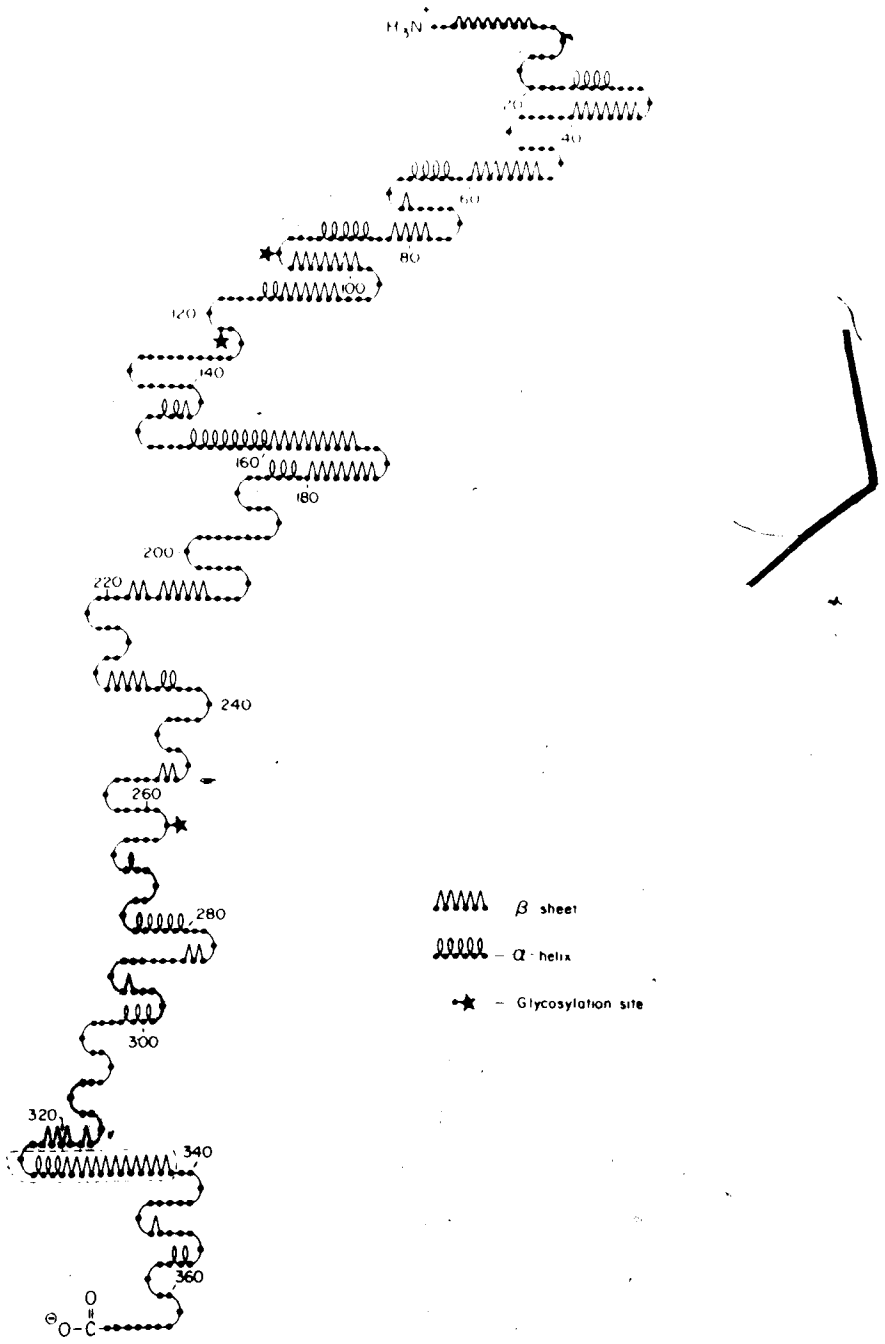
We have used the Surfaceplot predictive algorithm to select linear amino acid sequences in herpes simplex virus type 1 glycoprotein D which have a high probability of exterior location and are, therefore, potentially antigenic. Ten-residue peptides corresponding to eleven such sequences, and to two "negative controls" (*i.e.* sequences with very low probability of being on the surface of glycoprotein D) were synthesized, conjugated to KLH carrier protein and used to "immunize" rabbits. The polyclonal antisera thereby obtained was found, in each case, to react positively in ELISA with the peptide conjugated to BSA and with isolated glycoprotein D. These results demonstrated that all of the synthetic peptide-conjugates were immunogenic in rabbits and that glycoprotein D isolated by affinity chromatography and HPLC and bound to the ELISA plate is in a highly denatured state. In contrast, not all of the antisera reacted positively in ELISA with HSV virions; that is, with glycoprotein D *in situ*. Seven linear antigenic sites on HSV-1 virions were identified. These correspond to glycoprotein D sequences 12-21, 2-21, 121-130, 206-215, 267-276, 288-297 and 314-323, and were used to generate anti-peptide antibodies.

