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Anti-Idiotypic Studies of a Neutralizing Epitope on the Glycoprotein B Complex of Human Cytomegalovirus

A Thesis Submitted to the School of Graduate Studies

University of Ottawa

In Partial Fulfilment of the Requirements for the

Degree of Doctor of Philosophy

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Faculty of Medicine

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ABSTRACT

Human cytomegalovirus (HCMV) is an ubiquitous member of the herpesvirus family that is responsible for morbidity and mortality in immunocompromised individuals. Our understanding of the immune response to this complex pathogen is still incomplete, limiting our capacity to devise effective strategies for its control. The goal of the research described in this thesis was to use the idiotype (id) network of the immune response as a means of manipulating the response to HCMV infection. More specifically, the objectives were to generate anti-id antibodies that would mimic an epitope of the viral envelope glycoprotein complex B (gB), an immunodominant constituent of HCMV that is widely regarded as a potential vaccine candidate. The anti-id antibodies would then be evaluated for their ability to act as surrogate immunogens in stimulating an immune response to gB in naive hosts, as well as for their utility in developing improved HCMV diagnostics.

Anti-id antibodies (Ab2) were induced against the gB-specific neutralizing murine monoclonal antibody CMVB1 (mAb1). Two different series of mAb2 were generated, as well as rabbit polyclonal Ab2. Extensive analyses indicated that the Ab2 were directed to idiotopes closely associated with the combining site of mAb CMVB1, and thus might mimic the original reference epitope of gB. Purified mAb2 and rabbit Ab2 were therefore prepared and used to immunize mice. Resulting data showed that all mice mounted an anti-Ab2 (Ab3) response whereas mice immunized with appropriate controls did not. The Ab3 mouse sera were then further evaluated to determine if they exhibited antigen-specific reactivity for the

reference antigen, gB. Using assays previously developed to characterize the anti-HCMV activity of the Ab1 mAb CMVB1, we found no detectable antigen-specific activity in any of the Ab3 sera raised against the mAb2. However, the Ab3 sera raised against purified rabbit Ab2 did exhibit anti-HCMV activity, in a manner analogous to that of mAb CMVB1. This was manifested by binding to HCMV-infected cells, by immunoprecipitating viral proteins identified as gB, and by neutralizing viral activity in vitro. A comparison of the id profiles of the Ab1 and antigen-specific Ab3 showed that at least one idiotope was co-expressed on the Ab1 and antigen-specific Ab3 antibodies, suggesting some dominance of this recurrent id. However, the data also demonstrated that different subsets of B lymphocytes had been stimulated by gB compared to the rabbit Ab2. These results not only revealed mechanisms by which the id network may contribute to the generation of antibody diversity in the immune response, but also illustrated how subtle structural differences may influence the immune response that is induced, considerations of some relevance in the selection of id based vaccines and therapies.

The mAb2 were used to develop an innovative ELISA for detecting a laboratory strain of HCMV, based on the ability of viral antigen to inhibit the specific interaction between Ab1 (mAb CMVB1) and complementary mAb2. A linear dose-response curve was obtained for HCMV between 20 and 0.6 x 10^3 PFU/ml, with 50 % inhibition at approximately 3 x 10^3 PFU/ml. The principles of this immunoassay show promise not only for HCMV, but also for the rapid measurement of other infectious agents.

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aa	amino acid(s)
Ab1	first generation antibody against an epitope
Ab2	antibody directed against the id of Ab1
Ab3	antibody directed against the id of Ab2
ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	acquired immunodeficiency syndrome
BME	Basal Medium Eagle
BSA	bovine serum albumin
C	constant immunoglobulin domain
CDR	complementarity determining region
CTL	cytotoxic T lymphocytes
CPE	cytopathic effect
D	diversity segment
DMEM	Dulbecco's Modified Essential Medium
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate conjugate
GAT	glutamic acid-alanine-tyrosine
gB	glycoprotein B
gH	glycoprotein H
H	heavy

HA	hemagglutinin
HBsAg	hepatitis B surface antigen
HCMV	human cytomegalovirus
HFF	human foreskin fibroblasts
HHV-6	human herpesvirus-6
HHV-7	human herpesvirus-7
HSV	herpes simplex virus
id	idiotype(s), idiotope(s)
IDR	idiotope determining region
iE	immediate early
i.p.	intraperitoneal
IR	internal repeats
J	joining segment
kb	kilobase
KLH	keyhole limpet hemocyanin
L	light
mAb	monoclonal antibody
MHC I	class I major histocompatibility antigen
MHC II	class II major histocompatibility antigen
MOI	multiplicity of infection
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PEG	polyethylene glycol
PFU	plaque forming unit(s)
RF	rheumatoid factor
RIP	radioimmunoprecipitation
RT	room temperature
s.c.	subcutaneous
SLE	systemic lupus erythematosus
TR	terminal repeats
UL	unique !ong
US	unique short
V	variable
V_H	variable region/gene of the heavy chain
V_L	variable region/gene of the light chain
VZV	varicella zoster virus

CHAPTER 1: INTRODUCTION

1.1 Cytomegalovirus

1.1.1 Historical perspective. Manifestations of human cytomegalovirus (HCMV) infection were first recognized cytologically in 1904 as a result of the large cytoplasmic and nuclear inclusion bodies present in kidney, lung, and liver cells of stillborn infants (reviewed in 1). In 1925 similar prominent inclusions in liver cells were associated with lesions produced by other herpesviruses (2) and the causal relationship between viral infection and these inclusion bodies was proposed. In 1950 the term "generalized cytomegalic inclusion disease" was adopted to describe the morphological features found in congenitally infected infants (3), and viral particles in infected pancreatic cells were identified by electron microscopy shortly afterwards (4). In 1956-1957 investigators in three independent laboratories successfully isolated the virus from tissue cultures of human cells (1).

1.1.2 Structural and genomic properties. HCMV is a member of the Herpesviridae family, a group of large and biologically complex viruses defined primarily by morphological criteria (1). Complete virus particles are composed of four structural elements: the central core, the capsid, the tegument (also called the matrix), and the envelope. The core consists of linear double-stranded DNA and protein. Surrounding the core is the icosahedral capsid comprised of 162 capsomeres, or subunits. Beyond the capsid is the tegument, an area of globular proteins that anchors the nucleocapsid to the lipid envelope. Because of the variable thickness of the tegument and the irregularly shaped envelope, the mature virions are pleomorphic with diameters ranging from 150-300 nm. Herpesvirus

populations also include several typically aberrant forms such as naked particles which lack the envelope and tegument, and empty particles which lack the DNA and/or capsid core (1,5,6). Other human herpesviruses are herpes simplex virus (HSV)-1 and HSV-2, varicella zoster virus (VZV), Epstein-Barr virus (EBV), and the more recently identified human herpesviruses 6 (HHV-6) (7) and 7 (HHV-7) (8). Over the years various subclassification schemes have been proposed for the herpesviruses, including biological range of natural host specificity (1,9,10). Although none has been entirely satisfactory, there is still some usage of the latter system whereby HCMV is referred to as a betaherpesvirus.

HCMV is a complex virus. The 230 kb pairs that comprise the viral genome are organized into a unique long (UL) and a unique short (US) component that can invert, or isomerize, during replication (6,11). These two components are linked by sequences with internal repeats (IR), and their distal ends are additionally flanked by terminal repeats (TR). The TR sequences are a source of minor variation between the three prototype strains of HCMV, Towne, Davis, and AD169. Sequence diversity in the IR region is largely responsible for intrastrain variation (6,12) although variation has also been found throughout the entire genome (13). The existence of different HCMV strains has been recognized antigenically for over thirty years (14-16) and has been used as a means to differentiate reinfection of seropositive patients from reactivation of latent infection (17,18). In the past few years molecular techniques have been used to identify over twenty variant strains from clinical isolates (19). Nonetheless, an overall sequence similarity of 80% has been found between different strains and the prototype AD169 (1), and there is no

formalized system of classification into subtypes or serotypes.

The genomes of HCMV prototype strains have been sequenced and more than 200 open reading frames have been recognized (20,21). Like other herpesviruses, HCMV has a temporally regulated pattern of gene expression. The periods of immediate-early (IE), early, and late transcription relate to the appearance of viral mRNA or protein in productively infected cells. In general, IE genes are transcribed in the absence of viral protein synthesis and encode nuclear proteins that control later stages of transcription. During the early period viral DNA replication occurs. The early gene products also regulate subsequent gene expression but, like the IE proteins, are not themselves present in mature virions. It is primarily during the late period that the many structural proteins are synthesized and viral assembly occurs (6,22). Replication of HCMV is characteristically slow. With HCMV laboratory strains and at a high multiplicity of infection (MOI) DNA synthesis is not initiated until 12 hours post-infection, structural proteins appear after 36-48 hours and infectious virus after 48-72 hours (5,6). However, replication is often much slower for wild-type viruses. The nucleocapsids are assembled in the nucleus and virions acquire their bi-layered envelope by first budding from the nucleus and then being re-enveloped in the trans-Golgi network (23). From there some virus particles make their way out of the cell via intracytoplasmic channels but the majority remain cell-associated (23,24).

1.1.3 Epidemiology. Cytomegaloviruses are species specific, sc that humans are the only HCMV reservoir. Transmission of the virus occurs via body

secretions and blood. In pregnant women HCMV will cross the placenta, giving rise to in utero infection. Perinatal infection occurs via breast milk and cervical secretions, and because infants continue to shed HCMV into urine and respiratory secretions for several years after infection, the virus is easily transmitted to others (25,26). After puberty the common modes of infection are via oral/respiratory routes and sexual contact with actively infected individuals (1,9).

Infection with HCMV is ubiquitous. Non-industrialized countries generally have a higher incidence of infection, approaching 100% in adults, than industrialized nations (9). But various socio-economic factors also influence the epidemiology. Seropositivity of young female personnel in a Halifax children's hospital was 22%, but 49% for women attending that hospital's pre-natal clinic (27). In Edmonton, 42.5% of normal blood donors were seropositive (28), reflecting similar studies in the United States (29) and the United Kingdom (30). However the prevalence of infection in a small Inuit community in the Northwest Territories was 80% by six years of age, and 100% for those over 30 years of age (31). In all populations the percentage of infected individuals increases from infancy through later childhood and well into adulthood.

In otherwise healthy adolescents or adults, primary infection with HCMV is usually asymptomatic, only occasionally causing mononucleosis. However the frequency of infection is noteworthy in two populations susceptible to severe disease because of their impaired immune status. These are newborns and patients whose immune systems are compromised. The frequency of congenital infection in Western countries is estimated as 1% of live births (9,32), with about

10% of these infants exhibiting clinical infection and a further 5-17% of the initially asymptomatic infants later developing sequelae (32). Immunosuppressed patients may be divided into those receiving immunosuppressive therapy and those with acquired immunodeficiency syndrome (AIDS). In the former group average frequencies of 53% for primary infection and 85% for recurrent infection have been reported following kidney transplantation, with similar rates for other allograft patients (9,33-35). In AIDS patients seropositivity is 90-100% (9,36).

Epidemiological evidence supports the view that seropositive (but asymptomatic) blood donors may be a source of infectious virus (9,37-39), but numerous factors influence whether the recipient will or will not become infected. These include the immune status of the recipient, the number of infected white blood cells, which are the putative source of latent virus (40-42), and other as yet undefined factors that may affect viral reactivation in the new host (1,43-45). There is thus some debate about the need to provide blood products from seronegative donors to patient populations at risk for developing serious HCMV-associated disease (28,37,39). However a recent review concluded that such products were warranted for four immunosuppressed populations defined as follows: "[1] pregnant women who are cytomegalovirus seronegative, [2] premature infants of low birth weight who are born to cytomegalovirus-seronegative mothers, [3] cytomegalovirus-seronegative recipients of allogeneic bone marrow transplants from cytomegalovirus-seronegative donors, and [4] cytomegalovirus-seronegative patients with ... AIDS" (38). In healthy recipients blood transfusions are only rarely a source of active infection (38).

1.1.4 Pathogenesis. In severe disseminated disease HCMV can be found in almost all the body's organ systems, but ductal epithelial cells are the focus of infection (9). For reasons not entirely understood the virus preferentially infects different tissues in different patient populations, eg. the salivary glands in infants and young children, the lungs in many immunosuppressed transplant patients, the central nervous system in infants with cytomegalic inclusion disease and in AIDS patients. Kidney involvement, however, is found in almost all patients and results in the shedding of infectious virus into urine for prolonged periods of time (43-45). It has also been noted that the presence of virus in given cells is not always coincident with pathology or disease (46), and that immunopathologic mechanisms may be involved, particularly in immunosuppressed patients (43,45).

One of the hallmarks of infection by herpesviruses is that primary infection is followed by a latent and persistent infection for the lifetime of the host. The cells in which latent virus resides have not been identified with certainty, but white blood cells have been implicated by various investigators (9,39). During latency production of infectious virus cannot usually be demonstrated, but reactivation and consequent viral replication may occur under appropriate conditions. In the case of HCMV, reactivation is prompted by changes in the host's immune status, but the underlying mechanisms are not fully understood. Paradoxically, even in immunocompetent individuals the various cell-mediated and humoral immune responses triggered by infection only serve to restrict HCMV replication, but not to eradicate the virus from the host (22,45,47,48).

It is still unclear how HCMV binds to permissive cells and initiates infection.

Class I major histocompatibility complex (MHC I) molecules on the cell surface have been proposed as viral receptors, possibly by β2-microglobulin which coats the viral surface, displacing the β2-microglobulin normally bound to MHC I (49). Evidence to support this idea included the finding that β2-microglobulin-coated virus was more infective than uncoated virus (49). However, these conclusions were later disputed (50) and the significance of the earlier study was questioned even by the original authors (51). Several receptors distinct from MHC I have also been described, including membrane glycoprotein(s) in the 30-34 kDa range (52-55), and a 92.5 kDa receptor specific for the gp86 viral envelope glycoprotein, identified by anti-idiotype antibodies (56,57). Further characterization of this receptor suggested that it was involved in the fusion of HCMV to the membrane of permissive cells (58). But since a concurrent publication by the same authors found this receptor on both permissive and non-permissive cells, its significance remains unclear (59). It is also noteworthy that a non-receptor mediated mechanism of viral/cell adsorption has been demonstrated. Two recent papers (60,61) reported that HCMV, like various other herpesviruses, attached directly to the surface of cells via heparin, a membrane proteoglycan. The viral component mediating the binding was identified as the envelope glycoprotein gcII (60).

The mechanism by which β2-microglobulin might bind to HCMV was revealed in 1988 when homologies were found between the HCMV IE-1 protein and the heavy chain of MHC I (62-64). Other HCMV proteins with similarity to human gene products have also been found, eg. IE-2 sequences homologous to the β chain of MHC II proteins (65), three viral genes with homology to G-protein

coupled receptors (66), an HCMV-encoded 38 kDa protein homologous to a membrane component of human fibroblasts (67), and possibly molecules related to the T cell receptor (21,68). The role of these cellular homologues in HCMV disease pathogenesis is a source of great interest, but is still unclear (21,45).

1.1.5 Immune response to infection. The biological and structural sophistication of HCMV is reflected in the complexity of the immune response it triggers in infected hosts. Our understanding of this response remains incomplete, but great progress has been made over the past decade in identifying viral antigens and in defining the humoral and cellular immune responses directed against them. A short review of this subject is presented below.

1.1.5.1 Viral antigens. In the usual chicken-and-egg manner of antigens and antibodies, the HCMV proteins believed to be most immunologically relevant are those to which significant immune responses have been identified. In fact, most of the proteins specified by the virus seem to be antigenic, and several extensive reviews of this subject are available (24,69,70). In brief, the following proteins are recognized as most immunodominant: (1) **EI-1 pp72**, a non-structural phosphoprotein present in the nucleus of infected cells (71); (2) **late DNA binding protein p52**, the most antigenic of several DNA binding proteins; a non-structural phosphoprotein present in the nucleus and associated with the nuclear membrane (72); (3) matrix/tegument proteins: **basic matrix phosphoprotein pp150**, and **lower matrix phosphoprotein pp65** (range reported from 64-69 kDa); pp65 is the most abundant structural component and a major constituent of the nuclear and cytoplasmic dense bodies characteristic of HCMV-infected cells (73); (4)

nucleocapsid proteins: the **28 kDa nucleocapsid protein**, and the **major nucleocapsid protein, 150 kDa**, which represents 90% of the total protein of the nucleocapsid (24); (5) **envelope glycoprotein complexes: gB, gcII, and gH**, present on the surface of infected cells as well as on the virion surface. As one of these complexes, gB, is the target protein for immunological studies presented in this thesis, these glycoproteins will be described in some detail.

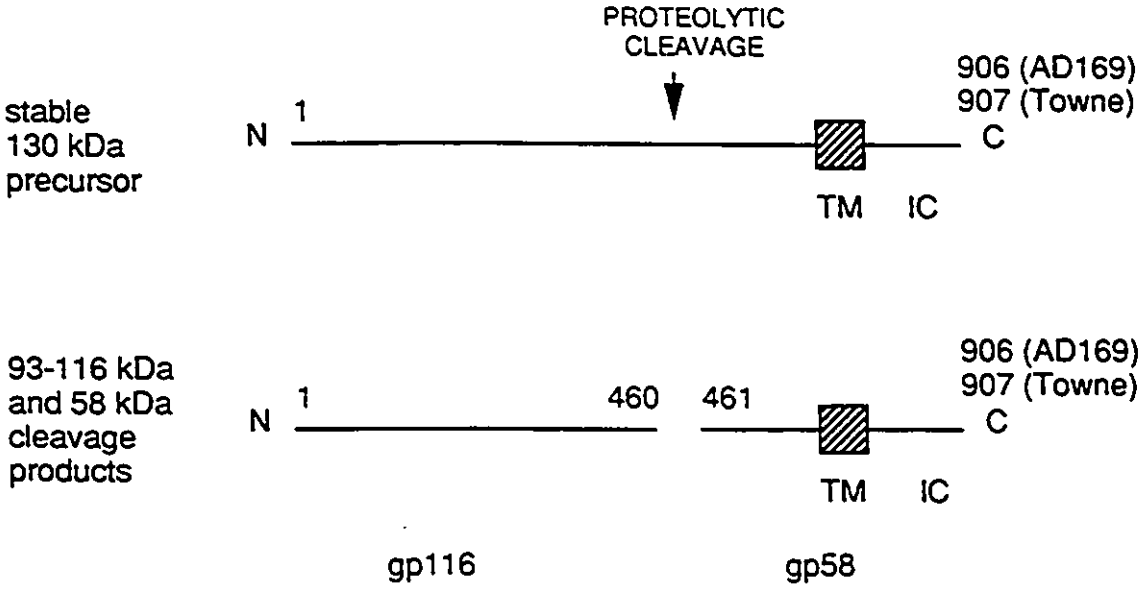
Reconstructing the identification of HCMV envelope glycoproteins can be confusing, because the variable glycosylation and precursor/product relationships led to a multiplicity of descriptions for the same gene product. Publications by Pereira et al. in 1982 and 1984 were the first to recognize several groups of immunologically related glycoproteins, and to outline the processing events that led to their mature forms (74-76). In 1988 an influential article consolidated the growing number of reported envelope glycoproteins into three distinct families of disulphide-linked glycoprotein complexes, designated gcI, gcII and gcIII (77). Of these complexes, gcI, more usually referred to as gB because of its homology with gB of HSV (78), has now been recognized as immunodominant (24,70,79,80). As a result much attention has recently been directed to gB, with a corresponding increase in our understanding of its structural and immunologic properties.

By electron microscopy and immunological probes, gB has been visualized on the distal end of 12 nm spikes projecting out from the virion envelope (81). Early descriptions of gB (75,76,82,83) were followed by cloning (78) and mapping (84) of the gB gene, which in turn allowed more precise studies of the intracellular processing events determining the glycoprotein's mature forms. The 130 kDa

stable gB precursor is a glycosylated protein comprised of 907 amino acids (aa) (Towne strain, cf. 906 aa for strain AD169) (85). As illustrated in **Figure 1**, the precursor is proteolyzed in the Golgi complex between aa 460 and 461 to yield cleavage products of 116 kDa and 58 kDa, representing the N-terminal and C-terminal portions, respectively (85-87). The two proteins are disulphide-linked (77,86,88) and both species (also referred to as gp116 and gp58) are heavily glycosylated (89,90). A hydrophobic transmembrane domain has been identified from residues 752-772 (85), and aa between this region and the C-terminus are intracellular (85,91). Targets for neutralizing and non-neutralizing antibodies have been found on both gp116 and gp58, but the major binding domain for neutralizing antibodies resides within gp58, adjacent to the cleavage site at residue 460 (85,92-96). The recent article by Qadri et al. (96) is particularly useful because it schematically integrates new information with existing data. This topographic map and its original legend are reproduced as **Figure 2**. In general, gB is well conserved between different clinical strains (97-99). One report showed that the frequency of variability was highest between aa 448-480, and on this basis four variant groups were defined (100).

Much less investigation has been done on gcII and gcIII. The gcII complex represents a heterogeneous family of multimeric glycoproteins with molecular masses of 93-300 kDa under non-reducing conditions, and 47-52 plus 48-200 kDa under reduced conditions (60,77,101,102). Unlike constituents of the gB complex which are processed from a single gene product, the polypeptides of the gcII complex are derived from several genes (101,103). As noted above, gcII

Figure 1. **Cleavage of HCMV gB precursor to gp116 and gp58.** The stable 130 kDa gB precursor molecule is cleaved in the Golgi complex by a cellular protease between aa 460 and 461, to yield two disulphide-linked products designated gp116 and gp58. These three polypeptides comprise the elements of the gB complex.



