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Monoclonal Anti-Idiotypes Induce Neutralizing Antibodies  
to Enterovirus-70 Conformational Epitopes

A Thesis Submitted to the School of Graduate Studies  
University of Ottawa

In Partial Fulfillment of the Requirements for the  
Degree of Doctor of Philosophy  
Department of Microbiology and Immunology  
School of Medicine

by

James A. Wiley

 James A. Wiley, Ottawa, Canada, 1992



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## Abstract

The model pathogen used in the development of the anti-idiotypic antibodies produced in this project was enterovirus - 70. This virus is the causative agent of acute hemorrhagic conjunctivitis. In the past twenty-five years, this virus has been responsible for two worldwide pandemics of acute hemorrhagic conjunctivitis.

Monoclonal antibodies (MAbs), directed against the prototype enterovirus-70 strain, J670/71, were generated and characterized in order to produce monoclonal anti-idiotypic antibodies (MAB2s) for use as surrogate immunogens. Radio-immunoprecipitation and western immunoblot assays suggested that all the monoclonal antibodies recognize conformational epitopes on the virion surface. A neutralizing monoclonal antibody, MAb/ev-12, was selected for the production of MAB2s. Five MAB2s were selected for their capacity to inhibit the interaction of MAb/ev-12 with EV-70 in dot immunobinding inhibition and immunofluorescence assays. These five MAB2s also inhibited virus neutralization mediated by MAb/ev-12 suggesting that each recognizes a paratope associated idiotope. In competition enzyme immunosorbent assays, none of the five MAB2s recognized other neutralizing and non-neutralizing enterovirus-70 specific MAbs, thus demonstrating that the MAB2s were specific for private idiotopes.

Immunization with each of the MAb2s was carried out for the production of anti-anti-idiotypic antibodies (Ab3). All five MAb2s induced an immune response. Moreover, results suggested that they share idiotopes since MAb2:MAb/ev-12 binding could be inhibited by homologous as well as heterologous Ab3. Ab3 sera were shown to possess antibodies capable of immunoprecipitating <sup>35</sup>S-labelled viral proteins in the same manner as MAb/ev-12. Nine of fifteen mice immunized with MAb2s demonstrated Ab3 neutralizing activity specific for the prototype EV-70 strain, J670/71. The potential application of MAb2s to serve as surrogate immunogens for conformational epitopes is substantiated by the results presented in this report.

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## DEDICATIONS

This work is dedicated to my parents whose encouragement and support made the completion of my doctorate a reality.

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## LIST OF ABBREVIATIONS

- Å - angstrom
- Ab1 - 1st generation antibody against original antigen
- Ab2 - anti-idiotypic antibody made against Ab1
- Ab3 - anti-anti-idiotypic antibody made against Ab2
- Ab2 $\alpha$  - anti-idiotypic antibody, alpha type
- Ab2 $\beta$  - anti-idiotypic antibody, beta type
- Ab2 $\epsilon$  - anti-idiotypic antibody, epsilon type
- Ab2 $\gamma$  - anti-idiotypic antibody, gamma type
- Ag - antigen
- AHC - acute hemorrhagic conjunctivitis
- Ala - alanine
- BME - basal medium Eagle
- BSA - bovine serum albumin
- C<sub>H</sub> - constant region heavy chain
- C<sub>K</sub> - constant region Kappa light chain
- C<sub>L</sub> - constant region light chain
- C <sub>$\lambda$</sub>  - constant region lambda light chain
- °C - degrees centigrade
- CDR - complementarity determining region
- cox - coxsackievirus
- CPE - cytopathic effect
- D<sub>H</sub> - diversity segment heavy chain
- DMEM - Dulbecco's modified Eagle medium

DTH - delayed type hypersensitivity  
EIA - enzyme immunosorbent assay  
EV-70 - enterovirus-70  
F1 - 1st generation offspring cross  
F(ab) - fragment antibody binding monovalent  
F(ab)'2 - fragment antibody binding bivalent  
F(c) - fragment crystallizable  
FMDV - foot and mouth disease virus  
g - grams  
Glu - glutamine  
HAT - hypoxanthine aminopterin thymidine  
HBsAg - hepatitis B surface antigen  
HRV - human rhinovirus  
Id - idiotope  
IdI - private idiotope  
IdX - public idiotope  
Ig - immunoglobulin  
J<sub>h</sub> - junctional gene of heavy chain  
J<sub>k</sub> - junctional gene of kappa light chain  
J<sub>λ</sub> - junctional gene of lambda light chain  
K - kilodaltons  
M - molar  
MAb - monoclonal antibody  
MAb1 - 1st generation MAb against original antigen