Considering the Surfaceplot predictions, sequences 121-130 and 314-323 had less than 5 contiguous surface residues (Table 9). If one examines a Chou-Fasman secondary structure representation (Chou and Fasman, 1974a, b; 1978) of glycoprotein D (Figure 31), the sequence 121-130 includes a glycosylation site (Asn 121) and parts of two β-turns. These criteria are obviously indicative of a surface segment, and this demonstrates that such features as glycosylation sites and β-turns should be considered along with Surfaceplot data in selecting potential antigenic regions. In the case of peptide sequence 314-323, Surfaceplot indicates positive values for residues 322 and 324 (Table 9), and the Chou-Fasman structure (Fig. 31) predicts β-turns for residues ~312-315 and ~323-324. The fact that at least part of the sequence 314-323, being antigenic, must find itself on the exterior of the viral envelope makes it necessary to revise

FIGURE 31

PREDICTED SECONDARY STRUCTURE OF GLYCOPROTEIN D GENERATED USING A CHOU-FASMAN (1978) -BASED COMPUTER PROGRAM

This program was generously donated by the Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan. The program initiates α-helix when 4 out of 6 residues are helix formers and β-sheet when 3 out of 5 residues are β-sheet formers. The major linear surfaceplot predicted sites, 12-21, 44-51, 83-93, 130-140, 206-213, 244-275, 299-313, 340-351 and 356-365 were found to correlate well with β-turns. Neutralizing antigenic sites are shaded. The proposed transmembrane region (residues 323-339) is outlined.


H_3N
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
ΘO-C
O
β sheet
α - helix
- Glycosylation site

the proposal by Watson *et al.* (1982) that the membrane-spanning region for glycoprotein D is residues 314-339 (or residues 340-364 of the glycoprotein D precursor protein). Since Eisenberg *et al.* (1985) have described an epitope within the sequence 340-356 in isolated glycoprotein D, we suggest that the membrane spanning region is most likely to follow the predicted β-turn at residues 323-324 and extend to approximately residue 340. There are no charged residues within this sequence; part of it (residues 326-330) may be helical and the remainder is probably random coil, even though Chou-Fasman predictions suggest β-sheet, there is no evidence for corresponding sheet to stabilize such a structure within the membrane.

Synthetic peptides corresponding to sequences 2-21, 12-21, 267-276, 288-297 and 314-323 elicited a virus-neutralizing immune response in rabbits. The long peptide encompassing residues 2-21 was found to comprise two epitopes. One of these lies within the sequence 12-21, which by itself elicits antibodies capable of neutralizing virions in the absence of complement. Peptide 2-21 retains the ability to generate complement-independent neutralizing antibody, and in addition elicits the production of rabbit antibodies which neutralize virus very efficiently in the presence of complement. These results are compatible with those obtained by Cohen *et al.* (1984) who demonstrated that rabbits immunized with synthetic peptide 8-23 developed HSV-1 neutralizing antibodies. Eisenberg *et al.* (1985) reported that antiserum to peptide 268-287 had no neutralizing activity; in contrast we found that antisera to peptide 267-276 showed the strongest complement-independent neutralizing activity of all of the five linear epitopes which we identified.