TM: transmembrane domain, aa 752-772
IC: intracellular domain

Figure 2. **Topographic map of gB antigenic domains.** This Figure and its legend are reprinted in their entirety from the article of Qadri et al. (96).

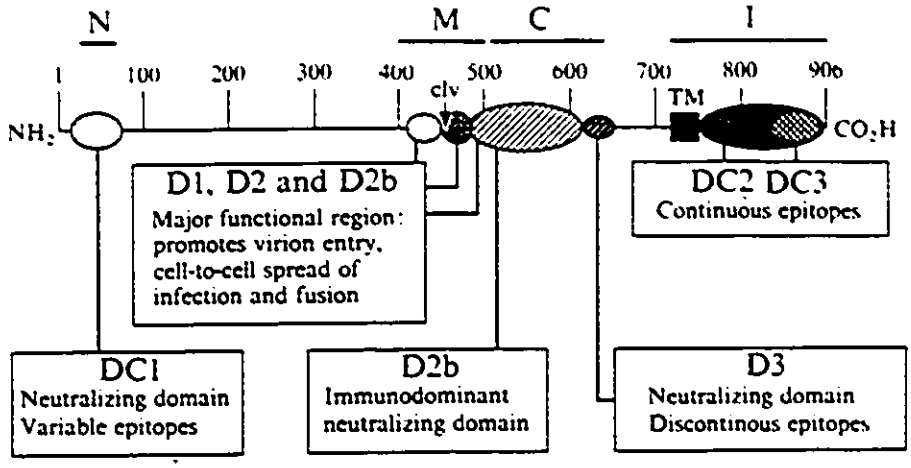


Fig. 6. Topographic map of the antigenic and functional domains mapped on CMV (AD169) gB. Antigenic domains are shown as ellipses. Domains DC1$_v$, DC2 and DC3 containing continuous epitopes were mapped previously (Basgoz *et al.*, 1992; Pereira *et al.*, 1991). Boxes show our designations for the antigenic regions of gB and list the functional properties that have been identified for certain neutralizing domains (D. Navarro *et al.*, unpublished data). Four antigenic regions of the gB molecule are indicated: N (amino terminus; positions 28 to 67), M (midregion; 411 to 476), C (carboxy terminus; 476 to 645) and I (intracellular; 719 to 906). The cleavage site is indicated by an arrow (clv) (Spaete *et al.*, 1988). The shaded box designates the hydrophobic transmembrane domain (Cranage *et al.*, 1986). Immunodominant neutralizing domain D2b and neutralizing domain D3 contain antigenic sites mapped by other laboratories (Kniess *et al.*, 1991; Liu *et al.*, 1991; Spaete *et al.*, 1988; Utz *et al.*, 1989).

glycoproteins have been reported to adsorb to cell surfaces via heparin and thus may be involved in initiating viral infection (60). The gcIII complex is usually referred to as gH because of its homology with glycoprotein H of HSV (104), and was first identified by precipitation by a murine monoclonal antibody (77,105). Under non-reducing conditions it is a disulphide-linked complex of 240 kDa, and when reduced is composed of 145 kDa and 86 kDa (gp86) polypeptides (77). Monoclonal anti-idiotype antibodies were used to mimic a neutralizing epitope on gp86 (106), and subsequently to identify a gp86 cellular receptor that may be involved in viral fusion (56,58,59). Only limited epitope mapping of gp86 has been carried out (107).

1.1.5.2 Humoral immune response. As the preceding section demonstrates, exposure to HCMV induces a humoral immune response against numerous viral determinants. In fact, antibodies directed to more than 20 different HCMV proteins, including both structural and non-structural elements, have been recognized in convalescent sera (24,69,70,108). However many factors are known to affect the nature of the immune response. These include the immune status of the host, the mode of viral transmission, primary or secondary infection, prior immunity, and host age (9,43-45). These differences are reflected in the relative proportion of different antibody classes, the kinetics and duration of the response, and the immunodominant target epitopes. A detailed account of these variations is beyond the scope of this review. In general, however, it can be said that primary infection in normal individuals usually results in a transient IgM response followed by a persistent, often lifelong, IgG response. In secondary infection,

defined as either re-infection with a different strain or reactivation of latent infection, the IgM response may or may not be present. IgA is detected in only some patients and more frequently during secondary infection. In immuno-suppressed patients these responses are similar, but exaggerated (9,22,70).

Both neutralizing and non-neutralizing antibodies are made. Ironically, despite extensive analysis, their role in protecting the host is not clear. For example, viral replication continues even in the presence of neutralizing antibodies, as does reactivation of latent virus (22,43,47,48,109). Nonetheless, humoral immunity clearly does influence the response to subsequent HCMV infection. The presence of maternal antibody to HCMV has long been correlated with reduced frequency and severity of congenital infection (29,32,110,111), and HCMV immune globulin moderates the severity of virus-associated disease in renal transplant patients (112-114). One study has suggested that an antibody response to the IE-1 protein immediately following the detection of HCMV in renal transplant patients was predictive of good graft function (115).

Characterization of antibodies directed to specific viral proteins has contributed to our knowledge of the interplay between the virus and the host, but it has been difficult to establish cause and effect between particular antibodies and a protective immune response. The late DNA binding protein p52 is highly immunogenic and induces an IgM response that is one of the first to be detected; it may be a marker for acute infection (24,70). The EI-1 pp72 phosphoprotein is an immunodominant antigen and, as noted above, an early antibody response to it was recently correlated with improved kidney graft survival (115). Antibodies to

the basic matrix phosphoprotein pp150 are widespread in individuals who have been exposed to the virus, and thus are useful as markers of HCMV seropositivity. The magnitude of the response to pp150 during acute infection varies in different studies (24,116). The abundant lower matrix phosphoprotein pp65 stimulates both IgM and IgG antibodies early in the primary immune response (117), but these may not be very effective in viral neutralization (24). Similarly the major nucleocapsid protein of 150 kDa, representing most of the nucleocapsid protein, is only weakly immunogenic (24). It should be noted, however, that many of these conclusions were drawn from studies in which the source of viral antigen was a prototype strain of HCMV. A recent article has demonstrated that immunoblotting patterns of sera from infected renal transplant patients differed depending on whether the viral antigen was the prototype AD169 or the homologous isolate (118). Thus some caution may be warranted when interpreting these data.

Given their location on the surface of the virus one would expect the envelope glycoproteins to be important targets for the immune response. Although the humoral immune response to gcII and gH is not very pronounced (24), there is no question about the immunodominance of gB. Recent studies have demonstrated that gB is the most abundant protein complex on the viral envelope, and that a neutralizing antibody response to it routinely occurs after infection (119-124). In fact, a substantial proportion of the neutralizing activity found in serum is directed against gB (70,122,123). In the single study reported, purified gB was administered to healthy subjects and induced a neutralizing anti-gB response in previously seronegative individuals (121). A response has also been induced in

animals immunized with recombinant gB (122,125).

1.1.5.3 Cell-mediated immune response. The cell-mediated immune response to HCMV has received much less attention than the humoral response. Nonetheless, it plays a critical role in maintaining the virus in its latent state after primary infection and in resolving active disease in immunosuppressed patients (47,48,126-128). The EI-1 pp72 phosphoprotein has long been recognized as an immunodominant target for cytotoxic T cells (CTL) (48,71,129). Some CTL directed against gB have also been identified (121,129,130) and T cell epitopes have been mapped to both gp116 and gp58 of the gB complex (48,130). An important paper by Riddell et al. in 1992 showed that the adoptive transfer of HCMV-specific CD8+ CTL into immunosuppressed bone marrow transplant patients reconstituted their HCMV immunity for over one month (131). Although the number of patients was small (n=3) this has significant implications for future therapeutic strategies.

1.1.5.4 Viral evasion of host immune mechanisms. The ability of HCMV to escape immune destruction in the normal host is illustrated by the latent infection it establishes. The mechanisms by which it achieves this are not fully appreciated, but several different strategies have been identified. First, HCMV infects lymphocytes, macrophages, and polynuclear blood cells, the very cells needed for mounting an effective immune response (40,41,45,132). This results in diminished immunocompetence of the host against HCMV, as well as a temporary and non-specific immunosuppression that is associated with active HCMV infection (22,132). Second, receptors for the Fc of IgG are induced in the

perinuclear region and on the surface of infected cells (133). The advantage, from the perspective of the virus, is that non-specific IgG then masks viral antigens and protects infected cells from subsequent binding by specific antibodies and/or CTL (22,134). Third, the virus confounds the immune system by binding to β2-microglobulin, a host protein normally non-covalently bound to MHC I molecules (135). Virus coated with β2-microglobulin was identified in urine of infected patients but could not be neutralized by antibodies, suggesting that the target antigens were masked by β2-microglobulin (136). More recently, however, another report failed to confirm these results, leaving their significance unclear (137). Finally, it has been suggested that HCMV may be subject to antibody pressure in vivo, as are certain other viruses, with some of the strain variations found in gB reflecting antibody escape mutants (99).

1.1.6 Control of infection. Two population groups are currently the major focus for disease control. These are pregnant women and immunosuppressed patients. Strategies to prevent HCMV infection may be grouped as follows: (a) elimination of viral transmission by blood transfusions, (b) passive immunotherapy, and (c) immunization. As noted above, four groups of seronegative and immunosuppressed patients were recently recognized as being at high risk of developing serious HCMV disease from seropositive blood products. A recommendation was thus made that these patients be given only products unable to transmit the virus; that is, either derived from seronegative donors, or washed and/or filtered to remove white blood cells (38). In addition, five other categories of patients were listed "for whom the risk is less well established but sufficient to merit

consideration" (38). In general, these were seronegative patients receiving autografts, allografts from donors of undefined HCMV status, or seronegative AIDS patients.

Attempts to prevent HCMV infection in transplant recipients by passively administering immune globulins have been carried out since the early 1980's (138) and many clinical trials have been conducted (112,113). Results have been organized into subgroups of patients who were seronegative versus seropositive prior to transplant; who received organs from seronegative versus seropositive donors; solid organs versus bone marrow, etc. However, such a detailed account is not relevant here. Suffice to say that this mode of therapy is valuable in reducing the gravity of HCMV-associated syndromes in many patients, and for preventing HCMV disease in some. This conclusion is most apparent for seronegative kidney transplant patients (1,113,139). For bone marrow transplant patients, who have a high mortality rate from HCMV-associated interstitial pneumonia, the studies are less convincing. A recent review of the subject concludes that no study proves that immune globulin reduces the incidence or severity of HCMV pneumonia (113). High dose therapy <u>may</u> provide some protection in conjunction with antiviral drugs, perhaps reflecting the complex immunopathology occurring in the lungs.

It is likely that two other products will also be available for passive immunotherapy and/or prophylaxis in the future: human monoclonal antibodies (mAb) to HCMV and cytotoxic T cells. Results of phase I studies in bone marrow transplant patients have recently demonstrated the safety of human mAb with

specificities for glycoproteins gB and gH (140,141), and preclinical evaluations have been conducted on another anti-gB neutralizing human mAb (142). HCMV-specific CTL also reconstituted viral immunity in bone marrow recipients (131). Utilization of these approaches will undoubtedly increase over the next years.

Of course, rather than the foregoing strategies, one would prefer an effective vaccine. Unfortunately, this objective has not been easy to realize. Two live vaccines were developed in the mid-1970's by attenuation of clinical strains (143,144). The second of these, the Towne vaccine, has continued as a focus of particular attention. Both humoral and cell-mediated responses have been stimulated in healthy volunteers, with no apparent adverse reactions (145). Subsequent clinical trials on renal transplant patients indicated that seronegative patients receiving a kidney from a seropositive donor benefited from prior immunization with the vaccine, developing significantly milder HCMV disease. The incidence of disease was not reduced, and seropositive recipients derived no benefit from the vaccine (146). However, in spite of these encouraging results, there has been a tremendous reluctance to use a live HCMV vaccine. This is due to concerns regarding HCMV latency, attenuation, oncogenicity, and the possibility of inducing immunosuppression, as well as questions about the duration of protection and the efficacy against other strains (9,80,111). The current consensus is that a non-infectious vaccine would unquestionably be preferable.

The most promising candidate for a non-infectious subunit vaccine is the viral envelope.complex gB. Possible strategies include purification of gB from viral preparations, DNA technologies to produce gB/viral recombinants or appropriate

polypeptides, and anti-idiotype vaccines mimicking an epitope of gB (111). In view of the research described in this thesis, it can now be stated that progress has been made with each of these approaches. The gB complex has been isolated from virus preparations and immunization of healthy volunteers has resulted in stimulation of both humoral and cellular responses (121). Recombinant gB has been expressed in vaccinia viruses (104,122,125) and in adenoviruses (123,147), inducing neutralizing antibodies against gB in experimental animals. The publications derived from the present research (148) are the first to describe anti-idiotype antibodies related to gB.

1.1.7 Methods for laboratory detection. Laboratory techniques to detect HCMV infection may be classified as those that identify viral antibodies, or those that identify the antigen. Each has advantages and disadvantages, in part related to different clinical situations. For example sometimes only the serological status of a patient may be needed, whereas at other times the diagnosis of active infection is imperative. The outstanding challenges today are to differentiate active infection from latent infection in seropositive individuals, and to develop rapid assays which are as sensitive and precise as more traditional techniques of viral isolation.

Serological tests for antibody are generally rapid. But since infection with HCMV is so prevalent and high antibody titres persist for years, they are considered useful only in limited circumstances. Moreover, even under these conditions, their significance may be difficult to interpret (9,149,150). Potentially valuable situations include screening blood donors and organ donors, identifying

seronegative individuals in patient populations at risk for developing serious HCMV-associated disease, monitoring seroconversion in populations at high risk, following the response of IgM and IgG in an immunocompetent host suspected of HCMV mononucleosis, screening neonates for the presence of IgM antibodies, and measuring antibodies to specific HCMV proteins as a means of elucidating the host's humoral immune response to the virus (1,124,149,151). For many years complement fixation assays were the gold standard of serological testing (1). However the many ELISA kits now commercially available are considered an improvement with regard to accuracy, speed, efficiency and sensitivity (149,152). Indirect fluorescent assays for detecting antibodies may be more sensitive than other techniques, particularly a modification of this test, the anti-complement immunofluorescent assay which greatly reduces non-specific reactions (1,150,151). Latex agglutination tests may not be as reliable as other methodologies but are extremely fast and thus practical for emergency screening of organ donors (153,154) or routine screening in blood banks (30). Assays such as immunoblotting to identify particular species of antibodies, and plaque reduction for neutralization, are generally considered too laborious for routine use (149).

In principle, isolation of the virus directly from clinical samples is preferable to serological assays. HCMV does grow in tissue culture, developing characteristic cytopathic effect (CPE). But viral growth is so slow that a definitive test result may not be reached for weeks -- an impossible delay for initiating antiviral therapy in high risk patients or those with potentially invasive disease (1,9,149,155). Fortunately, several modifications of the basic culture technique have been

developed, and are widely used today. In the "shell vial" assay (156,157) the test samples, often urine, are inoculated onto coverslips seeded with human fibroblasts within small shell vial tubes. Centrifugation is carried out to increase viral adsorption and after 16 hours the infected cells are reacted with a monoclonal antibody specific for an early or IE protein of HCMV. Binding to viral antigens is then visualized by FITC- or enzyme conjugated- second antibodies. Although the shell vial assay is a great advance over conventional culture, there are certain drawbacks. For example some clinical specimens are toxic for the fibroblast monolayer, and as for any immunoassay the specificity of the mAb is critical (158). Therefore neither the sensitivity nor the accuracy is considered as high as with conventional culture (1,150,155). Several other immunoassays for detecting viral antigen in clinical specimens are available, and are used to various extents in different clinical laboratories (1). Nonetheless, the numerous reports of "new and improved" HCMV assays in the current literature illustrate the ongoing demand for innovative reagents and/or approaches that will succeed in developing more rapid, accurate, and sensitive immunological tests.

In addition to identifying HCMV by cell culture, several techniques exist for identifying the virus directly in tissues or cells. The most promising of these are the antigenemia assay, nucleic acid hybridization, and DNA amplification by the polymerase chain reaction (PCR). The antigenemia assay detects viral antigens in leukocytes isolated from peripheral blood (159), and when used in conjunction with mAb specific for the lower matrix protein pp65 rather than for IE proteins, claims to identify active infection (160). However, the need to process specimens

immediately after collection is a practical limitation of the technique in many situations (161,162). Nucleic acid hybridization may be performed either on cell extracts or on histological specimens in situ, with a reported sensitivity of 1 pg viral DNA (155). Many probes are commercially available but false positives have been a consistent difficulty when compared with conventional culture. Whether this is a genuine problem or merely a reflection of the technique's increased sensitivity remains unclear (149). The enormous potential of PCR has also been applied to detecting HCMV in clinical samples, as witnessed by many recently published studies (162-167). It seems likely that this methodology will eventually find an accepted place in routine laboratories. But at present numerous difficulties such as false positivity, inhibitors present in biological samples, and concerns regarding optimal primers, still have the upper hand (13,149,162,165,168). Furthermore, given the increased sensitivity possible with PCR, reported as 0.001 pg DNA (155) or one infected cell in one million (167), latent infection may be detected as well as active infection. Thus, as for DNA hybridization techniques, at some point it will be necessary to determine the clinical significance of viral sequences identified in culture-negative or even seronegative samples (10,162).

1.2 Idiotypes

1.2.1 Historical perspective. The concept of antibodies formed against the combining region of other antibodies was first articulated by Ehrlich and Morgenroth in the early 1900's, even though the structure of antibodies at the time was completely unknown (reviewed in 169). It took another half century before studies provided experimental support for the concept. In 1955 Kunkel and his

colleagues identified unique antigenic determinants on myeloma proteins (170) and subsequently on human antibodies (171). Similar observations were made at the same time by Oudin and Michel, working with rabbit anti-salmonella antibodies (172). It was Oudin and Michel who coined the term "idiotypic determinant" to reflect the distinct nature of the antigen on individual antibody molecules (Greek "idios" = private, personal). This nomenclature has been maintained to the present. But, as our understanding of idiotypes has increased so too has the terminology evolved. Today "idiotype" refers to the set of many idiotopes present on a given molecule; and an "idiotope" is defined as a single determinant, analogous to an epitope, or functionally, the region of an immunoglobulin that makes contact with an anti-idiotypic antibody (169)[1].

1.2.2 Structural basis. Before the structural basis of idiotypes can be discussed, it will be necessary to review briefly the relevant structural elements of antibodies themselves[2]. The prototype antibody molecule is assembled from four disulphide-linked polypeptides, two identical heavy (H) chains and two identical light (L) chains. Both heavy (440 aa) and light (220 aa) chains are divided into globular domains of approximately 110 aa each. The sequences within these domains are either constant (C), ie, relatively conserved within species for each of the heavy and light chain classes, or variable (V). Light chains consist of a single V plus C region, heavy chains of a single V plus three or four C regions.

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[1] The abbreviation "id" will be used for both terms in this thesis, unless the intended meaning of idiotope vs. idiotype is not evident.

[2] This section describes id on antibodies. But id are similarly present on the membrane bound immunoglobulin receptors on B cells, and on T cell receptors.

The V regions are situated at the amino terminal ends of each polypeptide, such that digestion with the enzyme pepsin results in two cleavage products with distinct functions: the Fc portion, consisting entirely of C domains, effects biological functions such as complement fixation; and the divalent $F(ab')_2$ portion, consisting of two identical disulphide-linked L plus H chain fragments, binds antigen. Reduction of the $F(ab')_2$ results in two monovalent Fab fragments. Within the V domain of the heavy (V_H) and light (V_L) chains further patterns have been recognized. Each domain contains three short sections with extreme sequence variation, designated hypervariable or complementarity determining regions (CDR1, CDR2, CDR3), interspersed with more conserved framework regions. X-ray diffraction studies have allowed an appreciation of the three-dimensional nature of the V domain: seven polypeptide segments assume the conformation of two β-pleated sheets of three and four strands each, with a hydrophobic core. These constitute the framework region of the domain. Sequences linking the β strands form loops between them, and these generally represent the CDR sequences. It is the six CDR from one V_H plus one V_L that form an antibody combining site, directly contacting the antigen and defining that antibody's specificity (173,174).

An enormous number of antibody specificities can be made by the humoral immune system, and this repertoire is possible because of the unique molecular genetics of immunoglobulin synthesis. Not only are the H and L chains each encoded by more than one gene segment, but these segments may be selected from hundreds of possible variants. For example, for the extensively studied mouse immunoglobulins, each κ light chain is produced by three regions -- the V_κ

region, the joining (J) region, and the C_κ region -- which are ultimately rearranged and joined into a functional gene aligned as V-J-C. Since each V_κ segment is selected from over 200 germline variants, and the J segment from 4 functional variants, approximately 800 different V_κ polypeptides may be produced. Similar mechanisms, though with fewer possible variants, exist for the generation of λ light chains. For H chains further complexity exists in that one additional coding element is present between V and J, the diversity (D) region. The final gene is arranged as V-D-J-C, and over 7200 different heavy chains may be produced. It should be noted that CDR1 and CDR2 are wholly contained within the V gene segment, whereas CDR3 is formed by the junction of V-(D)-J, so that CDR3 often exhibits more variability than CDR1 or CDR2. In addition, there are several other means of generating V regions with different aa sequences, including junctional diversity, whereby extra sequences may be included between V-D or D-J, and some preferential combinations of V_H and V_L. In all, it has been estimated that over 1.6×10^7 combinations of gene-encoded variability are possible (173-175).

Somatic point mutations are one final mechanism for producing sequence diversity in the combining sites of antibodies. Somatic mutations occur randomly, after the DNA segments of the V region are rearranged, and are scattered throughout both V_H and V_L regions. The molecular mechanisms responsible for the mutations are not understood, but they do occur at a rate significantly higher than "background" mutation rates of non-immunoglobulin genes (175). Furthermore, although somatic mutation events may arise in antibodies early in the immune response, their frequency increases greatly as the immune response matures, so

that the memory response is dominated by lymphocytes that have undergone extensive mutation (176-178).