MAb2 - monoclonal anti-idiotypic antibody against MAb1  
MAb/ev-1 - 1st MAb against enterovirus-70  
MAb/ev-11 - 11th MAb against enterovirus-70  
MAb/ev-12 - 12th MAb against enterovirus-70  
mg - milligrams  
MHC - major histocompatibility complex  
mM - millimolar  
mL - millilitres  
ng - nanograms  
nts - nucleotides  
P1 - P1 genomic region of picornavirus genome  
P2 - P2 genomic region of picornavirus genome  
P3 - P3 genomic region of picornavirus genome  
PBS - phosphate buffered saline  
PEG - polyethylene glycol  
PFU - plaque forming units  
RIP - radio-immunoprecipitation  
RT - room temperature  
SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel  
          electrophoresis  
S-S - disulphide bond  
TMV - tobacco mosaic virus  
ts - temperature sensitive  
Tyr - tyrosine

uCi - microcuries  
ug - microgram  
um - micrometer  
uL - microlitre  
UTP - uridine tri-phosphate  
UTR - untranslated region  
V<sub>H</sub> - variable heavy chain region  
V<sub>k</sub> - variable gene of kappa light chain  
V<sub>L</sub> - variable light chain region  
V<sub>λ</sub> - variable gene of lambda light chain  
VP1 - viral protein 1  
VP2 - viral protein 2  
VP3 - viral protein 3  
VP4 - viral protein 4  
VPg-pU - viral protein-uracil phosphate complex  
v/v - volume per volume  
w/w - weight per weight  
xg - times gravity

## CHAPTER 1

### INTRODUCTION

#### 1.1 Picornaviruses

Within the *Picornaviridae* family there are four genera: i) *enterovirus*, ii) *rhinovirus*, iii) *aphthovirus*, and iv) *cardiovirus* (Palmenberg 1987). The enteroviruses include the polioviruses, echoviruses, coxsackieviruses (cox), simian, bovine, porcine and human enteroviruses. The human rhinoviruses (HRV) are comprised of over 100 different serotypes. The aphthoviruses are made up of 7 serotypes of foot-and-mouth-disease virus (FMDV) which infect cloven hoofed livestock. The cardioviruses are exemplified by mengovirus and encephalomyocarditis virus.

##### 1.1.1 Virion Structure

The three major capsid proteins, VP1, VP2 and VP3 are those which show the least degree of sequence homology between picornaviruses. However, their secondary and tertiary structures are very similar. Each protein takes on a conformation referred to as an eight stranded anti-parallel  $\beta$  barrel. Eight sequences of amino acid residues within each protein take on a  $\beta$  sheet configuration. These sequences or strands are joined by loops which protrude from the virion surface. Each strand lies in an anti-parallel sense relative

to the next. The  $\beta$  sheets make up the core of the protein structure and are stabilized via hydrogen bonds connecting adjacent strands. The barrel denotes the tertiary conformation of the strands. It is wedged shaped and oriented such that the narrowest point is at the 5 or 3 fold axis of symmetry. The smallest structural unit of a virion shell is the protomer which contains 1 copy of each capsid protein. As there are 60 copies of each protein there are thus 60 protomers. Groups of 5 protomers are assembled to produce the next structural subunit - a pentamer. The assembly of these pentamers produces the characteristic icosahedral (5:3:2) symmetry of picornaviruses (Rueckert 1990).

The loop structures linking the anti-parallel strands protrude from the virion surface. They are hydrophilic, highly diverse in their amino acid sequence, and correspond to the location of neutralization epitopes. Of the neutralization epitopes mapped onto HRV 14 (Sherry et al. 1986), the Sabin strain of poliovirus serotypes 1, 2 and 3 (Minor et al. 1986) and FMDV (Kitson et al. 1990) at least half or more are reliant on protein folding and interactions for their structure. Such epitopes are conformational as opposed to continuous epitopes which are contingent on amino acid sequence only. Mapping studies on HRV 14 and poliovirus have demonstrated that a significant degree of structural homology

exists between these viruses despite a relatively low degree of amino acid sequence homology in their capsid proteins. This structural homology has been exploited in the construction of viable chimeric viruses. The exchange of analogous epitopes between two poliovirus serotypes (Yeates et al. 1991, Murray et al. 1988) or between two different viruses, ie; neutralization epitope I of HRV 14 inserted to the same site on poliovirus (Altmeyer et al. 1990) are examples of this.