Dietzschold *et al.* (1984) raised polyclonal and monoclonal antibodies to glycoprotein D isolated from HSV-1 and HSV-2. Interaction of these antibodies with synthetic peptides (peptide 1-23 with citraconylated lysines 10 and 20, and peptides 8-23 and 11-23) suggested that one of the epitopes in the N-terminal 1-23 sequence is located between residues 11 and 19. We have confirmed this suggestion since antisera to

peptide 12-21 reacts with isolated glycoprotein D and with HSV-1 virions.

Eisenberg *et al.* (1988) identified another antigenic site in the sequence 268-287 by showing reactivity of this peptide with an anti glycoprotein D monoclonal antibody. This monoclonal antibody did neutralize HSV-1 at a dilution of 1:50, however, when peptide 268-287 was conjugated to KLH and used to immunize rabbits, the anti peptide antibodies did not react with either glycoprotein D or intact virions. We found, in contrast, that antibodies to the KLH conjugate of peptide 267-276 recognized both glycoprotein D and HSV-1 virions. They also neutralized. This difference might be explained on the basis that the longer peptide (268-287) adopts a conformation distinct from that of the native epitope, whereas the shorter peptide (267-276) is more flexible. An alternative explanation is that the method of conjugating the peptide to to KLH resulted in the differences in its immunogenicity. Eisenberg *et al.* coupled their peptide via a C terminal cysteine, whereas we coupled our peptide through an N terminal BB-gly. These results point out the importance of selection of cross linker and point of attachment of peptide to carrier protein in the design of peptide vaccines.

CHAPTER V

SUMMARY AND FUTURE PROSPECTS

The major focus of the work presented in this thesis has been the structure and immunology of human herpesviruses. We have applied a predictive algorithm for the detection of surface/antigenic sites to the primary amino acid of herpes simplex virus type I glycoprotein D. From our predictions we have described a number of linear epitopes that stimulate the production of neutralizing antisera when injected into rabbits. In studies on human cytomegalovirus, we have tapped the human immune system to reveal the identity of immunodominant virion proteins to which neutralizing antibodies are produced.

Each of the studies described is of interest to the field of vaccine research, however, a combination of the two approaches that we have utilized may yield a powerful tool for studies in human viral immunology. The inclusion of an assay for T lymphocyte response, for example the T cell proliferation assays of De Freitas *et al.* (1985) or Torseth *et al.*, (1987) would be important in order to assess the overall immune response (humoral and cell mediated responses) to viral or virus sub unit antigens.

The proposed system for synthetic vaccine development would be composed of three sections.

1. The Surfaceplot analysis of antigens for potential linear antigenic sites, and the construction of synthetic peptide conjugates of these sites.

2. Screening the peptide conjugates in a human context by sera analysis (ELISA), flow cytometry for B cell receptors, and T cell proliferation assays.

3. Production of human monoclonal cell lines and analysis of antibodies for neutralizing activity.

We have initiated the pilot studies into producing a synthetic peptide vaccine for herpes simplex virus; using peptide sequences from glycoprotein D. With the availability of nucleotide sequences from HIV (Ahzon *et al.*, 1986; Starcich *et al.*, 1986) varicella zoster virus (Davison & Scott, 1986), as well as HCMV (Weston & Barrel, 1986) and HSV 1 glycoproteins B (Bzik *et al.*, 1984) and C (Frink *et al.*, 1983) these studies may be extended to cover a number of medically important viral diseases. Indeed, the procedure is not restricted to viral sequences but may be applied to almost any human pathogen.

Epitope mapping currently relies greatly on the production of antibodies to the whole protein, in mice or rabbits, and the analysis of the interaction of such antibodies with fragments of the protein. We have applied the converse approach. From the sequence of proteins we can deduce potential linear antigenic sites, raise antisera to synthetic peptides of these regions, and test the antisera for recognition and neutralization of the intact protein/virus.

In the development of a human mapping system, the option to generate antisera is not available. However, antisera from patients suffering from viral illnesses is available and these may be used to assess the immunogenicity of specific peptides. The poor specificity of human sera towards any one or two peptide sequences, as we have observed in the case of HSV glycoprotein D (see Chapter IV), would require extensive testing of large numbers of human sera. Given that such testing can readily be carried out by ELISA, this task is not formidable.