At the sequence level, idiotopes are simply a further example of immunoglobulin diversity. But the structural relationship between idiotopes and the combining site of the antibody, termed the paratope (179), has always been enigmatic. Early studies indicated that many idiotopes were associated with the V region hypervariable segments (180), a conclusion confirmed by subsequent research. In some cases an idiotope is assembled from multiple CDRs of both V_H and V_L chains (181-183), whereas in others it is localized within a single CDR of either V_H or V_L (184,185). In fact, several reports have described systems in which idiotope integrity was altered by the substitution of a single aa (185-187). In addition to idiotopes associated with CDRs, some id are situated outside the paratope, on framework residues of V_H and V_L. This was first established by experiments showing that interactions between an anti-hapten antibody and complementary anti-idiotype antibodies were only partially inhibited by the hapten (188); and subsequently by showing that anti-id could be induced against regions of antibody other than the paratope (189). These data were reinforced by surface variability analysis, studies analogous to those that had defined the CDR. In this case, "idiotope determining regions" (IDR) were predicted on both CDR and framework portions of the V region (190). An estimated 15-20 idiotopes, some of which may be overlapping, may comprise the idiotype of a single molecule (191).

The three dimensional structure of an id/anti-id complex was recently revealed by crystallography (192). The idiotope was present on a mAb directed

against lysozyme, and was shown to consist of 13 aa assembled from all six CDR of the V_H and V_L, plus one framework region of V_L. Of equal interest was a correlation of these 13 aa with the residues formerly defined as the paratope of this antibody, ie., those making contact with the antigen. The comparison showed a partial overlap in composition of the idictope and the paratope, with 7 aa of the id (from both V_H and V_L) also contributing to the formation of the paratope.

Further insight into the structural relationship between the idiotype and the paratope has been provided by analyzing the frequency of idiotope expression. Originally idiotopes were thought to be unique markers for myeloma proteins, or antibodies of one specificity from one individual. But investigators soon recognized recurrent idiotopes in certain antibody populations (169), and then extensively characterized recurrent id in several model systems of mice immunized with various haptens. Studies such as these have demonstrated that recurrent id (also called cross-reactive, shared, or public id) are in fact phenotypic markers for germline V genes (176,193,194). It was found that an immune response to a given antigen is often dominated by the expression of a particular id, whether in different individuals, or even in different species. These data again implied some association between the expression of idiotopes and the antigen specificity of the paratope. On the other hand, recurrent id were also observed on antibodies of different antigenic specificities in a single individual, called parallel sets and implicated in autoimmune diseases, thus clouding the issue once again.

As several authors have noted, the emphasis on studying recurrent id should not detract from the fact that many idiotopes are private, not recurrent.

Private id reflect somatic mutation of V genes in single B cell clones which, as noted above, increase proportionately as the immune response matures. By definition, therefore, they are likely to be unique products, usually associated with the higher affinity antibodies typical of secondary and hyperimmune responses (176,191).

One additional class of idiotope pertinent to this discussion has been identified, the so-called "silent id". Silent id are usually encoded in B cells specific for an antigen that stimulates a dominant recurrent id so that, as the name implies, the silent clones are not expressed. Characteristically, if expression of the dominant id is suppressed experimentally, then the silent clones will emerge. This phenomenon has certain implications for therapeutic manipulation of the immune system by id and anti-id (195,196).

It has been suggested that the confusion in distinguishing idiotopes from the paratope is self-imposed, reflecting our operational definitions of them. That is, having identified these conformations by their distinct functions (paratopes interact with epitopes, id interact with anti-id), we became locked into conditioned ways of perceiving them (197). However given the presence of overlapping id, and of id that overlap antigen-binding specificities, it has been convincingly argued that no topographical distinction can, or should, be made between paratope and id. Rather the surface of the antibody V region should be viewed as a composite of multiple surfaces, with particular sequences able to interact with a variety of complementary structures at different times or under different circumstances (198). In fact this idea has been extended to include the possibility of multiple paratopes,

as well as multiple id, being expressed on a single antibody (199).

A great deal of information about the structural basis of idiotypy has been provided by analyzing antibodies generated against the V antibody domains. In 1982 Jerne and his colleagues introduced the terminology which became integral to this area of study. **Ab1** represented antibodies raised against the antigen of interest, expressing a particular id profile; **Ab2** was the population of antibodies raised against the id of Ab1 (anti-Ab1, anti-id); and **Ab3** the population of antibodies raised against the id of Ab2 (anti-Ab2, anti-anti-id) (197). The Ab2 antibodies were then subclassified to reflect the location of the Ab1 target idiotope with which they interacted. First, a fundamental distinction was made between Ab2 that bound id closely associated with the paratope of Ab1, designated Ab2β, versus Ab2 that bound id outside the paratope, associated with the framework residues of the V region and designated Ab2α. These were distinguished on the basis of the Ab2 being able to competitively inhibit interaction of Ab1 with antigen (197). Soon afterwards the original Ab2β designation was further refined and Ab2β versus Ab2γ antibodies were differentiated. Ab2γ were those targeted to id near but not in the paratope, such that Ab2/Ab1 binding sterically interfered with subsequent Ab1/antigen attachment. Conversely, the Ab2β were truly antibodies against paratope-associated id (200). A major corollary of these ideas was that Ab2β antibodies, by mirroring the conformation of the paratope which was itself complementary to the original epitope, would thus resemble the antigen, and in fact could represent the antigen's "internal image". This hypothesis had major theoretical and practical ramifications which are discussed below.

1.2.3 Immune regulation. In the early 1970's immunologists began to speculate on the biological significance of anti-id, prompted by the demonstration that anti-id could suppress the production of id-positive antibodies (see 198). This idea was then extended to the notion of anti-id as regulators of the immune response, operating within some kind of ordered system. Today considerable evidence supports the view that anti-id influence the regulation of the immune response, and that under particular circumstances may also contribute to disease.

1.2.3.1 Network theory The concept of an id network involved in regulating the immune system was first postulated by Lindenmann in 1973 (201), followed by Jerne in 1974 (179). However since Lindenmann acknowledged his inspiration from earlier statements of Jerne, it is Jerne who is credited with the theory. The original description of the network and Jerne's further development of it (197) contained several essential elements. The first was that its basic constituents were the id and the paratope, each present on antibodies and lymphocytes. These had dual functions in that they both recognized and were recognized themselves, and thus created the network's fundamental connections. The second point has already been alluded to in the description of Ab2β antibodies, above. That is, since anti-paratope antibodies were the internal images of external antigens, they would be recognized by B cells specific for those antigens. Thus, without any external antigenic stimulation, the immune system already contained internal representatives of all possible antigens. This idea was extended to include regulatory functions within the network, since both positive (immune) and negative (tolerant) responses could potentially be initiated by antigens. A third principle was

that this interacting system was normally maintained in a state of dynamic balance. When antigen entered into it the equilibrium was shifted to the "excess antigen" side, and the network responded by producing more antibody (Ab1), which in turn then stimulated greater production of anti-antibody (Ab2), then Ab3, etc., in responses of diminishing magnitude until an equilibrium was once more re-established. Jerne's original descriptions did not specify T cells as part of the id network, but today they are also generally included (195).

These original ideas were quite clearly offered as a contribution to immunological theory, with the expectation that they would encourage research proving or disproving its tenets. In fact, it did stimulate a great number of investigations over the past two decades, and accumulated data overwhelmingly support the general concepts of the id network. More current studies, however, have also prompted modifications to the original principles and raised some complex issues which remain unresolved. For example, the sequential stimulation of Ab2, Ab3, Ab4, etc., is now believed not to be open-ended but rather a mini-network loop, since Ab3 would interact with Ab2 in a manner similar to that of Ab1 (202). Also, it appears that only a subset of id are involved in regulatory functions, likely germline-encoded recurrent id on framework residues of the V region (203). No agreement seems to exist regarding the degree of "connectivity" between the network elements, nor whether the id on B and T cell receptors are identical (191,200), and the notion that anti-id may manipulate clonal expansion "has triggered among immunologists a controversy similar to a religious war of the Middle Ages" (203). There is also continued discussion over what constitutes a

true internal image antigen, ideas further examined in the context of anti-id as vaccines.

1.2.3.2 Idiotypes and autoimmunity. One confirmation of the id network in vivo comes from finding a reciprocal relationship between circulating levels of Ab1 and Ab2 after immunizing healthy individuals with antigen. However it has also been observed that under these circumstances, despite the reduction of id-positive antibodies (Ab1), the overall levels of antigen-specific antibody do not decline (200,203,204). Thus how much contribution the id network really makes to normal immune regulation is indeterminate. Another perspective from which to explore the issue may be obtained by examining pathologic conditions in which id/anti-id interactions are suspected of playing a role. As recently reviewed by Rodey (202), one can envisage at least three mechanisms by which disruptions to the id network could cause disease. These are (a) the failure to produce a regulatory anti-id, such that an autoantibody clone would expand uncontrollably; (b) sustained formation of Ab1/Ab2 immune complexes, leading to pathologic deposition of the complexes; and (c) Ab2β mimicking the binding site of a cellular receptor, resulting in persistent and aberrant activation of the receptor-mediated function. Each of these postulated mechanisms has some limited empirical support (202).

Several more obscure categories of id and anti-id have been particularly associated with autoimmunity. Parallel id/anti-id sets, referred to previously, are defined as those id expressed on antibodies of more than one specificity by a single individual. By definition, anti-id against these parallel sets fall within the Ab2α group, in which the target id are located on the framework of the Ab1 (198).

Another kind of anti-id has been termed an "epibody", or Ab2ε. Epibodies not only bind to an id on the Ab1, but to a similar sequence that is present on the antigen to which the Ab1 is directed. Studies suggest that epibodies, which are usually IgM, are derived from autoreactive clones of the immune repertoire (205). Finally, certain other autoreactive anti-id have been recognized in the well characterized mouse system involving the recurrent id T15, associated with the anti-phosphorylcholine response. In this case the "autobodies" are the Ab1 expressing the T15 id, that are able to bind to other identical Ab1. The binding site has been localized to the CDR2 and framework region of V_H (206). Similar naturally occurring autobodies were recently identified in human sera (207).

The autoimmune rheumatoid diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis have come under considerable scrutiny for involvement of the id network. Sequence analyses of autoantibodies at the nucleotide or protein level have not been able to find unique sequences or genes. But a small number of specific families of V genes are used preferentially, and the frequency of recurrent id is correspondingly high, at least in major subpopulations of autoimmune patients (202,208,209). Therefore id markers have been used as one means of identifying the participation of autoantibodies in these diseases. A well characterized system is that of id 16/6, a recurrent id associated with anti-DNA antibodies as well as with antibodies against certain bacteria (eg. Klebsiella and Mycobacterium tuberculosis). Evidence for some relationship between the expression of id 16/6 and SLE pathogenesis is drawn from studies showing raised levels of the antibodies in patients with active disease compared

to those in remission, the presence of id-positive antibodies in kidney and skin lesions, and inverse levels of id-positive and naturally occurring anti-id antibodies in a patient over several months (210,211). Furthermore, experimental SLE was induced in mice after immunization with a human monoclonal anti-DNA expressing id 16/6, but was transiently improved by prior immunization against id 16/6 antibodies (209,210). These studies have been taken as evidence that the 16/6 id network influences the autoimmune processes involved in SLE, and that anti-id may play a role in mediating the disease. For example, it has been suggested that bacterial infection may initially induce anti-bacterial antibodies (Ab1) that express id 16/6 but which also bind DNA (the parallel sets of id-positive antibodies); subsequent stimulation of Ab2 then ensues, some of which will resemble DNA (Ab2β). This sets in motion an autoimmune cycle that is sustained, in part, by the id network (210).

Similar involvement of the id network has been sought in the etiology of rheumatoid arthritis. Rheumatoid factors (RF) are autoantibodies directed against the Fc portion of autologous IgG, and are found in a majority of patients with rheumatoid arthritis and other systemic rheumatoid diseases. Many monoclonal IgM RF are derived from a restricted number of germline gene segments and express a recurrent id, termed Wa, that has been localized to CDR2 of κ light chains (212). This same recurrent id was identified on a human mAb specific for HCMV (213). Although the human RF used in this study exhibited no anti-HCMV reactivity, and the mAb had no RF activity, sequence analysis revealed a marked similarity in the germline gene composition of their V regions. This suggested to

the authors the possibility of an association between the immune response to HCMV infection and the etiology of rheumatoid arthritis. As for the SLE scenario, the expression of recurrent id would then contribute to the perpetuation of autoimmune reactivities. Implications of a viral etiology were similarly drawn from a study in which rabbit anti-RF antibodies were generated, and the rabbit antisera recognized HCMV early and late antigens (plus EBV antigens) in vitro, a result consistent with HCMV having been an initiating antigen in this network (214).

1.2.4 Applications. As the structural basis of id and anti-id became increasingly understood, certain applications became possible. Some of these were based on id as markers of V region genes, and others on the concept that Ab2β may mimic external antigens.

1.2.4.1 Idiotypes as genetic markers. The recognition of id as phenotypic markers of germline V region genes allowed them to be used in analyzing B cell clones. Id were instrumental in research demonstrating the dominance of secondary versus primary B cell clones, once an immune response was established. They have also been used to examine mechanisms involved in isotype switching and the maturation of the antibody response (169). For example, studies demonstrated that after one week an anti-hapten response in mice did not exhibit somatic mutation, but by the second week mutations were frequent, and were correlated with increased anti-hapten affinity (215). More recently they have been used to identify human B cell clones expressing an id associated with the response to human immunodeficiency virus (216).

The presence of a common id on the clonally derived B cells of lymphoma

patients suggested to investigators that anti-id therapy might be effective. One study reported that complete or partial remissions were induced in a majority of 28 patients after a course of treatment in which monoclonal anti-id were given either alone or in combination with α-interferon (217). To date, anti-id antibodies directed against recurrent id have been preferred given the difficulty and expense of generating antibodies against a private id. Although the mechanisms by which the anti-id exert their anti-tumour effect is not clear, several possibilities have been suggested, including antibody-dependent cell-mediated cytotoxicity (ADCC), complement-mediated lysis, and some form of immune regulation mediated by the id network (217,218). In some cases tumour regression continued long after treatment was complete, but in others the induced remissions were temporary, despite continued therapy. An explanation offered for this latter finding is that id-negative variants may slowly emerge once most of the tumour has been cleared (169). Other adaptations of the anti-id approach are being pursued, eg. tagging the anti-id with radioisotopes, or making bi-specific mAb consisting of anti-id plus anti-T cell antigens (219). In addition, a recent article reported the induction of an immune response in 7 of 9 lymphoma patients who were vaccinated with autologous id-positive tumour cells (220). Thus, the potential for id-based therapies in this disease seems promising.

1.2.4.2 Molecular mimicry: anti-id as internal images of antigen. In Jerne's network the existence of Ab2β internal image antibodies had been postulated largely on theoretical grounds, without benefit of much empirical support. Direct evidence for anti-id mimicry of antigen was first provided by Sege and Peterson

(221). Using bovine insulin they generated Ab1 in rats and then Ab2 in rabbits. The rabbit Ab2 not only interacted with insulin receptors on tissues but also stimulated an appropriate physiologic reaction in the cells, from which these authors concluded that the anti-id antibodies might resemble a portion of the insulin molecule itself. Similar conclusions were reached by Urbain et al. (222) working with rabbit anti-id to Ab1 against tobacco mosaic virus.

The idea that Ab2β might be useful as surrogate immunogens for inducing immunity against infectious diseases was arrived at independently by Nisonoff and Lamoyi (223) and Roitt et al. (224) in 1981. It immediately launched one of the most active areas of id research, with many scientists endeavouring to put the concept to practical use for vaccine development, and many others trying to define internal image antigens at the molecular level. Certainly many studies in animals over the past decade have supported the feasibility of anti-id vaccines against microbial pathogens. These include bacteria (eg. Escherichia coli, Streptococcus pneumoniae), viruses (eg. reovirus, hepatitis B), and parasites (eg. Trypanosoma rhodesiense, Schistosoma mansoni), and excellent reviews dealing with this subject area are available (196,198,225-227). A full discussion of each of these studies is not possible here, but the hepatitis B and reovirus systems will be described as they illustrate the potential and limitations of this approach.

In 1986 Kennedy et al. described an anti-id vaccine for hepatitis B (228). Human antibodies (Ab1) specific for the surface antigen of the virus (HBsAg) were used to generate rabbit anti-id (Ab2), which in turn were tested for their vaccine potential in two chimpanzees. In response to immunization with Ab2 the

chimpanzees produced specific anti-Ab2 (ie., Ab3) antibodies. More significantly, they were protected from infection when challenged with infectious virus, compared to control animals that developed active hepatitis. Titres of anti-HBsAg were still high three years after vaccination, suggesting that immunity was sustained (229). Related research has examined the expression of different id associated with the human response to HBsAg, the ability to modulate the immune response to the virus by prior immunization with anti-id, and the potential of non-internal image anti-id (Ab2α) as therapeutic agents (229,230). Unfortunately, with regard to the development of the vaccine itself progress has rather stalled, no doubt partly because a good vaccine for the virus already exists (169). Nonetheless, this research was noteworthy because it demonstrated that anti-id could provide protective immunity against a major human disease, in the relevant animal model.

Reovirus has also been the focus of molecular and immunological investigations utilizing anti-id. The reovirus type 3 hemagglutinin (HA) protein mediates viral attachment to permissive cells and also induces both B and T cell immune responses. Anti-id antibodies were utilized by Greene and his colleagues to identify the cellular receptor for the virus. Both polyclonal and monoclonal anti-id were made against antibodies specific for the HA. In a manner similar to the identification of insulin receptors, these Ab2 bound to the cellular receptor and also triggered cellular responses as did the original ligand (231,232). Moreover, in vitro incubation of cells with anti-id protected them from infection (233). The anti-id were then used to isolate and characterize the receptor from rodent lymphoid and neuronal cells, clearly demonstrating that conformations on the Ab2 functionally

mimicked those present on the HA (234).

Further analyses were carried out to explore the interactions between the binding domain of the HA, the receptor, the Ab1 and the monoclonal Ab2. First, the Ab2 V regions were sequenced, and a comparison made between the deduced aa and the sequenced reovirus HA protein. This revealed that the receptor-binding epitope of HA, a linear sequence from aa 317-332, was mimicked by portions of Ab2. The homologies were present in the V_H domain (corresponding to HA sequences 317-324) plus the V_L domain (HA sequences 323-332), and involved both CDR and framework residues of the Ab2 (235). Synthetic peptides corresponding to the homologous Ab2 sequences bound the Ab1, and competed with reovirus for binding to the cellular receptor (236).

Other research addressed the Ab2 vaccine possibilities. Since reovirus stimulates both humoral and cell-mediated immune responses, the ability of the Ab2 to induce similar responses was examined. Investigations showed that the Ab2 elicited neutralizing antibodies and delayed-type hypersensitivity responses in syngeneic mice, and that reovirus-specific CTL lyzed Ab2 hybridoma cells (237), confirming the biological mimicry of HA by Ab2. Using synthetic peptides, the response was then further defined by immunizing mice with peptides corresponding to the HA epitope or the Ab2 V_H and V_L homologous sequences. Antibodies induced against the HA peptide and against the linked V_H-V_L peptide were neutralizing, whereas those against the free V peptides were not, showing that both the H and L chains were necessary to produce the conformation of HA they mimicked (238).

Although there is abundant evidence for the functional mimicry of antigen by Ab2, its expression at the level of primary structure has rarely been found. In fact, the reovirus system described above is one of very few examples of its kind. Three others are (a) the system using as antigen the synthetic polymer glutamic acid-alanine-tyrosine (GAT), in which Ab2 were found to contain sequences similar to GAT (239); (b) that in which Ab2 mimicked the hormone thyrotropin, thereby identifying its cellular receptor (240); and (c) the identification of very similar V region sequences of a mAb1 against the hormone angiotensin II, and the mAb3 (241,242). Unfortunately, the majority of studies utilizing Ab2 as surrogate antigens have not dealt with reactants that were sequenced. Nonetheless, in several cases where sequencing has allowed some direct or indirect comparison to be made between the reference antigen and functional Ab2β, homologies were not detected (192,243-245). The net effect of this has been increased interest in understanding the structural basis for the functional mimicry that undeniably occurs with some Ab2β, as well as a re-evaluation of the significance of internal image anti-id.

Ongoing contributions to our understanding of these issues have been made by the group headed by Heinz Köhler in La Jolla, California. First, it would seem to be an oversimplification that the mimicry of antigen by Ab2β must be a result of shape similarity, and that this results from identity at the aa level (246). In fact, the many examples of anti-id functioning as surrogate antigens for non-proteinaceous moieties (247-249) and for conformational epitopes (250-252) are proof that other mechanisms exist. Instead, it is the net affinity between id and

anti-id that determines the biological outcome of their interaction. Although the contacts involved in binding may be influenced by gross shape complementarity, this resemblance is not imperative (246). Even in cases where sequence homology does exist, it has been pointed out that the way in which antigen and Ab1 interact will be quite different than the interactions of Ab1 and Ab2, or Ab3 and antigen, given the different molecular and three-dimensional contexts of each antigen-paratope or id-paratope pair. Second, the assumption that only Ab2β can function as effective surrogate antigens has been called into question. Köhler notes that many anti-id meeting criteria established for Ab2β as internal image antigens failed to induce antigen-specific Ab3, and conversely many non-internal image Ab2 did so. These findings, and others, prompted his formulation of a "revised" network theory (246). Its major thesis was embodied in a recommendation that the designations of Ab2α, Ab2β, and Ab2γ be abandoned, to be replaced by the concept of a "network antigen". This proposed change in nomenclature was more than just semantic. It reflected the view that any anti-id could potentially stimulate the immune system as would an external antigen, if the affinity of interaction with the complementary id was great enough. Network antigens thus differed from Ab2β in two critical respects. First, no conformational similarities were assumed between the nominal epitope and the network antigen. And second, there was no necessity for the network antigen to inhibit antigen/Ab1 interactions. Adopting this more inclusive approach, it was argued, would lead to more rational and effective strategies for selecting id-based therapies (246).