X-ray diffraction studies on HRV 14 (Rossmann et al. 1985), the Mahoney strain of poliovirus type 1 (Hogle et al. 1985), and mengovirus (Luo et al. 1987) have provided high resolution images of the surface features of these viruses. These images have revealed the presence of a canyon structure on the surface of HRV 14 and comparable surface depressions on polioviruses and mengovirus. The canyon is located at a constant radius from each 5 fold axis of symmetry. The rim is lined by the VP1 and VP3 amino acid residues which comprise the loop extensions joining the anti-parallel strands. The depth of the canyon is approximately 25Å. The canyon width ranges from 30Å at the entrance to 12Å at the canyon floor. This would preclude penetration to the canyon floor by F(ab) arms of neutralizing antibodies since they are 35Å in diameter (Rossmann et al. 1985).

Evidence for the location of the viral attachment site on

the canyon floor has been produced in mutagenesis studies. Random and site directed mutagenesis of selected canyon floor residues gave rise to mutants possessing altered affinity for their cellular receptors (Colonno et al. 1988).

The loop extensions protruding from the virion surface contain hypervariable amino acid sequences which comprise the neutralization epitopes and which come under continual immunological surveillance. These residues are not crucial for viral attachment to cellular structures. Since viruses take advantage of cellular structures for binding purposes, their attachment sites must remain conserved to ensure their specificity. This necessitates shielding or camouflaging the viral attachment site from immunological selective pressures (Rossmann and Palmenberg 1988). For HRV 14, poliovirus and mengovirus, the rim structures bear the burden of these continual selective pressures. The narrow entrance which precludes the F(ab) arm, shields the amino acids of the canyon floor thus conserving the viral attachment site.

An important corollary in this strategy is the selection of a cellular structure capable of penetrating to the attachment site on the floor of the canyon (White and Littman 1989). In the case of the major HRV group the first structural domain of the intercellular adhesion molecule-1, a member of the immunoglobulin (Ig) superfamily, contains the viral

receptor site (Greve et al. 1989; Lineberger et al. 1990). The receptor molecule used by all serotypes of polioviruses is also a member of the Ig super-family (Mendelsohn et al. 1989). The actual receptor site is also in the first structural domain of this molecule (Koike et al. 1991). All of this evidence accounts for the structure-function relationship of the canyon features relative to viral attachment and immune surveillance.

Proposed mechanisms of picornavirus neutralization have included virion aggregation by crosslinking with antibodies, conformational changes induced by antibody binding, blocking access to the viral attachment site, and inhibiting the release of the viral genome (Dimmock 1984). Verification of one of these mechanisms as the choice method is unlikely. Antibody binding relative to execution of any of the above mechanisms is difficult. The binding of antibodies may likely end in a crippling effect on the virion merely reducing the probability of successful infection as opposed to total inactivation of the virion (Rueckert 1990).

## **1.2 ENTEROVIRUS-70**

### **1.2.1 Enterovirus-70 Genome**

Enterovirus-70 (EV-70) is a member of the genus *enterovirus*, family *Picornaviridae*. This virus contains a positive sense

ssRNA genome with a molecular weight of  $2.5 \times 10^6$  (Yamazaki et al. 1974). The four structural proteins are VP1(35K), VP2(28K), VP3(27K) and VP4(9K) (Esposito and Obijeski 1976). These proteins have also been referred to as 1A(VP4), 1B(VP2), 1C(VP3), and 1D(VP1) in accordance with picornavirus genomic organization (Arnold et al. 1987). The genome contains a 5' untranslated region (UTR) of 726 nucleotides (ntd), an open reading frame containing 6582 ntd, a 3' non-coding region of 82 ntd, a 3' poly A tract and a 5' VPg protein (Ryan et al. 1990).

The 5' UTR possesses the sequence features of an internal ribosomal entry site for translation purposes. This site includes RNA secondary structures similar to those found in poliovirus and cox-A21. This region is homologous with other picornavirus 5' UTRs. In hybridization studies using a variety of enterovirus complementary DNA genomic probes, only the 5' UTR of EV-70 was recognized (Auvinen et al. 1989).

The genetic homology of the EV-70 P1 region with other enteroviruses is not restricted to one particular enterovirus. The VP4 protein is the most conserved of the capsid proteins and shows the greatest degree of similarity with polioviruses and cox-A21. The three principal capsid proteins are most homologous with bovine enterovirus (Ryan et al. 1990). Like other picornaviruses, the core sequences of these proteins,

which are responsible for the characteristic eight stranded anti-parallel  $\beta$  barrel, exhibit a higher degree of conservation than the connecting loop sequences. The P2 and P3 regions are most homologous with cox-B1, B2 and B3 serotypes. The 3' non-coding region possesses conserved sequences which are likely involved in stem-loop structures. Compensating mutations in the ntd of this region are likely for the purpose of maintaining these secondary structures.