Having selected a small number of potentially immunodominant sequences, it is necessary to determine the response of seronegative individuals to these sequences. Could these sequences elicit an immune response in humans? B lymphocyte recognition of a synthetic peptide antigen can be studied conveniently *in vitro* by flow cytometry using peptides conjugated to fluorescenated carrier proteins. On the surface of each B lymphocyte is a receptor of the same unique specificity as the antibodies which these

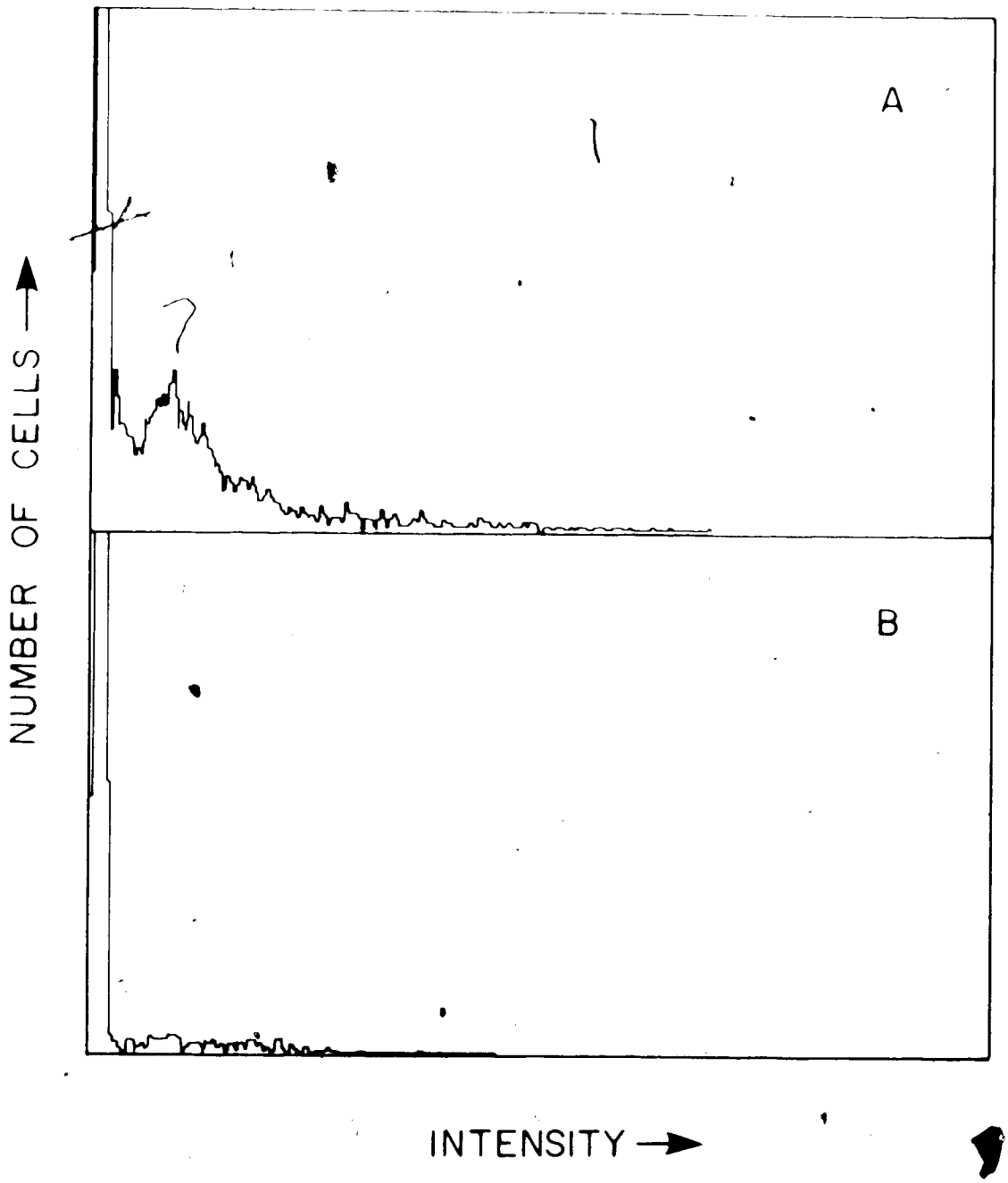
cells synthesize. It is through these receptors that B cells interact with antigens. By introducing fluorescence labeled antigen to B cells we are able to detect the presence of specific receptors on the B cell surface. Analytical flow cytometry may be used to assay the reactivity of B lymphocytes with the labeled antigen. For example, we have carried out a preliminary flow cytometric survey of lymphocytes from donors found to be seronegative against HSV 1 glycoprotein D peptide 12-21, a peptide which was found to elicit a neutralizing antibody response in rabbits. Figure 32 shows the results from analytical flow cytometry using peptide sequence 12-21 bound to fluoresceinated BSA carrier protein. For sample A, 21% of lymphocytes (enriched for specificity to sequence 12-21 by panning) bound to the fluoresceinated peptide conjugate, whereas in sample B, which was prepared in a manner identical to sample A, only 3% bound to the probe. Control experiments with carrier enriched lymphocytes showed the background binding for the assay to be ~2%. The number of cells in sample A bearing receptors for sequence 12-21 is greater than would be expected for a single epitope. It is therefore likely that this sequence contains more than one epitope, that together with binding to receptors of approximate specificity could account for the numbers of fluorescent cells that we have observed. It is certainly safe to assume from these results that this individual does carry B-lymphocytes with specific receptors for peptide sequence 12-21, and would be expected to mount an antibody response to that peptide-antigen. Since the B lymphocytes from sample B (and from six other samples from different individuals screened thus far) did not carry specific receptors for sequence 12-21, this individual would not mount an immune response to this peptide-antigen. De Freitas *et al.* (1985) also found glycoprotein D peptide 13-23 did not produce human T cell proliferation *in vitro*. It is therefore unlikely that peptide sequence 12-21 will be useful as a potential human vaccine for HSV-1.