Chapter 2. STATEMENT OF OBJECTIVES

The overall goal of my doctoral research was formulated within the context of the information presented in Chapter 1: To develop anti-idiotype antibodies (Ab2) that would function as network antigens in mimicking an immunodominant epitope of HCMV. The hypothesis for the study was that Ab2 thus generated have the capacity to function as surrogate antigens in stimulating a specific and neutralizing immune response to HCMV, and additionally are practical reagents for developing improved viral diagnostics. The specific objectives were further defined as follows:

1. Generate monoclonal and polyclonal Ab2 against an Ab1 mAb specific for an immunodominant epitope of HCMV.

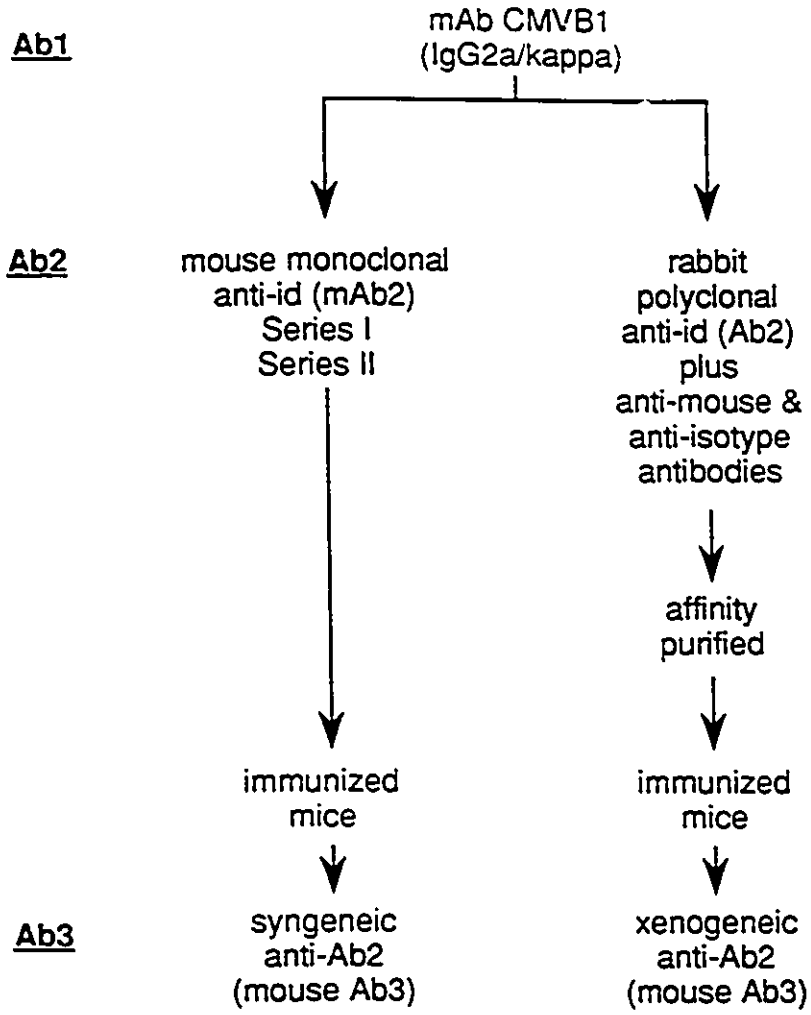
2. Evaluate whether these Ab2 mimicked the HCMV nominal epitope sufficiently to induce a specific immune response against HCMV.

3. Characterize the anti-HCMV response of the Ab3, if generated, with regard to the Ab1.

4. Determine if the monoclonal Ab2 would be useful reagents for developing improved HCMV diagnostics.

Since the nomenclature for the various antibodies described in the subsequent chapters may be confusing, a schematic diagram outlining the generation of Ab2 and Ab3 is shown in **Figure 3.**

Figure 3. Schematic diagram outlining generation of Ab2 and Ab3.


Ab1
mAb CMVB1
(IgG2a/kappa)
Ab2
mouse monoclonal
anti-id (mAb2)
Series I
Series II
rabbit
polyclonal
anti-id (Ab2)
plus
anti-mouse &
anti-isotype
antibodies
affinity
purified
immunized
mice
immunized
mice
Ab3
syngeneic
anti-Ab2
(mouse Ab3)
xenogeneic
anti-Ab2
(mouse Ab3)

Chapter 3. MATERIALS AND METHODS

3.1 Virology

3.1.1 Propagation of HCMV. The prototype HCMV strain AD169 was obtained from the Laboratory Centre for Disease Control, Ottawa, Ontario. HCMV stocks were propagated by infection of confluent human foreskin fibroblasts (HFF) at low MOI (0.2) in Basal Medium Eagle (BME, Gibco/BRL, Burlington, Ontario) containing 5% heat inactivated bovine calf serum (Hyclone Laboratories, Logan UT) and 2 mM L-glutamine. When the cells exhibited extensive CPE the supernatant was decanted and clarified of cellular debris by centrifugation at 1000 x g for 10 min, then aliquoted on ice and stored at -70°C until further use. Quantitation of infectious virus particles was performed by plaque titration assay as described elsewhere (253). The HCMV stock used throughout these studies had infectious titres of approximately 1 x 10^6 plaque forming units (PFU)/ml.

3.1.2 Plaque reduction assay. Viral neutralizing activity of antibodies was measured by a modified plaque reduction assay (253,254), as follows. Antibody samples diluted in BME were mixed with HCMV stock and guinea pig serum (as a source of complement) in the volume ratio of 45:45:10 and incubated for 60 min at 37°C. One-hundred and fifty µl of this mixture was then added to confluent HFF in 24-well culture plates at 37°C for a further 60 min, after which the cells were overlaid with 0.5% agar (Difco Laboratories, Detroit, MI) in nutrient medium. Ten days later the cells were stained with 1% (w/v) neutral red and after overnight incubation at RT the viral plaques were counted microscopically at 40X magnification. The concentration of virus used in each assay was adjusted such

that approximately 75 PFU were added to each well. Data were evaluated for levels of significance using Student's t test (255). When Ab3 mouse sera were tested, the sera were inactivated at 56°C for 30 min prior to assay. To determine whether the viral neutralizing activity of mAb CMVB1 was inhibited by mAb2, the assay was modified such that a concentration of purified mAb CMVB1 with only weak neutralizing activity was first incubated with the mAb2 for 90 min, and then residual CMVB1 neutralizing activity was measured as above.

3.2 Hybridomas and monoclonal antibodies

Splenocytes from immune mice (BALB/c, Charles River Laboratories, St-Constant, Quebec) were fused with the non-secreting murine plasmacytoma SP2/0 in the presence of 50% polyethylene glycol (PEG) 1450 (Eastman Kodak, Rochester, NY) as previously described (256). Hybridomas were initially cultured in Dulbecco's Modified Essential Medium (DMEM, Gibco/BRL) supplemented with 20% heat-inactivated bovine calf serum, 2 mM L-glutamine, 20 µg/ml gentamicin and the selective medium HAT (1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine, Sigma Chemical Co., St. Louis, MO). Over a period of several weeks the concentration of calf serum was gradually reduced to 10%, and after 3 weeks aminopterin was removed from the culture medium. Selected hybridomas were cloned at least twice by limiting dilution (257) and expanded and/or frozen and stored in liquid nitrogen as necessary. Ascitic fluid was produced in pristane-treated F1 mice (BALB/c x Swiss Webster, Animal Resources Division of Health Protection Branch, Health and Welfare Canada) as described by Brodeur and Tsang (258). Isotyping was accomplished by enzyme-linked

immunosorbent assay (ELISA) using class and subclass-specific anti-mouse immunoglobulins as the coating reagent (Fisher Biotech, Fisher Scientific, Orangeburg, NY).

3.3 Immunization protocols

3.3.1 Immunization of mice with mAb CMVB1 (Ab1). The methodology for producing mAb2 has been reported in detail elsewhere (250,259). Briefly, purified mAb CMVB1 was conjugated to keyhole limpet hemocyanin (KLH, Sigma) at a 1:1 (w/w) ratio in the presence of 0.05% glutaraldehyde, with agitation over a period of 2 h at RT. Syngeneic (BALB/c) mice were immunized s.c. at multiple sites with 75 µg protein and 25 µg of the adjuvant Quil-A (Cedarlane Laboratories, Hornby, Ontario). For series I mice, a booster injection of the same formulation was given s.c. 23 days later, and i.p injections without adjuvant were administered on days 69, 70 and 71, before the mouse was sacrificed to obtain the spleen on day 75. For series II mice, the s.c. boost was omitted and i.p. boosts were administered on days 9, 10, and 11 before the mouse was sacrificed on day 15. Trial bleeds were obtained retro-orbitally before immunization and approximately one week after each injection.

3.3.2 Immunization of rabbits with mAb CMVB1 (Ab1). Three NZW rabbits (Charles River Laboratories) were immunized s.c. at multiple sites with 200 µg of purified mAb CMVB1, emulsified in complete Freund's adjuvant. Trial bleeds were taken on days 10 and 17. Subsequent injections of antigen were administered on days 17 and 31 in incomplete Freund's adjuvant, and blood samples were obtained approximately one week after each booster injection.

3.3.3 Immunization of mice with mAb2. Groups of three to five BALB/c mice were immunized with each mAb2. The mAb2 were conjugated to KLH and given in conjunction with Quil-A (see section 3.3.1). Mice were immunized every 3-4 weeks and trial bleed samples were obtained one week after each injection. Analysis of mouse Ab3 utilized sera obtained after the first or second boost.

3.3.4 Immunization of mice with rabbit polyclonal Ab2. Three BALB/c mice per group were immunized s.c. with 30 µg purified rabbit Ab2, or 30 µg purified normal rabbit IgG, in conjunction with 25 µg Quil-A. Booster injections of the same formulation were administered on days 15 and 46, with blood samples withdrawn one week after each injection. The mice were exsanguinated 4 weeks after the last injection. Analysis of mouse Ab3 was carried out using sera obtained after the last trial bleed and/or exsanguination.

3.4 Immunoassays

3.4.1 General ELISA protocol. Unless otherwise specified, all ELISA procedures followed a similar protocol. Reagents were added in a volume of 100 µl/well except for the test sample which was contained in a volume of 50 µl/well. Ninety-six-well microtitre plates (Linbro/Titretek, ICN Biochemicals, Mississauga, Ontario) were coated with appropriate antibody diluted in PBS (pH 7.2). After overnight incubation at RT the wells were blocked with PBS containing 1% (w/v) bovine serum albumin (BSA) for 30 min at 37°C, followed by addition of test sample for 60 min at 37°C. Alkaline phosphatase conjugates were diluted in PBS containing 3% BSA, and added to the wells for a further 60 min incubation at 37°C. This was followed by addition of phosphatase substrate (1 mg/ml p-nitrophenyl

phosphate, Sigma) in 10% (v/v) diethanolamine, pH 9.6, for 60 min at RT, after which absorbance at 410 nm was measured spectrophotometrically (Dynatech MR7000 plate reader). Washing was carried out between each step of the procedure using PBS/Tween-20 (0.02%, v/v).

3.4.2 ELISA for screening mAb2. Supernatants of hybridoma clones were screened for specific anti-id reactivity by an ELISA which measured their binding to immobilized $F(ab')_2$ fragments of mAb CMVB1. Binding was detected by enzyme-conjugated anti-mouse Fc-specific IgG (Cappel/Organon Teknika, Durham, NC plus Jackson ImmunoResearch Laboratories, West Grove, PA). Inhibition of mAb2 binding to Ab1 by purified mAb CMVB1 or by other anti-HCMV murine mAb was tested by first incubating the inhibitor with the mAb2 for 60 min at 37°C, and then testing residual mAb2 binding as usual. Percent inhibition was calculated by comparison to a control in which PBS was substituted for the inhibitor.

3.4.3 ELISA for screening rabbit polyclonal Ab2. Ab2 antibodies in rabbit sera were identified by an ELISA which measured their binding to immobilized $F(ab')_2$ fragments of mAb CMVB1, compared to binding to $F(ab')_2$ of an unrelated isotype-matched control mAb (Hb-2). Binding was detected by enzyme-conjugated anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) in PBS containing 3% (w/v) nonfat dry milk.

3.4.4 Inhibition ELISA for detecting mouse Ab3 raised against mAb2. Anti-Ab2 antibodies (Ab3) in mouse sera were identified by an inhibition ELISA carried out as follows: 30 µl of mAb2 culture supernatant was mixed with 30 µl of mouse Ab3 serum diluted in PBS, in V-bottom microtitre wells. After agitation for 90 min

at RT, residual binding of mAb2 to Ab1 was measured by transferring 50 µl of the reaction mixture to wells of an ELISA plate already coated with $F(ab')_2$ fragments of mAb CMVB1. Incubation was allowed to proceed for 20 min at RT, whereupon binding of mAb2 to Ab1 was measured as described above (section 3.4.2). The concentration of each mAb2 used was established by prior titration, such that the control reaction mixture of mAb2 plus PBS gave an absorbance value of approximately 1.0. Ab3 titres were expressed as the maximum serum dilution resulting in 50% or greater inhibition of mAb2 binding. Percent inhibition was calculated by the formula [(A_{410} control - A_{410} test sample)/A_{410} control] X 100%.

3.4.5 Inhibition ELISA for detecting mouse Ab3 raised against rabbit polyclonal Ab2. Ab3 against rabbit polyclonal Ab2 were identified in a manner similar to that described in section 3.4.4, with the following modifications. Purified rabbit Ab2 was diluted in 5% normal rabbit serum and incubated with mouse Ab3 serum for 30 min at RT. Binding of residual Ab2 was then detected as described in section 3.4.3.

3.4.6 Inhibition ELISA for identifying shared mAb2 idiotopes. This inhibition ELISA was performed exactly as described in section 3.4.4, except that mAb2 supernatants were incubated with Ab3 sera induced by mAb2 other than themselves. These latter sera were operationally designated "heterologous Ab3" to distinguish them from "homologous Ab3" in which the inducing mAb2 and resulting Ab3 mouse antisera were paired.

3.4.7 ELISA for detecting the binding of mouse Ab3 to mAb2 $F(ab')_2$. The $F(ab')_2$ fragments of mAb2-3C5, mAb2-5C12, and the irrelevant control mAb Hb-2

were immobilized in microtitre wells. Mouse Ab3 sera diluted in 0.5% BSA/PBS were added, and binding was detected with enzyme-conjugated anti-mouse Fc-specific IgG.

3.4.8 Anti-id ELISA for measuring HCMV. Prior to use in the ELISA the HCMV stock was inactivated by heating at 95°C for 10 min. This step was included in order to minimize handling of infectious virus, after a comparison of the ELISA using inactivated or non-inactivated virus revealed that assay results were unaffected by treating HCMV in this manner. Virus samples for our experiments were prepared by spiking 100 µl HCMV (or BME medium as a control) into 1 ml normal urine. Preliminary investigations had shown that normal urine itself did not affect the assay results. Routinely, 4.4 ml of sample were used for each test. After removal of cellular debris the samples were centrifuged for 30 min at 10,000 g (Eppendorf model 5415C) and the pelleted virus immediately resuspended in 70 µl of 0.5% BSA in PBS containing biotinylated mAb CMVB1 at 12.5 ng/ml. This virus / biotinylated antibody mixture was then used in the anti-id ELISA for HCMV, as follows. Microtitre wells were coated with 0.5 µg/well purified mAb2, and blocked as usual. Fifty µl of virus / biotinylated antibody mixture (or control / biotinylated antibody mixture) which had been incubated together for 90 min at 37°C, was then added to the wells. After 20 min at RT the wells were washed, followed by addition of alkaline phosphatase-conjugated avidin (Cappel/Organon Teknika). All assays were done in triplicate. Virus present in the samples was quantitated by its inhibition of the binding of biotinylated mAb CMVB1 to immobilized mAb2, relative to the control, using the formula described above

(section 3.4.4). For biotinylation, mAb CMVB1 purified by protein-A Sepharose was labelled with biotin-X-NHS (Calbiochem, San Diego, CA) using a biotin:protein molar ratio of approximately 10:1 (174).

3.4.9 Indirect immunofluorescent assay (IFA). IFA was carried out on multitest slides (ICN Biomedicals, Horsham, PA) as previously described (253). In brief, confluent HFF were infected with HCMV at an MOI of 0.2 and fixed in 80% (v/v) ice-cold acetone when they exhibited extensive CPE. The fixed slides were stored at -70°C and could be kept for up to 18 months without deterioration. For the assay, wells were first blocked with 10% normal rabbit serum, and binding of mouse anti-HCMV was then visualized by fluorescein isothiocyanate (FITC)-conjugated goat antiserum to mouse IgG, IgA and IgM (Cappel/Organon Teknika). Positive titres were taken as the greatest dilution giving a reproducible fluorescent pattern above background samples, in which buffer (or appropriate sample diluent) was substituted for the test sample. For assays in which Ab3 mouse serum was tested, blocking of non-specificity was achieved with 10% HCMV-seronegative human serum and the test samples were diluted in 10% human serum (instead of PBS) for 30 min at 37°C before being used. A dilution of 1:20 mouse serum was the minimum tested to avoid non-specific fluorescence.

The IFA protocol was modified for studies examining the Ab2 inhibition of positive fluorescent reactivity. For testing mAb2 inhibition of mAb CMVB1, $F(ab')_2$ fragments of purified CMVB1 were diluted to a minimum concentration producing strong fluorescence and incubated with mAb2 culture supernatants for 60 min at RT. Residual CMVB1 reactivity was then tested by applying an aliquot of this

reaction mixture to the blocked wells for 20 min at RT, and continuing the assay as usual. For testing rabbit polyclonal Ab2 inhibition of mouse Ab3, a similar experimental design was used except that the Ab3 serum was incubated with purified rabbit Ab2 or rabbit IgG diluted in 10% HCMV-seronegative human serum before residual Ab3 reactivity was tested. All IFA were viewed with a Leitz Ortholux II fluorescence microscope equipped with a BP 450-490 excitation filter (Ernst Leitz Canada Ltd., Midland, Ontario).

3.4.10 Radioimmunoprecipitation (RIP) assay. The methodology for labelling of viral proteins with ^{35}S-methionine (Tran^{35}S-Label™, ICN Biomedicals Inc., Irvine, CA) and subsequent immunoprecipitation of the labelled cell lysates has been described elsewhere (250,253). In brief, lysates containing approximately 5 x 10^6 cpm from both HCMV-infected and mock-infected (control) cells were precleared with 5% normal serum (equal volumes of HCMV-seronegative human serum and non-immune mouse serum). Each lysate was then mixed with designated antibody, and after 90 min any insoluble material was removed by centrifugation (10 min x 10,000 g). Precipitation of the residual soluble antigen-antibody complexes was carried out with BSA-treated protein-A Sepharose after which samples were electrophoresed under reducing conditions through SDS-10% polyacrylamide discontinuous gels (260) using the Mini-Protean system (Bio-Rad, Richmond, CA). After electrophoresis the gels were fixed and prepared for autoradiography. The human anti-HCMV serum used as a control in the RIP assay was from a seropositive kidney transplant patient. ^{14}C-molecular weight standards (Gibco/BRL, Burlington, Ontario) were as follows: myosin

(200,000), phosphorylase B (97,400), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), β-lactoglobulin (18,400), lysozyme (14,300).

3.5 Antibody purification and analysis

3.5.1 Purification of mAb. mAb were routinely purified from ascitic fluid by precipitation with 45% (v/v) final concentration ammonium sulphate followed by chromatography over protein-A or protein-G Sepharose CL-4B (Pharmacia Ltd., Montreal, Quebec). Columns were equilibrated and run in PBS pH 7.2, and the mAb were eluted with 0.1 M glycine-HCl buffer, pH 3.0. All samples were dialyzed against PBS at 4°C before subsequent use. Protein concentrations were measured using the Bio-Rad Protein Assay according to the manufacturer's instructions.

3.5.2 Generation of $F(ab')_2$. $F(ab')_2$ fragments were generated by pepsin digestion of ammonium sulphate precipitated material derived from ascitic fluid (see section 3.5.1) as originally reported by Parham (261), using immobilized pepsin (Pierce, Rockford, IL). Purification of the $F(ab')_2$ fragments was then accomplished by passage over protein-A Sepharose. The antibody reactivity of all $F(ab')_2$ fragments was verified before further use.

3.5.3 Affinity purification of rabbit polyclonal Ab2. Ab2 was purified from rabbit antiserum in batches of 2 ml each by a series of sequential affinity columns. The first column was run in 0.1 M Tris-HCl (pH 8.0); all other columns were run in PBS. In all cases specific elution from the affinity matrix was effected with 0.1 M glycine-HCl buffer (pH 3.0). The glycine-eluted fractions were then dialyzed against PBS before further purification or analysis. (i) The pH of whole antiserum

was adjusted to 8.0 with one-tenth volume 1 M Tris, then passed through a 0.7 x 15 cm column of protein-A Sepharose CL-4B to obtain the IgG enriched fraction. (ii) This material was applied to an isotype affinity column, consisting of CNBr-activated Sepharose 4B (Pharmacia Ltd.) to which 10 mg of two purified irrelevant murine mAb (P2.13 and P2.16, both IgG2a/κ) had been bound according to the manufacturer's instructions. This step was designed to remove rabbit antibodies directed to epitopes on murine IgG2a or κ chains, while not adsorbing specific anti-id antibodies. The applied sample was recirculated through the column ten times before fractions were collected. (iii) Flow-through fractions were recovered and transferred to the third affinity column for which the ligand was 10.5 mg purified mAb CMVB1 (Ab1). Anti-id antibodies binding to this matrix were eluted in the glycine fraction. (iv) Concentration of purified Ab2 was accomplished by additional chromatography over protein-A Sepharose, and the recovered material was utilized for all subsequent experiments.

3.5.4 Purification of normal rabbit IgG. Normal rabbit IgG was prepared by fractionating rabbit serum over protein-A Sepharose as described above (section 3.5.3) and retaining the glycine-eluted material.

Chapter 4. RESULTS

4.1 Characterization of purified mAb CMVB1 (Ab1)

CMVB1 is an HCMV-specific murine mAb previously developed in our laboratory, and its partial characterization was reported before the current research was initiated (262). mAb CMVB1 is an IgG2a/κ antibody that recognizes a late viral antigen in cells infected with HCMV prototype strains AD169, Towne, and Davis. By IFA CMVB1 was shown to have no reactivity with other human herpesviruses (HSV-1, HSV-2, EBV or VZV), but recognized 24 of 24 clinical isolates obtained from patients in different regions of Canada over a five-year period. For the present studies, further analyses of purified mAb CMVB1 were performed. These showed that it was highly neutralizing in the presence of complement, with less than 28 ng/ml causing a 50% decrease in PFU by plaque reduction assay. Moreover by RIP assay mAb CMVB1 precipitated viral proteins of 58, 93-116, and 130 kDa (**Figure 4**, lane 2), consistent with the pattern established for the envelope glycoprotein complex gB. These same viral proteins were also precipitated by a neutralizing human serum from a renal transplant patient infected with HCMV (**Figure 4**, lane 4).

4.2 Generation of syngeneic monoclonal Ab2

Screening of over 775 clones from the series I fusion identified more than 35 with anti-id ELISA values greater than 1.2. Over several weeks eight of these mAb2 clones were selected for expansion and further study, based on their stability, good secretion, preliminary characterization suggesting they might be directed to the Ab1 paratope, and varied isotypes (**Table 1**). As illustrated in

Figure 4. **RIP assay of HCMV viral proteins recognized by mAb CMVB1.** Cell lysates from ^{35}S-radiolabelled HCMV-infected (lanes 2 and 4) or ^{35}S-radiolabelled control mock-infected (lanes 3 and 5) human foreskin fibroblasts were precipitated with mAb CMVB1 or a seropositive human serum, then analyzed by SDS PAGE. Lanes: 1, ^{14}C-molecular weight markers in kDa; 2,3, precipitation by mAb CMVB1 ascitic fluid diluted 1:1000; 4,5, precipitation by HCMV-seropositive human serum diluted 1:1200.

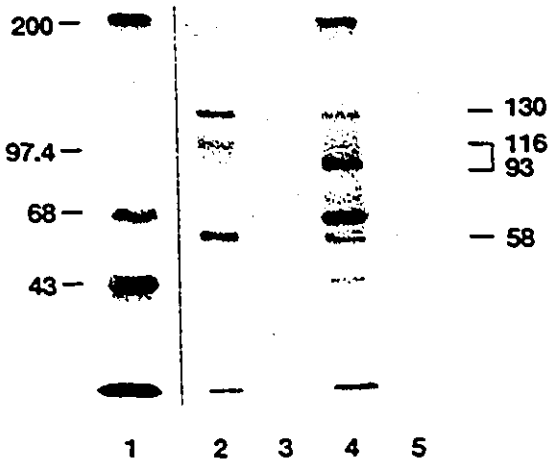

200
97.4
68
43
130
116
93
58
1
2
3
4
5

TABLE 1

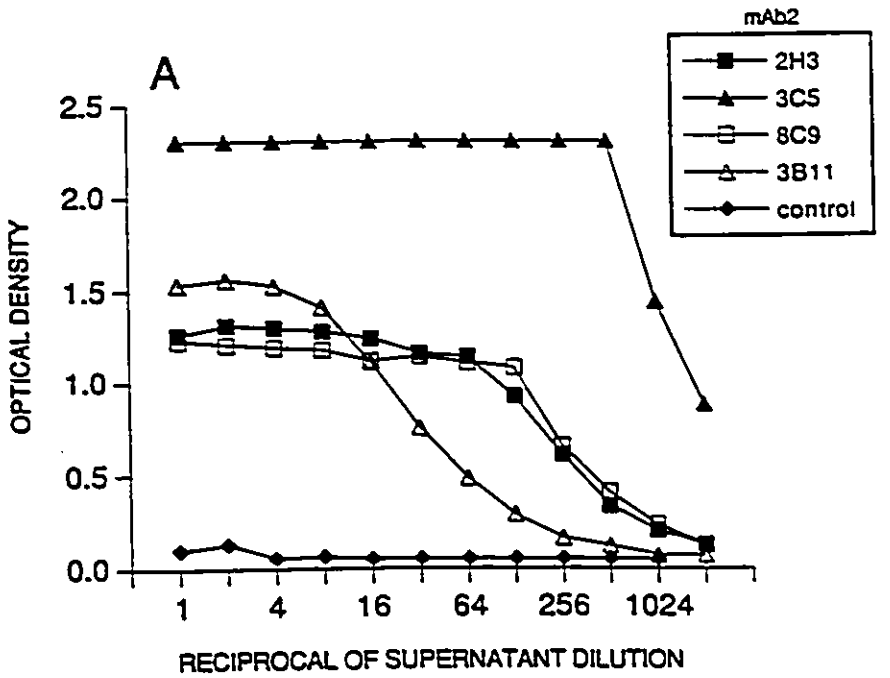
Isotypes of Series I mAb2

mAb2 Clone	Isotype
mAb2-2H3	IgG2b/κ
mAb2-3C5	IgG1/κ
mAb2-8C9	IgG2a/κ
mAb2-3B11	IgG2a/κ
mAb2-3F3	IgG2b/κ
mAb2-3F9	IgG2b/κ
mAb2-5C12	IgG2a/λ
mAb2-9B2	IgG2a/κ

Figure 5, the supernatants of all mAb2 exhibited a high degree of binding to the $F(ab')_2$ fragments of mAb CMVB1, with dilution causing a dose-dependent decline of reactivity. An irrelevant control anti-id mAb showed no binding to mAb CMVB1, and when wells were coated with the $F(ab')_2$ fragments of an unrelated IgG2a/κ mAb no binding of any mAb2 was observed (data not shown). To ensure that these observations represented specific binding of mAb2 to Ab1, inhibition experiments were carried out. Data in **Figure 6** demonstrate that purified mAb CMVB1 at concentrations of 10 μg/ml or more inhibited the interaction between all mAb2 and immobilized mAb CMVB1 by over 84%. CMVB1 had no effect on the binding between control id/anti-id in a parallel system. Furthermore, when several anti-HCMV mAb other than CMVB1 were similarly tested, no inhibition between the mAb2 and CMVB1 was observed. Collectively, these results indicated that the mAb2 were directed specifically against the id of mAb CMVB1.

Experiments were carried out to examine the relationship between the target id of mAb2 and the paratope of mAb CMVB1. The approach taken was to see if

Figure 5. **Dose-dependent binding of mAb2 to Ab1 (A and B).** Dilutions of hybridoma culture supernatants were screened for Ab2 reactivity by their ability to bind to immobilized $F(ab')_2$ fragments of purified mAb CMVB1, detected by enzyme-conjugated anti-mouse Fc-specific IgG.


A
mAb2
2H3
3C5
8C9
3B11
control
OPTICAL DENSITY
2.5
2.0
1.5
1.0
0.5
0.0
1
4
16
64
256
1024
RECIPROCAL OF SUPERNATANT DILUTION

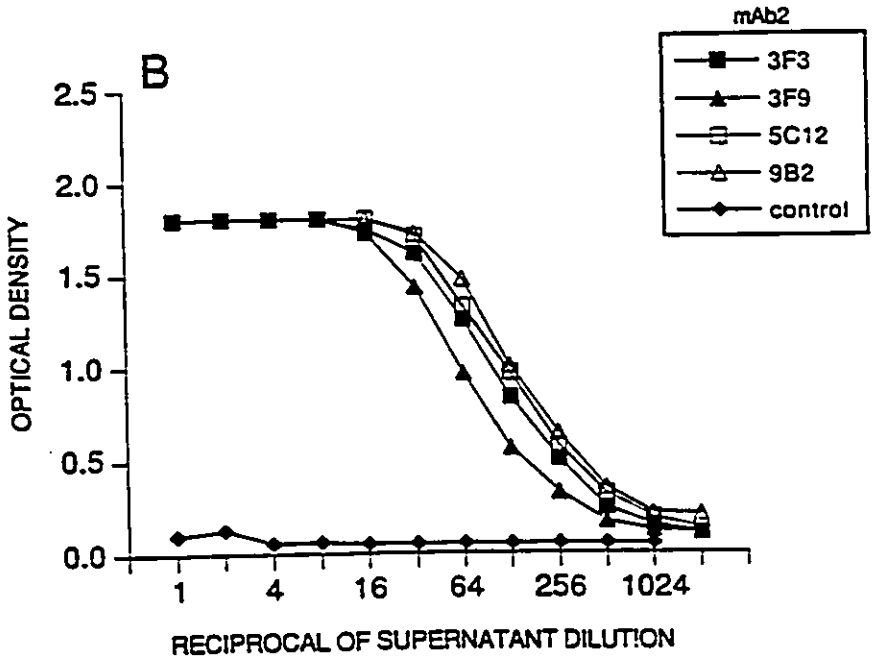
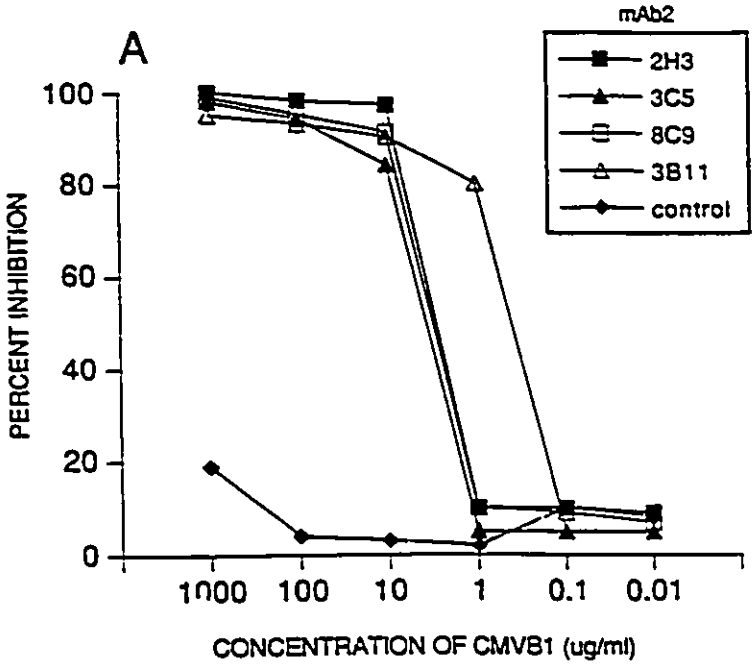
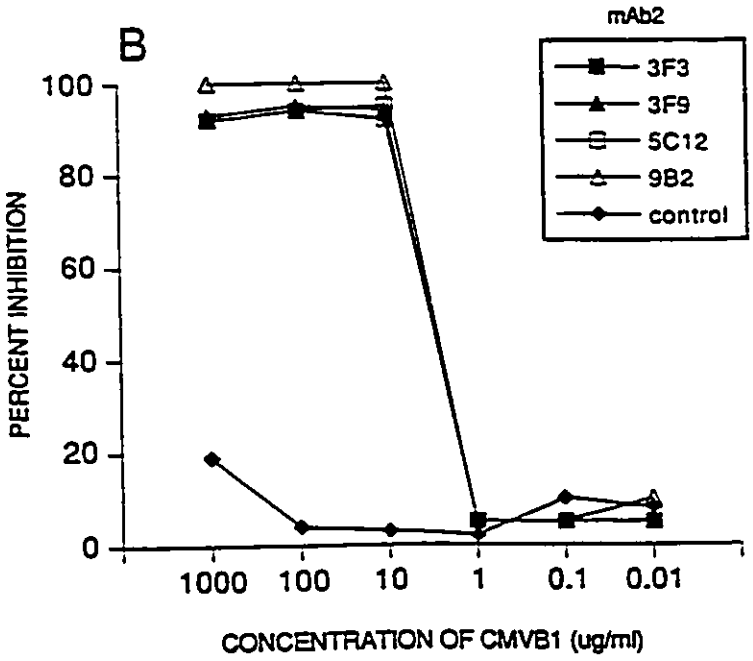

B
mAb2
3F3
3F9
5C12
9B2
control
OPTICAL DENSITY
2.5
2.0
1.5
1.0
0.5
0.0
1
4
16
64
256
1024
RECIPROCAL OF SUPERNATANT DILUTION

Figure 6. **Dose-dependent inhibition of mAb2 binding to Ab1 (A and B).** Undiluted mAb2 supernatants were incubated with increasing concentrations of purified mAb CMVB1, after which residual binding to immobilized CMVB1 was measured. Percent inhibition was calculated by comparison to a control in which PBS was substituted for the soluble CMVB1. Control values represent the percent inhibition of mAb CMVB1 in a parallel but unrelated Ab1/mAb2 system.


A
mAb2
2H3
3C5
8C9
3B11
control
100
80
60
40
20
0
PERCENT INHIBITION
1000
100
10
1
0.1
0.01
CONCENTRATION OF CMVB1 (ug/ml)


B
mAb2
3F3
3F9
5C12
9B2
control
100
80
60
40
20
0
PERCENT INHIBITION
1000
100
10
1
0.1
0.01
CONCENTRATION OF CMVB1 (ug/ml)

the normal interaction between mAb CMVB1 and HCMV could be blocked by mAb2. First, we showed that all eight mAb2 blocked the IFA reactivity of CMVB1. Representative results are shown in **Figure 7** and demonstrate that the fluorescence exhibited by purified mAb CMVB1 in the presence of medium (**Figure 7a)** was completely inhibited by prior incubation with mAb2-2H3, -3C5, and -8C9 (**Figures 7d,e,f).** Conversely no inhibition was observed after pre-incubation of mAb CMVB1 with an unrelated anti-id mAb (**Figure 7b)** or with an anti-id mAb2 not used in the current study (**Figure 7c).** In similarly designed experiments, we found that prior incubation of the mAb2 with mAb CMVB1 resulted in 100% inhibition of the viral neutralizing activity of CMVB1. The clear ability of the mAb2 to block anti-HCMV reactivity of mAb CMVB1 in these assays suggested that the anti-id mAb2 were directed to paratope-related sites on CMVB1, and supported the possibility that they mimicked the reference epitope of gB.

Given these encouraging results, purified mAb2 were used to immunize syngeneic mice. Anti-mAb2 (Ab3) in mouse sera were detected by an inhibition ELISA in which Ab3 bound Ab2 in liquid phase, thereby blocking subsequent binding of Ab2 to immobilized Ab1. All mice immunized with mAb2 mounted an anti-Ab2 response, with inhibition titres ranging from 200 to 1600. In addition, the binding to Ab2 of Ab3 raised against mAb2-3C5 and mAb2-5C12 was measured directly, using the $F(ab')_2$ fragments of these mAb2 to coat microtitre plates. Titres of 10^3 and 10^4 were observed for two Ab3 sera against mAb2-3C5 **(Figure 8A),** and titres of 10^4 were found for two Ab3 sera against mAb2-5C12 **(Figure 8B).** No binding was observed against the control $F(ab')_2$ nor when pre-immune mouse

Figure 7. **mAb2 inhibition of the IFA reactivity of Ab1**. IFA was carried out on fixed HCMV-infected human foreskin fibroblasts. $F(ab')_2$ fragments of purified mAb CMVB1 were diluted to the minimum concentration resulting in strong IFA reactivity, then incubated with supernatants of mAb2-2H3 (**d**), mAb2-3C5 (**e**), mAb2-8C9 (**f**), or as controls medium (**a**), an unrelated anti-id mAb (**b**), or a mAb2 not used in the current study (**c**). Assay of residual IFA reactivity of mAb CMVB1 was then carried out, visualized with FITC-conjugated anti-mouse IgG, IgA, IgM.

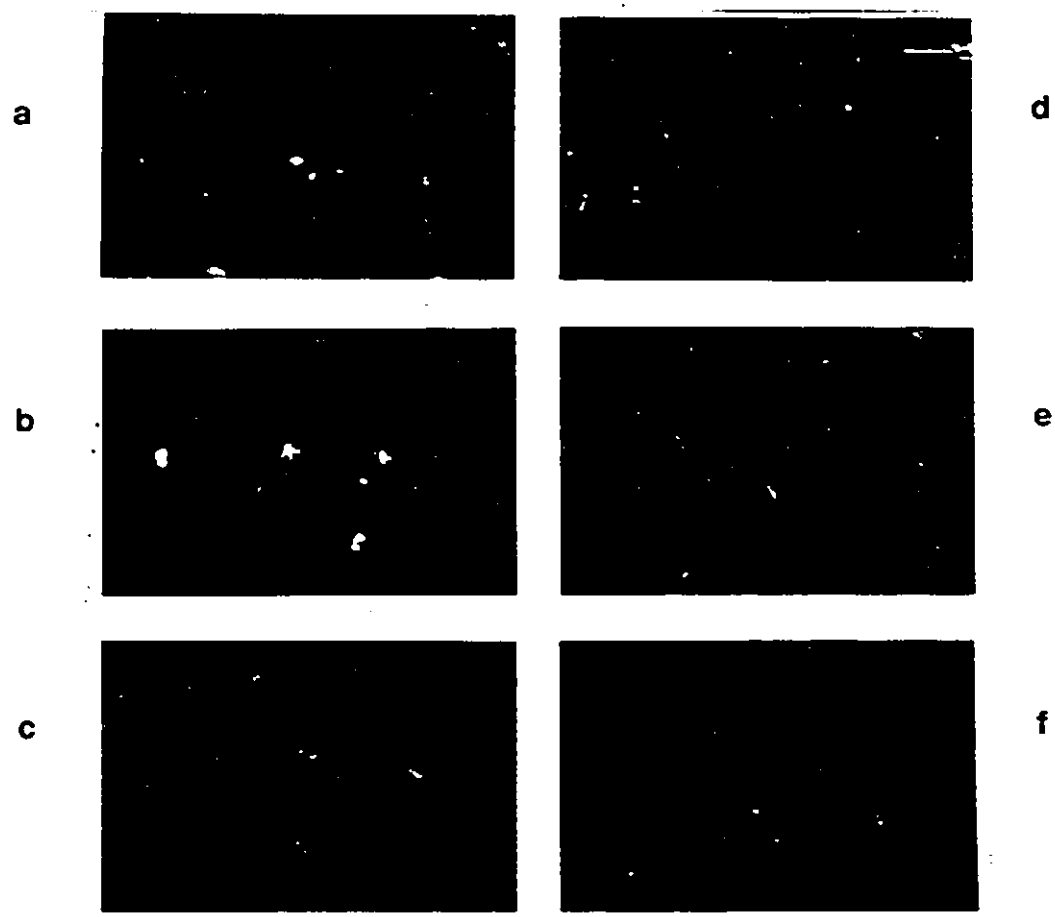
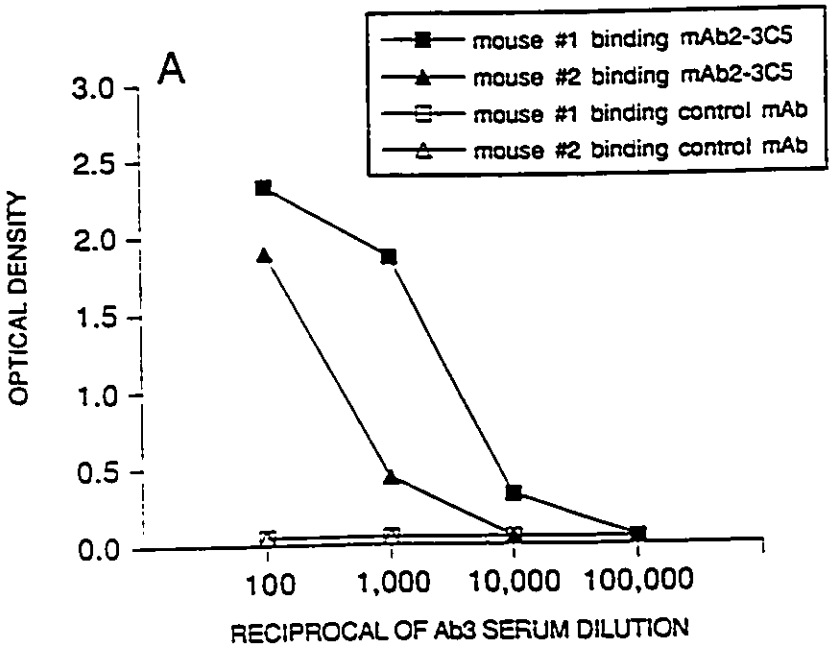
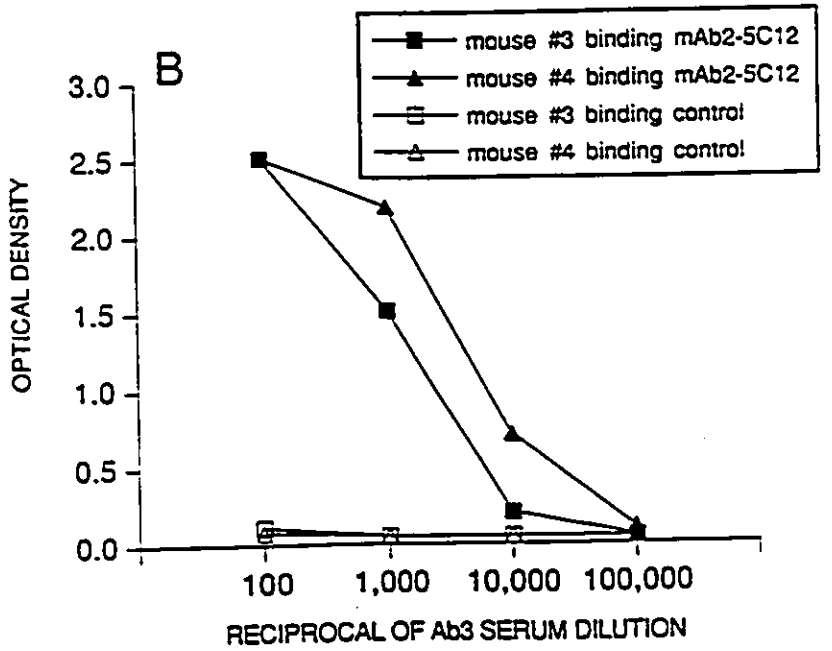

a
b
c
d
e
f

Figure 8. **Ab3 mouse sera raised against mAb2 bind to mAb2.** Microtitre wells were coated with the $F(ab')_2$ fragments of mAb2-3C5, mAb2-5C12, and an unrelated control mAb. Binding of mouse Ab3 sera was detected by enzyme-conjugated anti-mouse Fc-specific IgG. (**A**) Ab3 sera of mice #1 and #2 immunized with mAb2-3C5; (**B**) Ab3 sera of mice #3 and #4 immunized with mAb2-5C12.