It is natural for our work with synthetic peptides of glycoprotein D sequences to now progress into the human monoclonal antibody production and analysis stage. We

FIGURE 32

STATISTICAL ANALYSIS BY FLOW CYTOMETRY OF TWO POPULATIONS OF B-LYMPHOCYTES USING HSV-1 GLYCOPROTEIN D SYNTHETIC PEPTIDE 12-21 CONJUGATED TO FLUORESCENATED BSA AS RECEPTOR PROBE

In sample A, 21% of cells carried probe whereas in sample B only 3% of cells were marker positive. Both samples were taken from donors who were seronegative for this antigen. The lymphocyte samples were depleted of T-lymphocytes and monocytes (see Appendix 2 and 5 for methodologies) and then enriched for specificity towards the peptide antigen by panning procedures. Following reincubation with fluorescenated antigen they were subjected to analytical flow cytometry on a Coulter EPICS V flow cytometer. (Coulter Electronics Inc., Hialech, Fl).


A
B
NUMBER OF CELLS
INTENSITY

already have indications that glycoprotein D peptide sequences 2-21 and 267-276 are of importance immunologically, so these would be prime candidates for such analysis. The sequence of HSV-1 glycoprotein B has also been reported (Bzik *et al.*, 1984). Glycoprotein B is also involved in the initial stages of HSV-1 infection, and neutralizing antibodies have been raised to this protein in mice. What we wish to do is to test those peptides from glycoproteins D and B which are recognized by B lymphocytes (see above) for their ability to elicit the production of monoclonal antibody secreting cells *in vitro* (using the methods described in Chapter III). The monoclonal antibodies would then be analyzed for neutralizing capacity. Ideally we would hope to find a combination of peptides from gD and gB which would: 1) interact with B cell receptors, 2) elicit the production of neutralizing antibodies *in vitro*, and 3) stimulate T cell proliferation *in vitro*. Such a combination peptide should have excellent vaccine potential in humans.

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APPENDIX I

HUMAN LYMPHOCYTE PREPARATION

The lymphocyte preparations that we have used were derived from buffy-coats obtained from the Canadian Red Cross. The buffy-coats arrive in the plastic collection bags minus the red cells and plasma, although there are large numbers of red cells contaminating the sample. I suggest that all handling of human cells be carried out under level B biohazard containment. prior to the AIDS epidemic I was always conscious of the risks of hepatitis. Testing of all blood samples obtained through the Red Cross is carried out, but the processing of tests takes a couple of days. Since you will be handling fresh samples take every precaution.