A
mouse #1 binding mAb2-3C5
mouse #2 binding mAb2-3C5
mouse #1 binding control mAb
mouse #2 binding control mAb
OPTICAL DENSITY
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
1,000
10,000
100,000
RECIPROCAL OF Ab3 SERUM DILUTION


B
mouse #3 binding mAb2-5C12
mouse #4 binding mAb2-5C12
mouse #3 binding control
mouse #4 binding control
OPTICAL DENSITY
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
1,000
10,000
100,000
RECIPROCAL OF Ab3 SERUM DILUTION

serum was substituted for Ab3 serum.

To determine whether the mAb2 were each derived from distinct clones, inhibition ELISA were carried out by incubating the mAb2 with either heterologous or homologous Ab3 sera, and then measuring percent inhibition of binding of mAb2 to Ab1. Several aspects of the results of these experiments, shown in **Table 2**, are noteworthy. First, several of the mAb2 induced Ab3 with identical anti-id reactivities, and these have been grouped together in the Table. Only one of each of these sets was used in subsequent studies. Second, of the five distinct mAb2 remaining, significant binding of heterologous Ab3 only occurred against mAb2-5C12. In no case was inhibition seen with pre-immune mouse serum or an unrelated Ab3 mouse serum substituted for immune Ab3 serum.

To answer the critical question of whether the Ab3 were similar to mAb CMVB1 in their ability to recognize the reference antigen, HCMV, the Ab3 sera were tested using assays previously developed to characterize the Ab1. These included IFA to detect binding to HCMV-infected cells, plaque reduction assays to test viral neutralizing activity, and RIP assays to identify viral proteins targeted by the Ab3 antibodies. Despite considerable effort to maximize the sensitivity of these assays, all results were negative compared to appropriate controls.

In view of these results a second series of mAb2 were generated, adopting a brief immunization schedule so that fusion of the immune spenocytes was carried out only 15 days after initial challenge with mAb1 CMVB1. Screening of approximately 450 hybridoma supernatants by ELISA again identified many anti-id clones, from which seven mAb2 were selected. Six of these clones inhibited the

Table 2

Ab3 Sera Inhibit Binding of mAb2 to Ab1 (Series I)

mAb2 Binding	% Inhibition by Ab3 Mouse Sera Against mAb2-							
	2H3	3F3	3F9	3C5	8C9	9B2	3B11	5C12
2H3	89	93	89	0	1	4	0	3
3F3	93	87	88	2	8	8	0	3
3F9	49	87	89	8	8	12	0	0
3C5	0	0	0	96	0	0	0	0
8C9	2	9	10	4	99	91	0	0
9B2	6	9	11	3	42	87	0	0
3B11	0	0	0	0	0	5	81	0
5C12	43	4	50	49	7	24	0	84

Equal volumes of mAb2 supernatant and Ab3 mouse sera were incubated for 90 minutes, whereupon residual binding of mAb2 to Ab1 was measured by ELISA. Percent inhibition was calculated by comparison to samples in which PBS was substituted for Ab3 serum. Results represent the mean of triplicate values, derived from one mouse per Ab3 group.

binding of mAb CMVB1 to antigen (by IFA), suggesting they were directed to paratope-asscciated id, and one clone did not (mAb2-4F9) but was retained as a control. Isotypes of these mAb2 are listed in **Table 3**.

TABLE 3

Isotypes of Series II mAb2

mAb2 Clone	Isotype
mAb2-3H5	IgG1/κ
mAb2-4A8	IgG1/κ
mAb2-4H9	IgG1/κ
mAb2-5A8	IgG1/κ
mAb2-5B4	IgG1/κ
mAb2-5H3	IgG2b/κ
mAb2-4F9	IgG1/κ

Syngeneic mice were immunized with mAb2 purified from ascitic fluid. Anti-mAb2 were induced against all six of the mAb2 that were directed to paratope-associated id of Ab1, with titres similar to those previously measured for the series I mAb2. Conversely, only one of five mice immunized with mAb2-4F9 made a detectable response, with a weak titre of 100.

The mouse Ab3 sera were then used to ascertain whether the seven mAb2 clones were distinct. Again, the mAb2 were incubated with either heterologous or homologous Ab3 sera, and percent inhibition of binding of mAb2 to Ab1 was subsequently measured. Results of this study can be seen in **Table 4.** Each of the seven mAb2 is different than the others, however some degree of cross inhibition (28%, 30%) is observed between the heterologous mouse Ab3 sera raised against mAb2-4A8 and mAb2-5B4, suggesting that these mAb2 may share one or more idiotopes. No inhibition was observed when normal mouse serum was

Table 4

Ab3 Sera Inhibit Binding of mAb2 to Ab1 (Series II)

mAb2 Binding	% Inhibition by Ab3 Mouse Sera Against mAb2-						
	3H5	4A8	4H9	5A8	5B4	5H3	4F9
3H5	99	7	5	5	7	7	7
4A8	0	100	0	8	28	3	3
4H9	3	0	96	3	9	9	0
5A8	3	23	0	95	1	9	2
5B4	6	30	0	0	98	1	12
5H3	7	12	18	11	7	96	0
4F9	3	16	14	10	9	19	87

Equal volumes of mAb2 supernatant and Ab3 mouse sera were incubated for 90 minutes, whereupon residual binding of mAb2 to Ab1 was measured by ELISA. Percent inhibition was calculated by comparison to samples in which PBS was substituted for Ab3 serum. Results represent the mean of triplicate values, derived from one mouse per Ab3 group.

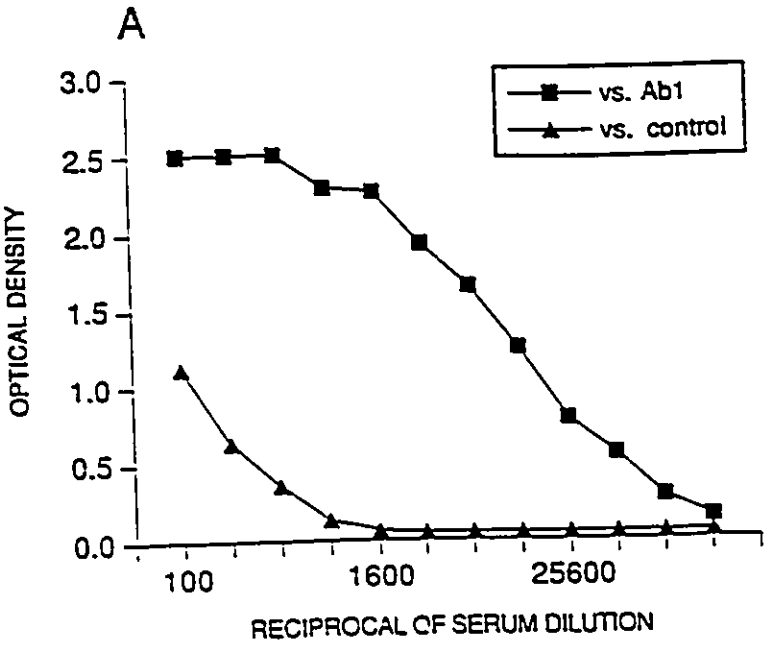
substituted for Ab3 serum in these experiments. To investigate whether the series I and series II mAb2 expressed any id in common, inhibition ELISA were repeated with homologous and heterologous Ab3 mouse sera in a similar manner. Significant inhibition (greater than 25%) was only observed in the binding of mAb2-5C12 to Ab1 using Ab3 sera raised against mAb2-4A8 (47%) and against mAb2-5B4 (34%). All other results and controls were negative.

The Ab3 mouse sera against series II mAb2 were next evaluated to determine if they possessed anti-HCMV properties similar to mAb CMVB1. Experiments were conducted to identify IFA reactivity, viral neutralizing activity, and precipitation of viral proteins by RIP assay. Once again, none of these assays identified any anti-HCMV activity in the Ab3 sera.

4.3 Generation of rabbit polyclonal Ab2

Rabbits immunized with mAb CMVB1 produced specific Ab2 which could be detected on days 10 and 17 after the initial challenge. The Ab2 were identified by simultaneous ELISA screening against the $F(ab')_2$ fragments of mAb CMVB1 (Ab1) and an isotype-matched unrelated murine mAb (**Figure 9**). Since analysis of the later trial bleeds revealed a proportionately increasing titre against the control anti-IgG2a/κ mAb compared to the Ab1 mAb, for this study rabbit Ab2 was purified from 10 ml of pooled serum collected on days 10 and 17. The rabbit polyclonal Ab2 was purified and concentrated by a series of four sequential affinity columns as described in section 3.5.3. This protocol yielded 1.3 mg of purified rabbit Ab2 (665 µg/ml) that exhibited an ELISA titre of 32,000 against the Ab1 mAb, but showed no reactivity against the control mAb (**Table 5**).

Figure 9. **Specificity of rabbit polyclonal Ab2 binding to Ab1.** Dilutions of rabbit antiserum were screened for Ab2 reactivity by binding to immobilized $F(ab')_2$ of purified mAb CMVB1, or to an unrelated but isotype-matched control mAb. Binding of Ab2 was detected by enzyme-conjugated anti-rabbit IgG. Trial bleed sera were obtained 10 days (**A**) or 17 days (**B**) after immunization.


A
vs. Ab1
vs. control
OPTICAL DENSITY
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
1600
25600
RECIPROCAL OF SERUM DILUTION

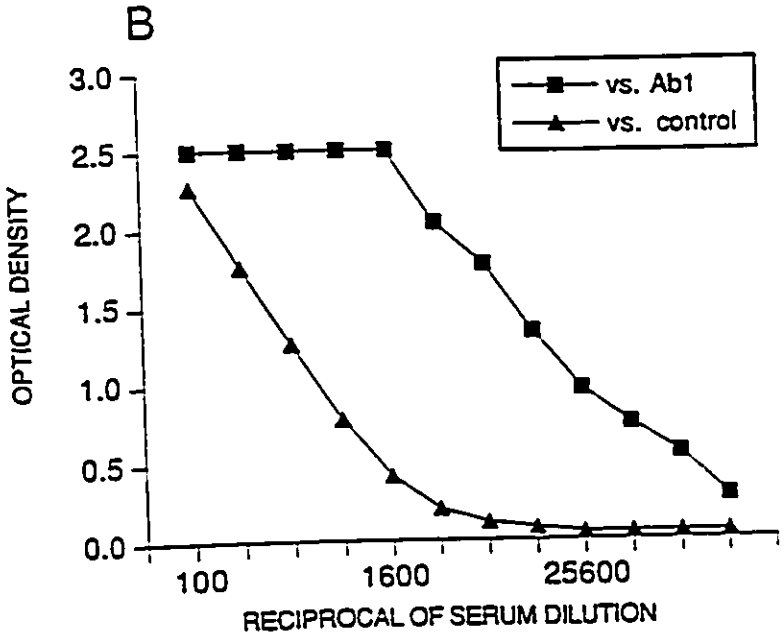

B
vs. Ab1
vs. control
OPTICAL DENSITY
3.0
2.5
2.0
1.5
1.0
0.5
0.0
100
1600
25600
RECIPROCAL OF SERUM DILUTION

TABLE 5

Purification of Polyclonal Ab2 from Rabbit Antiserum

Sample	ELISA Titre vs. CMVB1 (Ab1) (IgG2a/kappa)	ELISA Titre vs. Control mAb (IgG2a/kappa)
Rabbit serum pre-immunization	negative[a]	negative[a]
Pooled antiserum pre-purification	32,000	800
Purified Ab2 (665 µg/ml)	32,000	negative[b]

a highest concentration tested was 1:100 whole serum
b highest concentration tested was 6.65 µg/ml

Mice were then immunized with the purified rabbit Ab2 (mice #1-3) or an equal amount of purified normal rabbit IgG (mice #4-6). Trial bleed sera were assayed for the presence of anti-Ab2 (Ab3) antibodies by an inhibition ELISA in which Ab3 bound purified Ab2 in liquid phase, thereby blocking subsequent binding of Ab2 to immobilized Ab1. As shown in **Figure 10**, all Ab2-immunized mice produced Ab3 that inhibited the binding of Ab2 to Ab1 in a dose-dependent manner. The strongest Ab3 response was seen in the serum of mouse #1. Conversely, the sera of mice immunized with normal rabbit IgG caused no significant inhibition.

4.4 Characterization of Ab3 raised against rabbit polyclonal Ab2

In a manner analogous to the evaluation of our monoclonal Ab2, the sera of mice immunized with rabbit polyclonal Ab2 were then examined to see if they contained Ab3 antibodies specifically directed against HCMV viral antigens, as did mAb CMVB1. First, IFA studies were carried out. Results showed that Ab3 sera from mice #1 and #2 bound to HCMV-infected cells, with titres of 640 and 160, respectively. As seen in **Figure 11** the pattern of fluorescence was typical of antibodies to late HCMV antigens, with a prominent perinuclear inclusion body in the cytoplasm (**Figure 11a**), and was similar to that produced by mAb CMVB1 (**Figure 11d**). No reactivity was detected in serum from mouse #3 or with sera from mice #4, 5, or 6 (**Figure 11b**). To verify that the fluorescence we observed in the sera of mice #1 and #2 was truly the result of Ab3, blocking experiments were performed. In these studies, diluted Ab3 mouse serum was pre-incubated with either purified rabbit Ab2, an equal amount of purified rabbit IgG, or diluent

Figure 10. **Ab3 mouse sera raised against rabbit polyclonal Ab2 bind Ab2.** Equal volumes of diluted Ab3 mouse sera were incubated with purified rabbit Ab2, and residual binding activity of Ab2 for the $F(ab')_2$ fragments of Ab1 was measured. Percent inhibition was determined by comparison with wells in which PBS was substituted for mouse serum. Mice #1-3 had been immunized with purified rabbit Ab2, and mice #4-6 with an equivalent amount of purified normal rabbit IgG.

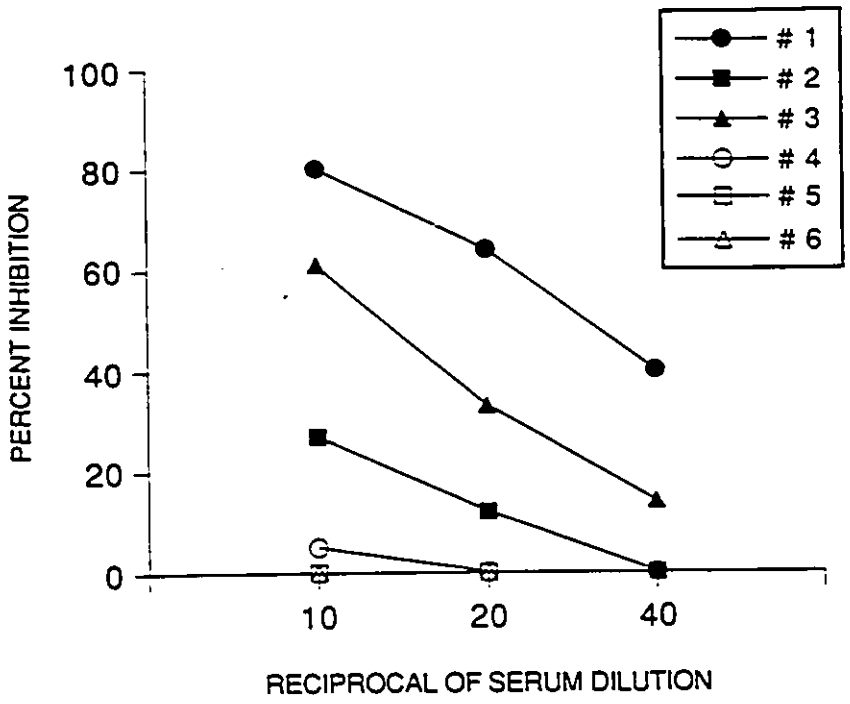
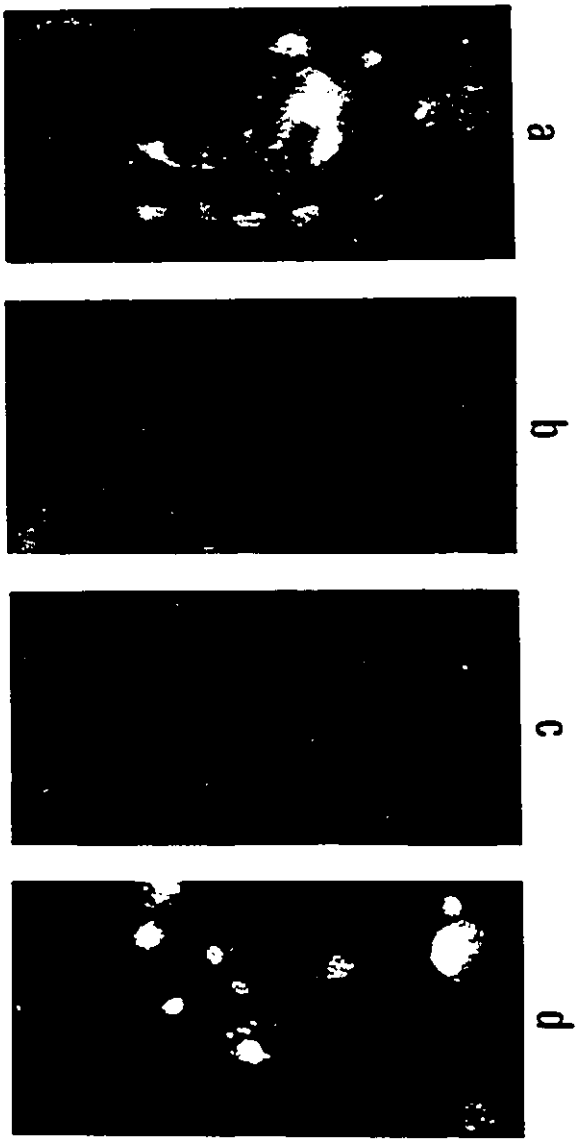

100
80
60
40
20
0
PERCENT INHIBITION
10
20
40
RECIPROCAL OF SERUM DILUTION
1
2
3
4
5
6

Figure 11. IFA studies of Ab3 mouse sera raised against rabbit polyclonal Ab2. IFA was carried out on fixed HCMV-infected human foreskin fibroblasts, visualized with FITC-conjugated anti-mouse IgG, IgA, IgM. (**a**) Serum from mouse #1 (1:80 dilution) immunized with purified rabbit Ab2; (**b**) serum from mouse #4 (1:80 dilution) immunized with normal rabbit IgG; (**c**) inhibition of the Ab3 immunofluorescent reactivity in (a) by pre-incubation with rabbit Ab2; (**d**) immunofluorescent pattern of purified mAb CMVB1.


a
b
c
d

(10% HCMV-seronegative human serum), after which residual IFA reactivity of the mouse serum was tested. Under these conditions 5 μg purified rabbit Ab2 completely inhibited subsequent reactivity of the Ab3 (**Figure 11c**), whereas normal rabbit IgG at 5 μg and at 20 μg resulted in no inhibition.

As a means of identifying the Ab3 target epitope(s) of HCMV more precisely, radioimmunoprecipitation experiments were carried out. Results from RIP assays are illustrated in **Figure 12**. They reveal that serum from mouse #1 (lane A3) precipitated viral proteins with the same molecular weight as those precipitated by mAb CMVB1 (lane A7), at 58, 93-116, and 130 kDa. No viral proteins were precipitated by serum from mouse #4, immunized with normal rabbit IgG (lane A5); and no bands were present in lanes from control mock-infected cells (lanes A4, A6, A8). An additional experiment was then performed to confirm these results and to compare the viral proteins precipitated by the Ab3 serum with those precipitated by a convalescent patient serum. As illustrated in **Figure 12B**, the same HCMV proteins precipitated by the Ab3 serum (lane B4) have also been recognized by the seropositive patient serum (lane B2).

Since the Ab1 mAb neutralized viral infectivity in vitro, we next investigated whether the Ab3 mouse serum possessed similar biological activity. For these studies the number of PFU in the Ab3 test wells were compared to those in which an equal dilution of pre-immune mouse serum had been substituted. Representative data in **Table 6** show that serum from mouse #1 neutralized HCMV at dilutions from 1:5 to 1:40. There was also an indication of some neutralizing activity in serum from mouse #3 at a dilution of 1:5, although this was not

Figure 12. **RIP assay of HCMV viral proteins recognized by mouse Ab3.** Cell lysates from ^{35}S-radiolabelled HCMV-infected cells (lanes A3, A5, A7, B2, B4) or ^{35}S-radiolabelled control mock-infected cells (lanes A4, A6, A8, B3, B5) were precipitated with sera derived from mice immunized with purified rabbit Ab2 or normal rabbit IgG, then analyzed by SDS PAGE as described in Materials and Methods. Lanes: **A1,B1**, ^{14}C-molecular weight markers in kDa; **A3,A4,B4,B5**, precipitation by mouse #1 serum (immunized with purified rabbit Ab2) diluted 1:40; **A5,A6**, precipitation by mouse #4 serum (immunized with normal rabbit IgG) diluted 1:40; **A7,A8**, precipitation by mAb CMVB1 ascitic fluid diluted 1:1000; **B2,B3**, precipitation by HCMV-seropositive human serum diluted 1:400.

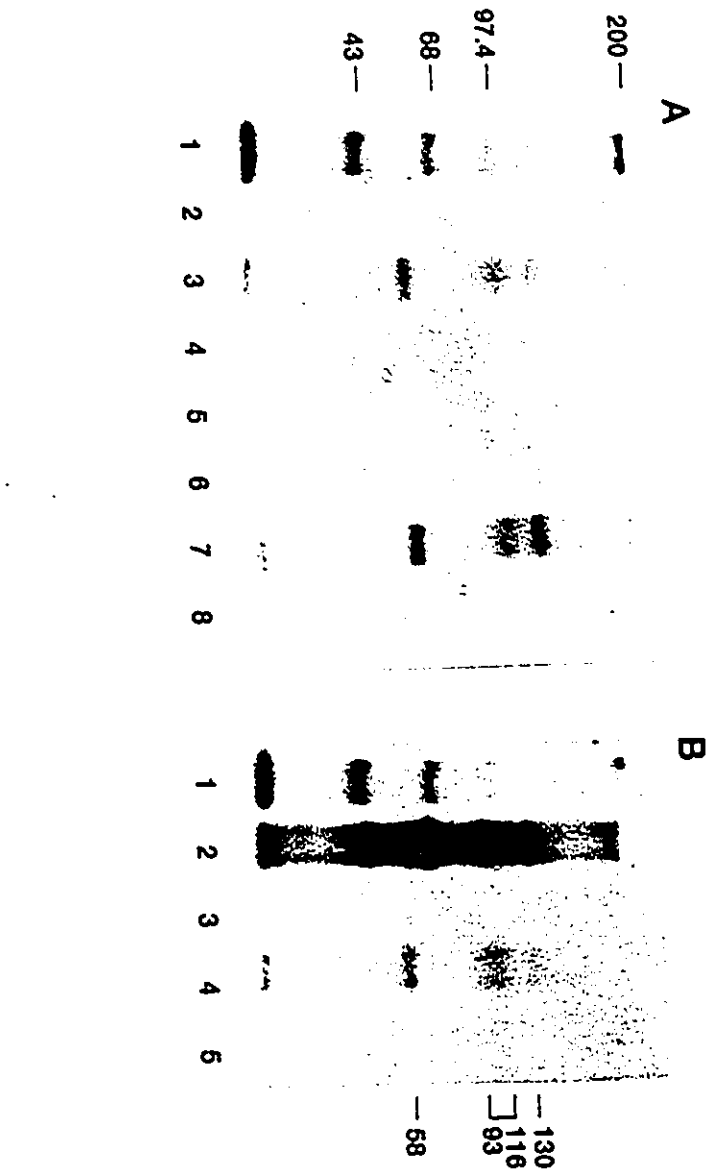

A
200—
97.4—
68—
43—
1 2 3 4 5 6 7 8
B
1 2 3 4 5
130
116
93
58

TABLE 6

Viral Neutralizing Activity of Mouse Ab3 Sera

Mouse Serum[b]	Plaques Formed in Presence of Diluted Mouse Serum[a]			
	1:5	1:10	1:20	1:40
#1	4 (2,3,7)[c]	19 (14,19,2[illegible][c]	26 (22,26,31)[c]	28 (28,27,30)[c]
#3	19 (18,20,20)	45 (33,46,55)	nd	nd
Pre-immune[d]	30 (24,29,36)	47 (43,44,54)	54 (44,48,71)	50 (57,47,46)

a average number of PFU per well (triplicate wells)
b all sera were inactivated at 56°C for 30 min before being tested
c statistically different than pre-immune serum at the same dilution ($p < 0.05$, Student's t test)
d pool of mice 1-6 used in current study

statistically significant ($p < 0.05$). No neutralization was observed with serum from mouse #2 or with sera from mice #4, #5 or #6 immunized with normal rabbit IgG.

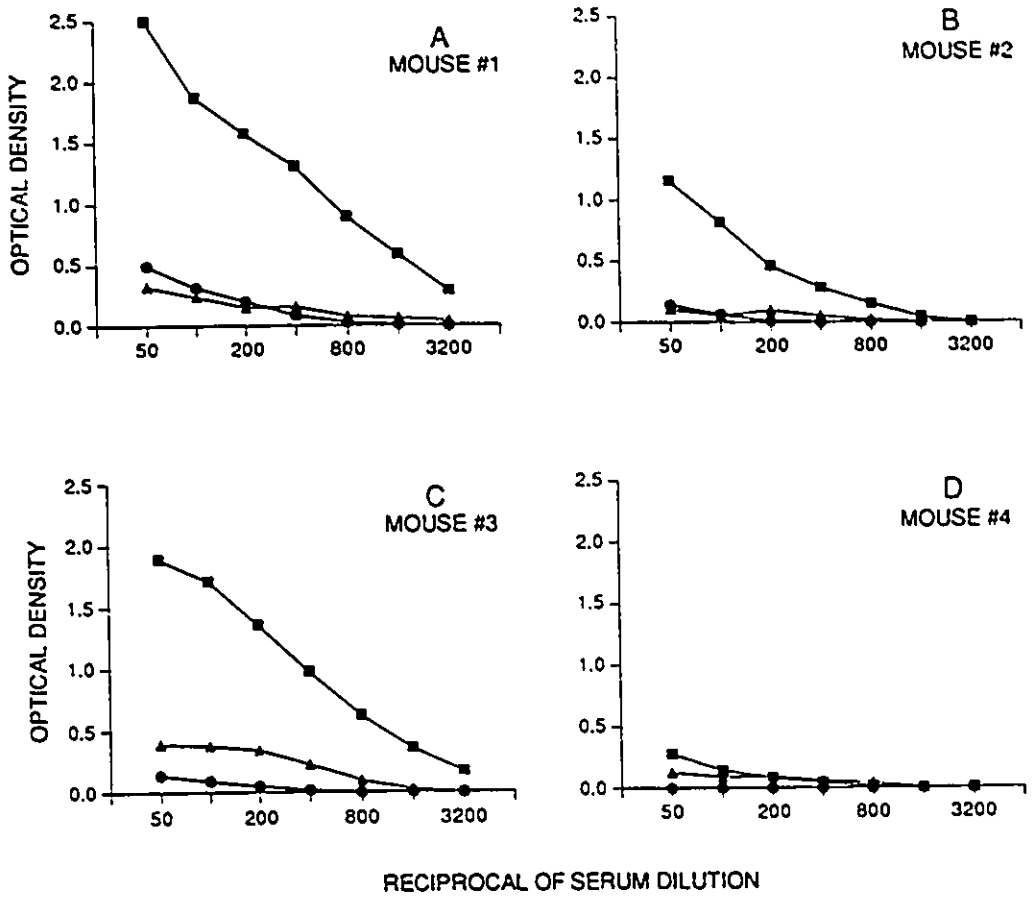
The idiotypic expression of the Ab3 antisera raised against polyclonal Ab2 was further examined by utilizing mAb2-3C5 and mAb2-5C12. As described above, these mAb2 had distinct specificities, but both appeared to be directed to paratope-associated id of mAb CMVB1. ELISA were set up to determine if the mouse Ab3 raised against rabbit polyclonal Ab2 would bind to the immobilized $F(ab')_2$ fragments of mAb2-3C5 or mAb2-5C12. Results presented in **Figure 13** demonstrate that sera from all mice immunized with rabbit Ab2 bound to mAb2-5C12, with serum from mouse #1 exhibiting the highest titre (**Figure 13A**). No significant binding to either mAb2-3C5 or the control mAb was seen (**Figures 13A,B,C**) and no binding was observed with serum from mice immunized with normal rabbit IgG (**Figure 13D**) or with normal mouse serum.

4.5 Anti-id ELISA for HCMV

The series I mAb2 described above were used to develop a unique immunoassay for detecting HCMV in vitro. We postulated that mAb2 engineered against an anti-HCMV antibody could be used as the basis of an assay for rapid viral detection. We reasoned that if viral antigen was first allowed to bind to anti-HCMV (Ab1 mAb CMVB1), then subsequent interaction between the Ab1 and complementary mAb2 would be inhibited. This inhibition could then be quantitated relative to appropriate controls. As a first step towards achieving this goal we developed an inhibition ELISA for measuring a laboratory strain of HCMV, AD169.

Initial experiments compared coating microtitre wells with either a single

Figure 13. **Binding of Ab3 mouse sera raised against rabbit Ab2 to mAb2.** The $F(ab')_2$ fragments of mAb2-3C5 (●), mAb2-5C12 (■) and an irrelevant control mAb (▲) were immobilized in microtitre wells. Binding of mouse Ab3 sera was detected with enzyme-conjugated anti-mouse Fc-specific IgG. Sera were derived from mice immunized with purified rabbit Ab2 (**A,B,C**) or an equivalent amount of normal rabbit IgG (**D**).


A
MOUSE #1
B
MOUSE #2
C
MOUSE #3
D
MOUSE #4
OPTICAL DENSITY
OPTICAL DENSITY
2.5
2.0
1.5
1.0
0.5
0.0
50
200
800
3200
RECIPROCAL OF SERUM DILUTION

anti-id (mAb2-8C9) or a pool of the five distinct series I mAb2, to see if one reagent or the other would prove advantageous. The data in **Table 7** show that there was over 90% inhibition of CMVB1 binding to either mAb2-8C9 or the pooled mAb2, when HCMV at 20 x 10^3 PFU/ml was employed in the assay, and significantly less inhibition (48% and 53%) when virus at 5 x 10^3 PFU/ml was used.

TABLE 7

Percent Inhibition by HCMV of Ab1/mAb2 Binding

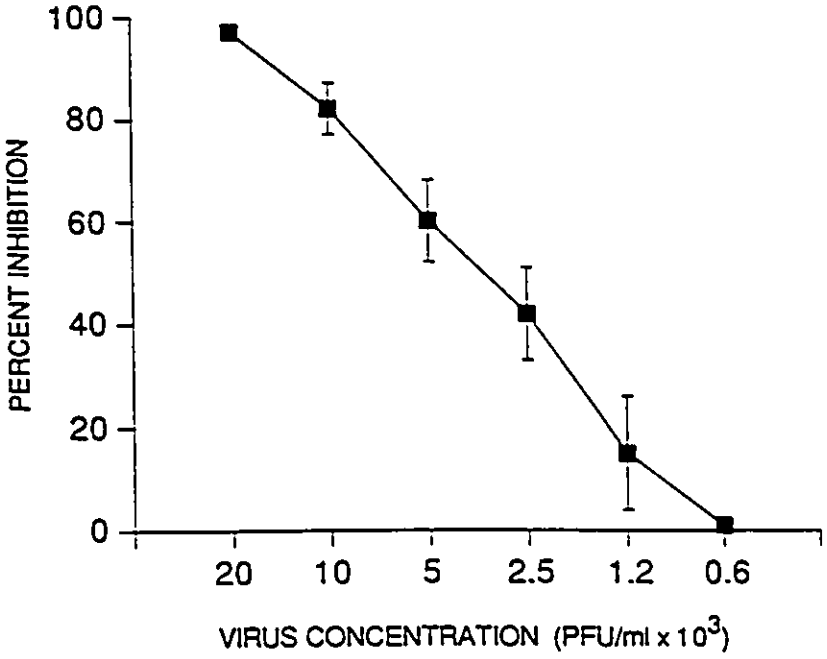
Virus concentration (PFU/ml x 10^3)	Anti-id mAb2 coating microtitre wells	
	mAb2-8C9	Pool[a]
20	91 ± 1	92 ± 1
5	48 ± 5	53 ± 6

a A pool of five series I mAb2, including mAb2-8C9 (n=3, mean ± S.D.)

No binding of mAb CMVB1 to an unrelated control anti-id mAb was observed. As a result of these data we concluded that the sensitivity of the assay was not improved by using the pooled mAb2 compared to mAb2-8C9 alone, and only mAb2-8C9 was used for coating wells in subsequent assays.

We next analyzed the concentration of virus for which a linear dose-response curve could be generated, and examined the sensitivity of the assay. Results of representative experiments are illustrated in **Figure 14**. When two-fold dilutions of the HCMV samples were made, there was a concentration-dependent reduction in the amount of viral antigen measured. The assay was linear over the range tested, 20 to 0.6 x 10^3 PFU/ml. Furthermore, if 50% inhibition was taken to be the cutoff for positive detection, then the assay sensitivity was established as approximately 3 x 10^3 PFU/ml.

Figure 14. **Dose-dependent measurement of HCMV by anti-id ELISA.** Virus samples were diluted two-fold and tested by the anti-id ELISA. Measurement of viral antigen was based on its ability to inhibit the binding of anti-id mAb2-8C9 to mAb CMVB1, relative to a control. (n=3, mean ± S.D.)


100
80
60
40
20
0
PERCENT INHIBITION
20
10
5
2.5
1.2
0.6
VIRUS CONCENTRATION (PFU/ml x 10^3)

Chapter 5. DISCUSSION

Most of the research described in this thesis relates to the generation and characterization of Ab2 that were induced by a mAb specific for an immunodominant glycoprotein of HCMV. The murine mAb CMVB1 was selected as the initiating Ab1 for the study because it was highly effective in neutralizing the infectivity of HCMV in vitro, and was directed against an epitope conserved on HCMV clinical strains. More specifically, by RIP assay it was shown to bind the glycoproteins of the envelope complex gB. This viral constituent is responsible for stimulating significant humoral and cell-mediated immune responses in infected hosts, and is widely regarded as one of the major candidates for vaccine development (80,114).

The Ab2 initially generated for these studies were monoclonal and syngeneic. Although Ab2β have been successfully developed in both monoclonal and polyclonal systems (196,225) the rationale for choosing a monoclonal strategy was that purified mAb2 of defined specificity would be the most valuable for the analytical and preparative aspects of the research. Furthermore, by working in a mouse syngeneic system only the id of each antibody would be seen as immunogenic when inducing the Ab2 and Ab3 antibodies. It has also been suggested that a more vigorous anti-id response may be made against monoclonal rather than polyclonal antibodies (263). A potential disadvantage of a monoclonal approach was that in selecting relatively few hybridomas to work with, some relevant clones might be excluded from the study. To minimize this risk the mAb2 were chosen with a view to obtaining a panel of anti-id derived from distinct clones,

using immunoglobulin class and subclass as an indirect basis of selection.

This strategy proved to be quite successful. When the id-specificities of the mAb2 were defined by competitive ELISA with mouse Ab3 sera, we found that five of the eight series I mAb2 were quite distinct from each other. There was also some evidence for the presence of a recurrent id on mAb2-5C12, since Ab3 mouse sera raised against several clones other than mAb2-5C12 also bound to it. Similarly, all seven of the series II mAb2 were distinct from each other, despite five of the six antibodies being IgG1/κ. Again, there was some suggestion of partial id sharing between two of the clones, mAb2-4A8 and mAb2-5B4. To complete the identification of Ab2 specificities, the id of series I and series II mAb2 were compared. This revealed no duplication between the mAb2 of these separate fusion events. However, there was an indication of idiotopes co-expressed on both the series I mAb2-5C12, and the series II mAb2-4A8 and mAb2-5B4. Since mAb2-5C12 was the only anti-id with λ light chains, the data suggested that this idiotope was localized on the V_H domain of the mAb2. Collectively, these results demonstrated that at least eleven idiotopes were clustered in and/or around the Ab1 paratope and contributed to the id profile of mAb CMVB1, an observation in keeping with existing views of idiotypic complexity (198,226,246).

The mAb2 used for these studies were shown to be specific for the idiotype of the immunizing Ab1 mAb CMVB1 in that they bound to the immobilized $F(ab')_2$ of the Ab1, but not to the $F(ab')_2$ of unrelated controls. Moreover, the binding could be specifically inhibited by soluble Ab1. Except for the control mAb2-4F9,

all mAb2 were classified as either Ab2β or Ab2γ. That is, the target idiotopes for the mAb2 were associated with the paratope of Ab1, since their interaction with Ab1 prevented subsequent binding of Ab1 to antigen. For both series I and II mAb2 this was demonstrated by the ability of the mAb2 to completely block the IFA reactivity of mAb CMVB1, and additionally for the series I mAb2, to block its viral neutralizing activity. Ultimately, however, we had to classify each mAb2 as either Ab2β or Ab2γ. To do this, Ab3 mouse sera were raised against each purified anti-id, and the Ab3 sera were analyzed. We found that all mAb2 were immunogenic and stimulated an easily detectable anti-Ab2 response. But when we tested the Ab3 sera for the same anti-HCMV reactivities that the mAb CMVB1 exhibited, that is IFA, neutralizing activity, and binding to viral proteins by RIP assay, we were unable to find any antigen-specific Ab3 reactivity whatsoever.

The inability of any of the mAb2 to induce a detectable antigen-specific Ab3 response in immunized mice most probably indicates that the mAb2 were Ab2γ, rather than Ab2β. This result was certainly disappointing, but perhaps not altogether surprising. Only a fraction of anti-id will be true internal image antigens, the traditional candidates for inducing antigen-specific responses. Moreover, a monoclonal Ab2β corresponds to only a single epitope of the nominal antigen, and in some cases this epitope may be part of a multideterminant antigen needed to induce immunity (264). One of the implications of the classic Ab2β concept is that the Ab2 should bind to a wide variety of antibodies from different species that are specific for the reference epitope, since the Ab2β and the antigen are presumably interchangeable in this regard. In fact, some workers have used this property as

a means of differentiating Ab2β from Ab2γ (169,265). Although this criterion is not absolute, particularly in the case of a private Ab1 id, the mAb2 of the present study had nonetheless been examined in this way. Both rabbit anti-HCMV raised against purified virus, and positive sera from renal transplant patients, were used as otner sources of anti-HCMV. However, all results had been negative (unpublished observations).

It is also important to note that even when anti-id appear to fulfill traditional Ab2β criteria, failure to induce antigen-specific Ab3 often occurs and may be difficult to explain. For example in each of three studies in which Ab2β were generated and induced antigen-specific Ab3, several other apparently bona fide Ab2β were unsuccessful. These studies involved rabies virus (266), human respiratory syncytial virus (267), and influenza virus (268). Conversely, there have been reports of anti-id that were <u>not</u> Ab2β but which did induce Ab3 with Ab1-like activity. For example, Zhou et al. (269) immunized mice with a syngeneic monoclonal Ab2α, and generated an Ab3 response that recognized the nominal antigen, in this case a synthetic peptide corresponding to gp41 of human immunodeficiency virus. Similarly, Suné et al. (270) reported that a monoclonal anti-id classified as an Ab2γ was able to induce neutralizing antibodies in mice against transmissible gastroenteritis coronavirus, the reference antigen in this system. Perplexing results such as these in addition to many other unpublished studies (see 246,271) were instrumental in prompting a re-evaluation of the different categories of Ab2 in the id network, as discussed in Chapter 1.

Immunization schedules for inducing monoclonal anti-id are different in each

laboratory. However, our protocol for producing the series I mAb2 was similar to that used by several investigators who had successfully induced internal image mAb in syngeneic mouse systems (106,265,270,272). When our analysis of mouse Ab3 sera against the series I mAb2 showed that no antigen-specific Ab3 had been induced, we decided to try a very short immunization protocol for the series II mAb2. This was based upon an observation in the literature suggesting that a more vigorous anti-id response might be made earlier in the immune response (263). Not unexpectedly, almost all of the mAb2 generated in series II were IgG1, compared to the more varied isotypes represented in series I (IgG1, IgG2a, IgG2b). This reflected the fact that proliferating B cells progressively switch the immunoglobulin class of the antibody they are producing while maintaining the same antigen specificity. For mouse H chains the progression of the isotype switch is fixed in the following order: μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, ε, α (273). It is of interest that the literature does not indicate any relationship between the isotype of mAb1 or mAb2 and the ability to generate internal image anti-id, even though both isotype switch and somatic mutations of the rearranged immunoglobulin gene are correlated with the maturation of the immune response. Therefore although we did attempt to have a variety of different isotypes represented in the mAb2 selected for study, this was only an indirect means for trying to identify mAb2 clones of different anti-id specificity.

In contrast to monoclonal systems, the advantages of polyclonal Ab2 are that even if internal image Ab2 represents only a tiny proportion of the immune response, as is usually the case, selective purification of this population may yield

a useful reagent. However, as with all polyclonal reagents it is impossible to define the exact antibodies that are responsible for the measured effects, or to generate identical antibodies in large quantities.