Ask for a serum sample from each donor so you can screen for antibodies to target antigens.

Protocol:

1. Set up 3 x 50 ml centrifuge tubes for each buffy-coat to be processed.
2. Pipet 20 ml of Ficoll-Hypaque (Pharmacia) into each tube.
3. Prepare a sterile 250 ml conical flask for each buffy-coat. You will be diluting the buffy-coats in this before loading the Ficoll-Hypaque tubes.
4. Wipe the bag containing the buffy-coats with Kleenex saturated in 70% EtOH. Sterilize all instruments, *e.g.* scissors that are to come into contact with the bag/buffy-coat.
5. Snip one of the bags outlet tubes and decant the buffy-coat into the 250 ml flask. Dispose of bag in Biohazard waste.
6. Dilute buffy-coat 1:1 with RPMI-1640 containing 25-40 μg/ml Gentamycin.

7. Overlay the blood/medium mixture over the Ficoll, taking care not to let the two phases mix.

8. Centrifuge at 1500g for 20 minutes, RT.

9. Gently aspirate media, leaving 1-2 ml above the lymphocyte layer.

10. Add [redacted] of RPMI-1640 containing 10% FCS into 15 ml tubes.

11. With a pasteur pipette, aspirate the lymphocyte layer, taking in some Ficoll and medium, and transfer to 15 ml tubes.

12. Top tubes off with medium and centrifuge at 500 g for 10 min.

13. Aspirate supernatant, leaving cell pellet intact, then resuspend cells in a couple of ml of RPMI-1640 and top up the tube. Centrifuge for 7 min at 500 g. Take a sample of cells to count then repeat the wash once more.

14. Leave tubes on ice for 5-10 mins.

15. Aliquot and freeze cells at a density of 1×10^6 cells/ml, in freezing medium: 10% DMSO, 20% FCS, in RPMI-1640.

16. Transfer cells to -70° freezer as quickly as possible.

APPENDIX II

T-CELL DEPLETION

T-cells are depleted from both potential feeder layers and from cells about to be transformed. T-cells are depleted by rosetting with AET- (2-aminoethylisothiouronium bromide hydrobromide) coated sheep red blood cells (SRBC) and separation over Percoll.

To prepare AET-SRBC:

1. Standard recipe = 402 mg AET/10 ml ddH_2O
2. Adjust pH to 9.0 with 5M NaOH (100-120 drops). Do not overshoot. Do not re-adjust pH with HCl.
3. Filter through 0.2 μm filter.
4. Wash SRBC x 3 with RPMI-1640.
5. Add 4 vol. AET to 1 vol SRBC and mix thoroughly.
6. Incubate at 37° for 30 min. mixing occasionally.
7. Wash 5x with RPMI-1640.
8. Make up to 4% v/v with RPMI-1640 + 10% FCS.

AET-ARBC will last up to 7 days at 4°, but wash once before use.

To prepare Percoll for T-cell rosetting:

1. Take 9 volumes Percoll (Pharmacia) + 1 volume 10x PBS (1:9 dilution → isotonic Percoll).

   N.B. Percoll can only be autoclaved <u>before</u> addition of PBS, so handle with care sterile technique.

2. Weigh density bottle

   Weigh density bottle + distilled water

   Carefully dry density bottle and re-weigh

   Weigh density bottle + Percoll-PBS

   Clean and dry density bottle and reweigh

   Weight of Percoll-PBS = weight of Percoll-PBS + bottle minus bottle alone

   Weight of distilled water = weight of water and bottle minus bottle

   Specific gravity (density) = weight of Percoll-PBS/weight of water

3. Weigh density bottle

   Weigh density bottle + RPMI-1640 with 5% FCS

   Clean and dry density bottle and reweigh

   Specific gravity (density) = weight of RPMI-1640 with 5% FCS/weight of water

4. Calculate the density required (1.080 g/ml) according to the formula:

$$x = \frac{SV - Vd}{(D - d)}$$

   D = density of Percoll-PBS

   d = density of medium

   S = density required (1.08)

   V = volume required (100 ml)

   x = ml of Percoll-PBS

N.B. It is very important to be accurate when weighing. The proportion of Percoll-PBS to medium should always roughly be 60:40.

P for stock iso-osmotic percoll in saline (SIP) = 1.123 g/ml.

T-cell depletion:

1. In a round bottomed tube (e.g. Nuncatom universal container, 25 ml with screw-cap) for each 10^7 PBL add 1 ml 4% AET-SRBC in 5% FCS. Use 10^8 cells maximum/tube.
2. Centrifuge 10 min at 400 g. Let sit on ice for 1 Hour
3. Aspirate supernatant but leave enough to form a workable meniscus
4. Resuspend pellet by gentle horizontal twisting inversion. This may take up to 15 min
5. Underlayer Percoll (1.08 g/ml) at a ratio 2 vol. cell 1 vol. Percoll
6. Centrifuge 20 min at 1000 g.
7. Collect interphase cells; let macroscopic RBC clumps settle for ~5 min and remove carefully by pipette.
8. Dilute mononuclear suspension in RMPI-1640, pellet at 750 g for 10 min and wash once.

Recovery should be 10-30% of input PBL.

APPENDIX III

SPECIFIC PRECURSOR ENRICHMENT (PANNING)

We have used this method for human monoclonal antibody production and antigen screening by flow cytometry.

Procedure:

1. Adhere sterile antigen to 6 well Costar Plate (5-10 μg/ml, overnight).
2. Wash antigen-bound plate 3x and block it with 100% FCS for 1 hour at 4°.
3. Under stringent 4° conditions, add 5 x 10^6 B lymphocytes/well in 2 ml RPMI-1640 containing 5% FCS.
4. Allow to settle for 2-3 hours.
5. Gently swirl and aspirate unbound cells. Wash by slowly adding cold RPMI-1640 with 5% FCS down one side of well, and gently swirl. One wash may be sufficient to remove more than 95% of non-specific lymphocytes.
6. Remove bound cells by vigorous direct pipetting with 4° RPMI-1640 with 5% FCS.

APPENDIX IV

EBV TRANSFORMATION

1. Resuspend up to 10^6 B-lymphocytes or precursor enriched cells in 1 ml of EBV at 10^6 transforming units/ml of RPMI-1640 containing 20% FCS.

2. Incubate at 37° for 2-3 hours, no longer or the virus will kill the lymphocytes.

3. Centrifuge 5 minutes at 400 g.

4. Aspirate supernatants observing MRC biohazard guidelines.

5. Resuspend cells to desired concentrations of cells in RPMI-1640 containing 20% FCS.

6. Plate out using limiting dilution culture technique, starting density 10^5 decreasing stepwise; 5×10^4; 10^4; 5×10^3; 10^3; 5×10^2; 10^2 cells/well. Use a feeder layer of 1×10^5 γ-irradiated B cells/well (irradiated with 2000 rads on ice).

Do not disturb cultures for 9-11 days.

APPENDIX V

MONOCYTE DEPLETION

Although not strictly necessary for the production of human monoclonals, this is required for B lymphocyte analysis by flow cytometry. Monocytes are removed by incubation with RPMI-1640 containing Carbonyl Iron and Gum Arabic (CIGA). Monocytes phagocytose iron, increasing their density, which allows separation from the B lymphocytes by centrifugation over Percoll.

1. Prepare CIGA stock solution of 5 g Carbonyl Iron (Sigma) and 5 g Gum Arabic (Sigma). Have sterilized by irradiation.

2. Prepare a working solution of 10% CIGA stock in RPMI-1640 medium with or without 5% FCS.

3. Incubate 10^8 lymphocyte/monocyte cells in 10 ml working CIGA solution at 37° for 45 min with constant mixing.

4. Underlayer 1.08 g/ml Percoll, ratio 2 vol medium:1 vol Percoll.

5. Centrifuge at 1,500-g for 20 min.

The B lymphocytes appear as an interphase band while the monocytes will pellet. Flow cytometric analysis of the monocyte depleted B lymphocytes is shown in Figure 33.