Results of the present study demonstrate that the rabbit polyclonal Ab2 do have the capacity to function as network antigens for a neutralizing epitope of gB. Evidence to support this claim comes primarily from analyzing the sera of Ab2-immunized mice, compared to control mice immunized with an equivalent amount of IgG from normal rabbits. First, we showed that an immune response to the id of Ab2 was induced, since the sera from all Ab2-immunized mice were able to inhibit subsequent binding of Ab2 to immobilized Ab1. Second, we found that the Ab3 sera exhibited anti-HCMV activity similar to that of the Ab1. IFA reactivity was observed with the mouse Ab3 sera and could be inhibited by pre-incubation with Ab2, confirming its specificity. In addition, the mouse Ab3 sera manifested biological activity, demonstrated by neutralizing HCMV infectivity in vitro. Neither of these reactivities was found in the sera from control mice. Therefore the conclusion drawn from this series of experiments was that the combining site of certain subsets of the rabbit polyclonal Ab2 population likely bore some complementarity to gB, the reference antigen. This interpretation was confirmed by RIP assay, by identifying the viral proteins that the mouse Ab3 were directed against. The bands at 58, 93-116, and 130 kDa were the same molecular weight as those precipitated by the Ab1 mAb, and proved that specific anti-gB antibodies had been generated in the Ab2-immunized mice. Moreover, proteins of this same apparent molecular weight were precipitated by serum from an HCMV-seropositive

patient, not an unexpected result given the immunodominance of gB in natural infection (70,123).

The mouse Ab3 generated against rabbit Ab2 not only recognized the reference antigen, but also expressed at least one idiotope in common with the initiating Ab1. This was demonstrated by the binding of mouse Ab3 sera to one of the monoclonal anti-id of series I (mAb2-5C12). This reactivity indicated that the Ab1 idiotope defined by mAb2-5C12 was recurrent, rather than unique, since it was carried on both the murine Ab1 and on some proportion of the mouse Ab3 antibodies, and in fact confirmed the earlier observations we had made when characterizing the id of series I and II mAb2. Moreover, these data demonstrated some dominance in the expression of the id defined by mAb2-5C12, since it was expressed in response to immunization not only by gB, but by Ab2 functioning as network antigens substituting for gB. Conversely, the Ab1 id defined by mAb2-3C5 was not detectable in the mouse Ab3 sera. From these studies we concluded that the idiotype of Ab1 and Ab3 were not identical. Although antigen-specific antibodies were generated in the mice immunized with rabbit Ab2, the Ab2 stimulated different subsets of mouse B cells, compared to those activated by gB of HCMV. These results are consistent with those described in other studies, reporting a similar fine variability between the Ab1 and the antigen-reactive Ab3 immune response. For example Mariani et al. (274) described the diversity of the anti-HLA-DR Ab3 response induced by mAb2 directed to different id co-expressed on the same Ab1. The study by Viale et al. (275) demonstrated that although an Ab3 response was induced that was similar to mAb1 with respect to binding a

tumour-associated saccharide epitope, the fine immunoreactivity of the two antibodies was not identical. This observation was further explored by sequence analysis of the Ab1 and Ab3, which showed that different germline genes had been utilized for portions of the V regions of the two antibodies (248). Anti-id studies related to the hemagglutinin of influenza virus found that immunization with polyclonal Ab2 induced distinctive subpopulations of Ab3 antiviral antibodies, generating a shift in the epitopes recognized by Ab3 compared to Ab1 (268). Similar observations were made in the immune response to anti-id corresponding to epitopes of human respiratory syncytial virus (267). Additionally, Keay et al. (106) generated syngeneic mAb2 to the 86 kDa glycoprotein (gp86) of HCMV. The mAb2 appeared to mimic the reference epitope, and were subsequently used to identify a cellular receptor for gp86 (56). However, in addition to binding to the initiating Ab1, the mAb2 also bound to another mAb that was specific for the HCMV glycoprotein p130/55, suggesting a shared id on these distinct anti-HCMV mAb. These investigations in complex antigen systems, including the present study, illustrate the contribution of id network mechanisms to the generation of diversity in the immune response. They also indicate that subtle differences in the properties of anti-id may stimulate distinct B cell populations in responding hosts. These considerations will be relevant to the selection of anti-id for use as effective surrogate immunogens or therapeutic agents.

The concentration of anti-HCMV antibodies in the Ab3 mouse sera was not high compared to the Ab1, with a maximum IFA titre of 640 and a maximum neutralization titre of 40, in both cases exhibited by the serum of mouse #1. A

similar low magnitude Ab3 response has been noted in other studies and could result from numerous factors. First, as noted previously, the id network itself reacts to perturbation with antibody responses of Ab1, Ab2, Ab3, etc., of decreasing magnitude. Second, the amount of internal image antigen present in the immunizing dose of Ab2 is likely very small, even when the polyclonal Ab2 has been extensively purified. One study showed that when the dose of polyclonal Ab2 was increased, a correspondingly higher Ab3 response was obtained (276). Quantitation of the low level of antigen-specific Ab3 was described in a recent paper by Chattopadhyay et al. (277). When xenogeneic mouse anti-id were used to induce Ab3 in monkeys, it was estimated that 4-8% of the antibodies in the Ab3 serum were anti-Ab2, but only 1.4% of the Ab3 actually exhibited Ab1-like activity. Third, the molecular context of the Ab2 immunogen is different than the original antigen. In the present case mice were immunized with an infected-cell lysate and whole viral particles to generate the Ab1 mAb CMVB1, whereas the immunogen of interest for inducing Ab3 in mice was the id of rabbit antibodies. Fourth, a functional antibody assay such as neutralization is probably quite stringent with regard to the structural requirements demanded of the antibody, whereas a binding assay would likely be more forgiving. An example of this was demonstrated in a study of bovine herpesvirus. When Ab3 were induced in mice against monoclonal bovine Ab2, the Ab3 titre was higher than Ab1 in radioimmunoassays, but lower than Ab1 by viral neutralization assays (278). Fifth, Ab3 antibodies of lower affinity than Ab1 may have been induced. If the rabbit Ab2 failed to mimic the entire original gB epitope, then Ab3 antibodies with only partial identity to the Ab1 would

be generated, certainly resulting in an altered affinity. Lastly, since xenogeneic Ab2 were used, it has been suggested that the dominant immune response of the mice may be against rabbit-specific determinants, at the expense of the anti-id response (270,276,278).

The magnitude and predictability of the Ab3 response are major concerns for those interested in practical aspects of Ab2 vaccines. In experimental models the importance of different immunization schedules, doses, adjuvants, carriers, routes of immunization, etc. have been examined sporadically, but given the variability between each system it is difficult to draw generalized conclusions (251,276,279). Two issues related to anti-id immunogenicity illustrate the difficulties likely to be encountered in this area. On the one hand, the generally low immunogenicity of anti-id is such that even in xenogeneic systems, where the immunizing Ab2 should be highly immunogenic, it seems that adjuvants and possibly carrier molecules will be needed. In the case of the vaccine for hepatitis B, for example, chimpanzees each received four injections of purified rabbit Ab2 adsorbed to alum before an antigen-specific Ab3 response was detected (228). Since adjuvants for human use are limited, this may be a problematic issue (280). On the other hand, when a vigourous anti-id response is stimulated after multiple Ab2 injections, the rapid clearance of immunogen may curtail the impact of the booster injections (226,281). Therefore in situations where repeated boosters are needed for effective anti-id immunization, a balance between these two potentially conflicting responses will need to be found.

Recent investigations have identified many antigenic domains for stimulating

B and T cell immunity on the 130 kDa precursor glycoprotein and 58/116 kDa cleavage products that comprise the gB family. Of particular note is that both continuous and discontinuous epitopes have been defined, with a recent report of a discontinuous epitope assembled from a consecutive sequence of 70 amino acids (282). Although the gB epitope defined by mAb CMVB1 has not yet been characterized, preliminary experiments from our laboratory suggest that it may reside on the 58 kDa moiety of the glycoprotein (unpublished observations). Whether it is a linear or discontinuous epitope is still unknown. Nonetheless, anti-id are particularly well suited to mimic complex and/or conformational determinants, as demonstrated in several systems including the S glycoprotein of murine coronavirus (251), a neutralizing epitope of enterovirus-70 (250), and gp120 of human immunodeficiency virus (252).

One way to approach controlling the pathogenesis of HCMV is to understand how the immune response to the virus is regulated. Although mechanisms related to the pathogenesis of HCMV disease remain unclear, since severe disease occurs almost exclusively in individuals with impaired immune systems, immunologic regulation undoubtedly plays a significant role (45,48). Antigen-specific cell-mediated responses may further be responsible for immunopathologic processes that occur in certain HCMV-related conditions. Several studies have proposed that the pneumonitis following allogeneic transplantation is largely a T cell response to a virally-induced antigen, likely an IE or early viral product (43,44), and a similar mechanism may be operating in HCMV-associated kidney disease (45). A relationship between HCMV disease and

graft rejection has been postulated, but this link is less obvious. In fact, though an epidemiologic correlation between increased graft rejection and active HCMV disease has been demonstrated, no causal relationship has been firmly established (33,44,45,283,284). Proposed mechanisms responsible for the immunopathology range from indirect, with HCMV stimulating increased MHC II expression on graft cells, to more direct, with the virus stimulating cross-reactive immune responses to cellular antigens (1,44,45,284). Anti-id antibodies corresponding to immunodominant antigens are one additional tool which may contribute to clarifying the complexities underlying these immunological phenomena.

The potential applications of anti-id as immunizing agents against infectious diseases are very versatile. Since the first suggestion that they might be practical in this way (223,224) most studies have focused on their use as direct surrogate immunogens. These would be substituted for conventional live vaccines when the latter might not be suitable, as for immunocompromised hosts, or acceptable, as for HCMV at the present time. And certainly under a variety of experimental circumstances anti-id have successfully induced humoral, cellular, and protective immunity. However, the distinctive nature of anti-id vaccines is now recognized to have potential beyond these initial boundaries, extending into areas that will likely attract increasing attention in the future. Ab2 have been utilized to modify the immune response, for example by inducing an antigen-specific response in hosts unresponsive to the nominal antigen (204,277,285). A recent paper described experiments in which monkeys did not mount a humoral immune response against a melanoma-associated proteoglycan antigen. However, when

they were immunized with xenogeneic mAb2 that appeared to mimic the antigen, a specific antibody response to the proteoglycan was induced and the Ab3 antibodies exhibited biological activity against melanoma cells in vitro (277). Perinatal vaccination with anti-id also shows promise, particularly for immunizing against bacterial polysaccharides such as those of Neisseria meningitidis, Haemophilus influenzae, and Escherichia coli (286,287). Furthermore, fetal immunity may be influenced via transfer of id and anti-id from maternal circulation, as recently demonstrated in studies of children born to mothers with the parasitic infections causing Chagas' disease and schistosomiasis (288); and in a model system of anti-id transfer from laying hens to embryos generating antibodies to BSA, the nominal antigen (289). It has also been suggested that anti-id vaccines may be particularly valuable against infectious agents that exhibit a high degree of antigenic variation. For example antibodies against sequentially induced influenza virus variants were found to express common id, encoded by similar germline V genes (290). In addition, a recent paper by Su et al. (249) demonstrated that Ab2 mimicking the lipid A region of bacterial lipopolysaccharide succeeded in inducing an antigen-specific Ab3 response in different species without a corresponding stimulation of endotoxin-associated side effects. These studies all affirm the unique possibilities that anti-id may offer for beneficial manipulation of the immune response against pathogenic organisms, in situations where a distinct advantage may be gained compared to other vaccine candidates.

Immunoassays are well established as practical, rapid tests for detecting many infectious agents in the clinical laboratory. Most immunoassays utilize

primary antibodies, either polyclonal or monoclonal, directed against specific epitopes of the infectious agent, and then measure the amount of primary antibody bound via a second antibody that is tagged with an easily identifiable marker. Despite the widespread success of this approach, effective immunoassays for certain infectious agents have been difficult to develop, encouraging the search for alternatives in the design of rapid immunological tests. With particular regard to HCMV, the development of an immunoassay as accurate and sensitive as traditional viral isolation techniques would be a genuine advance over existing technologies.

Despite the considerable interest in id and anti-id as potential vaccines, therapeutic agents, probes for receptors, and participants in immune regulation, there has been only minimal interest in employing anti-id for the improved diagnosis of infectious diseases (291,292). In view of the present and ongoing demands for better immunological assays for HCMV, we therefore sought to exploit the uniqueness of id/anti-id interactions for developing an ELISA specific for this virus. Particular details of immunoassay formats may be varied considerably, but the essence of an anti-id immunoassay lies in the conformational similarity between the anti-id and the target epitope for the Ab1, such that one may substitute for the other in binding to the Ab1 antibody. In an inhibition assay such as the one we developed, antigen present in the test sample is first allowed to bind to specific Ab1. Then subsequent interaction between the Ab1 and complementary anti-id will be blocked, compared to a control in which no antigen is present. The inhibition of binding can be quantitated and will reflect the concentration of antigen

present in the test sample.

Given the variety of existing immunological assays for infectious agents, what advantages might be expected from anti-id assays? First, there are many complex microbial antigens which are difficult to obtain in purified form. By generating anti-id antibodies, purified protein substitutes for these antigens can be utilized, and in the case of monoclonal anti-id, may be generated in quantity. Second, small antigens or haptens may have few exposed epitopes to any particular antibody, such that are not amenable to conventional sandwich immunoassays. And third, in certain configurations such as competitive assays, it may be possible to achieve greater sensitivity using anti-id antibodies than is otherwise possible (293).

The series I mAb2 of the present study were used to develop a rapid ELISA for detecting the prototype HCMV strain AD169 in normal urine (294). At the time this assay was set up it was unclear whether the mAb2 were Ab2β or Ab2γ. But in fact this distinction was quite irrelevant, since both categories of anti-id are equally effective in blocking the binding of Ab1 and antigen, interactions which form the basis of the assay. In our assay we were able to detect HCMV in urine in a concentration-dependent manner to a sensitivity of approximately 3×10^3 PFU/ml. These viral concentrations are relevant to those found in the urine of patients with active infection, which ranges from approximately 10^2 to 10^6 PFU/mL (295). Ironically, it is rather difficult to compare this level of sensitivity with that of other immunoassays reported in the literature, since most accounts have described the sensitivity of a new assay in relative terms with regard to other standard

techniques. However, a recent indirect ELISA described by Yamanaka et al. (296) does allow a comparison to be made. In this report 20 ml of virus-spiked urine was ultracentrifuged and the treated pellet applied to microtitre wells coated with a mAb specific for a 150 kDa viral protein, likely the pp150 basic matrix phosphoprotein. Binding of virus was detected by addition of human anti-HCMV serum, followed by conjugated anti-human IgG. This assay could accurately quantitate HCMV at concentrations of 10^4 PFU/ml or greater, and detected the virus at concentrations as low as 10^3 PFU/ml. Attempts to increase the sensitivity beyond these levels only resulted in higher non-specificity of control samples.

The anti-id ELISA for HCMV described herein has not yet been evaluated using clinical samples. However, given (a) the encouraging results obtained with detecting the laboratory strain of virus, (b) the need for improved immunoassays for HCMV, and (c) the potential advantages of exploiting id/anti-id interactions, further assessment of this ELISA will be undertaken. Moreover, the implications of these results are not limited to the detection of HCMV. Immunological assays utilizing these same principles may be applied to the measurement of other infectious agents, as has already been demonstrated in a recent publication from our laboratory (297).

Chapter 6. CONCLUSION

The studies described in this thesis and the resulting publications are the first account of anti-id antibodies (Ab2) related to the immune response to the gB complex of HCMV. This envelope glycoprotein is one of the most immunodominant constituents of the virus, and as such is the focus of considerable interest as a candidate for inclusion in future vaccines.

The goal of this study was to utilize the id network as a means of gaining insight into the immune response to HCMV. The major hypothesis of the research was that Ab2 could be generated that would mimic a gB neutralizing epitope sufficiently to function as a surrogate antigen for gB. Both syngeneic monoclonal and rabbit polyclonal Ab2 were developed against the highly neutralizing anti-HCMV murine mAb CMVB1 (Ab1), and two series of monoclonal Ab2 directed to paratope-associated id of Ab1 were successfully produced. However, the inability of the mAb2 to induce a detectable antigen-specific Ab3 response in mice led us to conclude that they were likely Ab2γ, rather than the internal image Ab2β we had sought. Nonetheless, the mAb2 were valuable in defining eleven distinct idiotopes in and around the paratope of the Ab1, confirming our current views with regard to idiotypic complexity.

On the other hand, polyclonal Ab2 purified from rabbit antiserum raised against mAb CMVB1 were capable of inducing a measurable anti-HCMV response in naive mice. The mouse Ab3 response was antigen-specific in that it bound to HCMV-infected cells and immunoprecipitated proteins of the gB complex. The Ab3 antisera also exhibited biological activity against HCMV, neutralizing viral infectivity

in vitro. The conclusions drawn from these experiments were that the polyclonal rabbit Ab2 sufficiently mimicked gB to function as a network antigen in this experimental system. Furthermore, a comparison of id profiles of the Ab1 and antigen-specific Ab3 could be made, utilizing the mouse mAb2. Results identified a recurrent id expressed in response to gB and the rabbit Ab2. They also indicated that different subsets of B cells were involved in the Ab1 and Ab3 responses, since only some id expressed on the Ab1 were also expressed on antibodies within the Ab3 population. These data suggested that subtle differences in Ab2 composition influence the Ab3 response, and that these distinctions should be taken into account in the selection of anti-id as therapeutic agents and/or vaccines.

The mAb2 were used to develop a novel anti-id ELISA for detecting HCMV in vitro. The assay was based on the ability of viral antigen to inhibit the binding of Ab1 to complementary mAb2. The ELISA detected a laboratory strain of HCMV in a concentration-dependent manner from 20 to 0.6 x 10^3 PFU/ml, with 50% inhibition at approximately 3 x 10^3 PFU/ml. These results not only demonstrated the promise of the assay for measuring HCMV, but also illustrated the potential of Ab2 antibodies as a basis for developing simple, rapid diagnostic tests for other infectious agents.

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APPENDIX:
LIST OF PUBLICATIONS AND ABSTRACTS DERIVED FROM THE CURRENT RESEARCH

A. Publications

1. Larose, Y., E.S. Tackaberry and B.R. Brodeur. 1991. Human Monoclonal Antibodies to Cytomegalovirus Recognize Viral Epitopes on the Surface of Virus-Infected Cells. Human Antibodies and Hybridomas 2:65-73.

2. Tackaberry, E.S., J. Hamel, Y. Larose, R. Kuhl and B.R. Brodeur. 1992. Monoclonal Anti-Idiotypes for the Rapid Detection of Human Cytomegalovirus. J. Virol. Meth. 40:175-182.

3. Brodeur, B.R., J. Hamel and E.S. Tackaberry. 1992. Anti-Idiotype Antibodies for the Diagnosis of Infectious Diseases. Can. J. Infect. Dis. 3:319-320.

4. Laferrière, C., R. Peeling, E.S. Tackaberry, J. Hamel, J.R. Dillon and B.R. Brodeur. 1993. A Novel Approach to the Laboratory Diagnosis of Chlamydia trachomatis Infections Using Monoclonal Anti-Idiotype Antibodies. J. Immunol. Meth. 163:123-131.

5. Tackaberry, E.S., J. Hamel, Y. Larose and B.R. Brodeur. 1993. Anti-Idiotypic Mimicry of a Neutralizing Epitope on the Glycoprotein B Complex of Human Cytomegalovirus. J. Virol. In press.

B. Abstracts

1. Larose, Y., E.S. Tackaberry and B.R. Brodeur. 1989. Heteromyelomas Secreting Human Neutralizing Monoclonal Antibodies Against Cytomegalovirus. Troisième Colloque de Biotechnologie de l'Université de Montréal. Montreal, Quebec.

2. Larose, Y., E.S. Tackaberry and B.R. Brodeur. 1990. Establishment of Hybridomas Secreting Human Monoclonal Antibodies Against Cytomegalovirus Virion Proteins. Canadian Society for Immunology. Mont Gabriel, Quebec.

3. Larose, Y., E.S. Tackaberry, S. Montplaisir and B.R. Brodeur. 1990. Generation of Human Monoclonal Antibodies to Cytomegalovirus Virion

Proteins. International Congress for Infectious Diseases. Montreal, Quebec.

4. Tackaberry, E.S., J. Hamel, Y. Larose and B.R. Brodeur. 1991. Syngeneic Anti-Idiotypes Against a Monoclonal Anti-Cytomegalovirus Antibody. Canadian Society for Immunology. Lake Louise, Alberta.

5. Larose, Y., E.S. Tackaberry and B.R. Brodeur. 1991. Human Monoclonal Antibodies to Human Cytomegalovirus Recognize Epitopes on the Surface of Virus Infected Cells. Third International Cytomegalovirus Workshop. Bologna, Italy.

6. Tackaberry, E.S., J. Hamel, Y. Larose and B.R. Brodeur. 1991. Induction of Anti-Idiotypes Against a Neutralizing Cytomegalovirus Antibody. Third International Cytomegalovirus Workshop. Bologna, Italy.

7. Tackaberry, E.S., J. Hamel, Y. Larose and B.R. Brodeur. 1992. Idiotypic Analysis of a Neutralizing Monoclonal Antibody to Human Cytomegalovirus. Federation of American Societies of Experimental Biologists (FASEB). Anaheim, California.

8. Tackaberry, E.S., J. Hamel, Y. Larose, R. Kuhl and B.R. Brodeur. 1992. Idiotype/Anti-Idiotype Antibodies: A Novel Approach for Diagnosis. Bio-Recognition: International Industrial Biotechnology Conference. Montreal, Quebec.