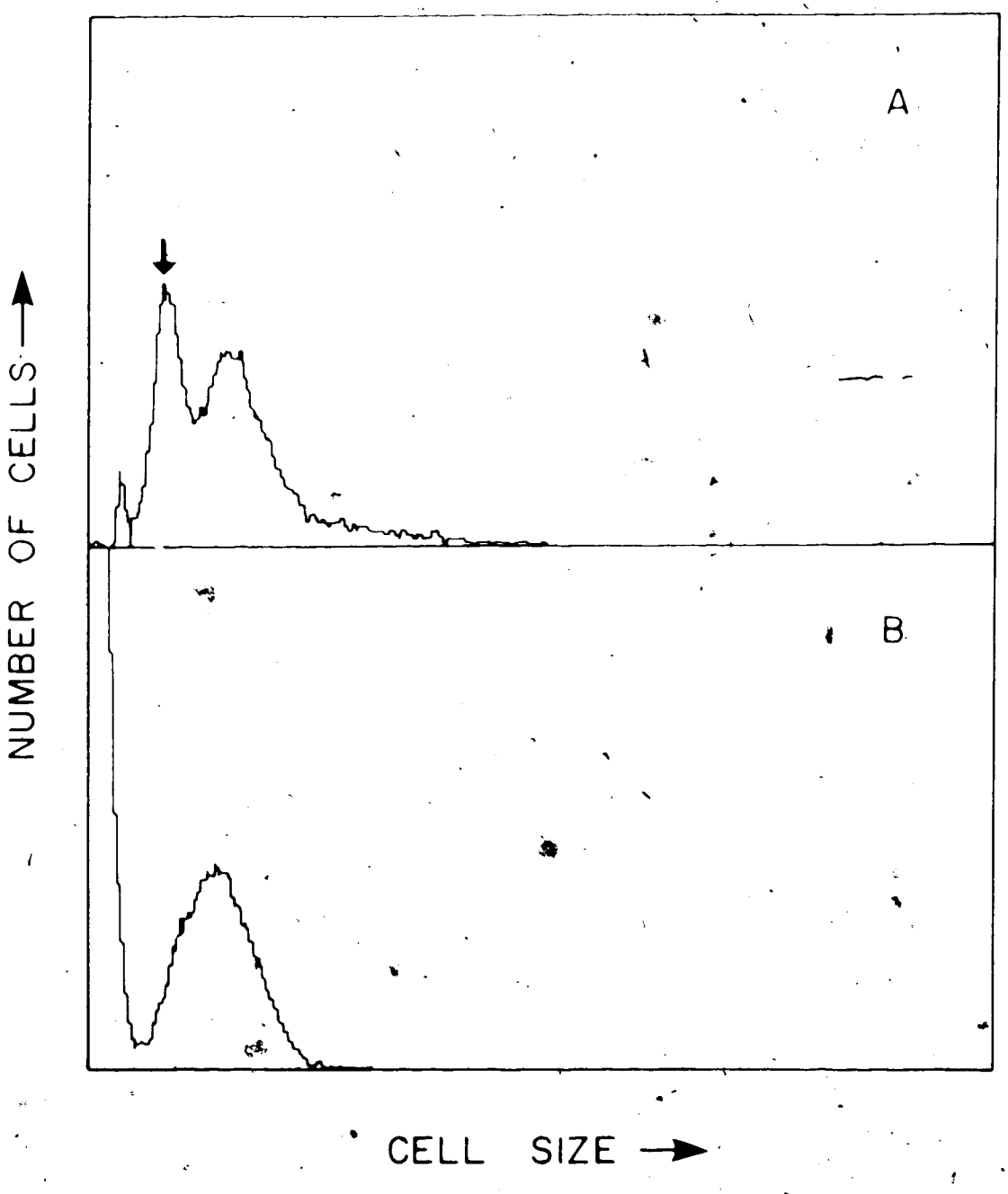
A
B
NUMBER OF CELLS
CELL SIZE

FIGURE 33

ANALYSIS OF THE SIZES OF CELL POPULATIONS BY FLOW CYTOMETRY

The cell types present in a sample of peripheral blood cells depleted of erythrocytes, platelets and T lymphocytes were analyzed. Panel A indicate the presence of two cell populations, monocytes indicated by the arrow, and B lymphocytes. As panel B shows, the monocytes may be removed by incubation with carbonyl iron and gum arabic followed by separation over Percoll gradients.