### 1.2.2 Replication of Enterovirus-70

The replication of EV-70 is unique since it is the only enterovirus known to be a natural temperature sensitive (ts) mutant. The optimal in vitro growth temperature is 32°-34°C. Under normal in vitro conditions, a single growth cycle is completed in approximately 7 hours (Takeda 1989). This includes a two hour latent period presumably required for adsorption, penetration, and uncoating of the virus. In an effort to deduce the mechanism of this ts mutation, temperature shift experiments were carried out at permissive (33°C) and non-permissive (39°C) temperatures (Takeda et al. 1982). If the shift to 39°C occurred within the latent period, no viral RNA synthesis was noted. If this shift to 39°C occurred following the latent period, then viral RNA synthesis gradually declined and ceased to exist 1 hour later. Within

2 hours of returning to 33°C, viral RNA synthesis returned to its maximal rate. It was also noted that 6-7 hours post infection, at either 33°C or 39°C, host cell protein and RNA synthesis were shut off. In [<sup>35</sup>S]methionine pulse labelling experiments at 39°C, all of the viral protein products could be detected except VP2 and VP4. The detection of most viral proteins at 39°C implied that viral RNA translation and processing of the polypeptide were taking place by use of the infecting genome. The lack of viral RNA synthesis in spite of active viral protein synthesis at 39°C suggested that the ts defect lay in the transcription of the viral RNA. The use of [<sup>32</sup>P]UTP demonstrated that the 5'VPg-pU complex was produced at 33°C but not at 39°C. Previous research had already shown that elongation of nascent viral RNA strands was not inhibited at 39°C, implying that the defect may lie in the initiation of viral RNA synthesis (Miyamura et al. 1984). These results suggested that the ts defect involved the synthesis of the 5'VPg-pU complex which may play a role in the initiation of viral RNA transcription. Evidence from poliovirus studies have supported the role of the 5'VPg-pU as a primer for viral RNA transcription (Takeda et al. 1986). It is of interest to note that the ts replication of EV-70 restricts its in vivo tissue tropism to a moisture laden environment which is maintained at temperatures lower than the body's core temperature of 37°C

ie; the eye. In addition, despite its ts replication, EV-70 shuts down host cell protein synthesis by the same mechanism as seen with other enteroviruses (Lloyd et al. 1988).

### **1.2.3 Etiology of Acute Hemorrhagic Conjunctivitis**

The disease caused by EV-70 is known as acute hemorrhagic conjunctivitis (AHC). This infection involves a variety of symptoms which include subconjunctival hemorrhage, preauricular lymph node enlargement, ocular pain, foreign body sensation and transient corneal epithelial keratitis (Uchida 1989). The incubation period is approximately 24 hours. The duration of the infection is generally 7 days (Mirkovic et al. 1973). AHC is also caused by another enterovirus, cox-A24. The symptoms of AHC caused by these two viruses are clinically indistinguishable. However, the serology of these viruses differs greatly and there exists little genetic homology between them. Thus differentiation of EV-70 and cox-A24 AHC can be made on these grounds (Natori et al. 1984).

In addition to the ocular symptoms of AHC, neurological sequelae have also been reported, the incidence of which has been estimated to be as high as 1/10000 AHC cases (Hung 1989). The symptoms of these neurological sequelae are similar to those observed after central nervous system inoculations of attenuated poliovirus into non-human primates (Uchida et al.

1989). These symptoms were classified as spinal, cranial or a combination of both (Wadia et al. 1983). The spinal form is characterized by acute asymmetrical flaccid motor paralysis of one or more limbs followed by atrophy of the affected muscles. The cranial form involves acute motor cranial nerve palsy (Vejjajiva 1989).

Diagnosis of EV-70 has relied primarily on serological evaluation of acute and convalescent phase sera. Successful virus isolations have come from conjunctival swabs taken within 4 days of the onset of symptoms (Nakazono and Kondo 1989). No virus has been isolated from cerebral spinal fluid.

Therapeutic treatments such as zinc-chloride ( $ZnCl_2$ ) application and the use of arildone and flavone have been suggested. Zinc ( $Zn^{+2}$ ) is known to block post-translational cleavage of poliovirus proteins. Arildone blocks the uncoating step of poliovirus and is known to act in the same manner on EV-70. Flavone inhibits a step in picornavirus replication for which EV-70 seems to be very susceptible in vitro (Yamazaki and Miyamura 1989). The use of interferon has also been proposed based on its efficacy in in vitro testing (Stanton et al. 1977). Neutralizing monoclonal antibodies (MAbs) may also provide a viable biological alternative. All of these therapies are faced with the lack of an adequate EV-70 animal model in which to test their efficacy.

#### 1.2.4 Epidemiology of AHC due to Enterovirus-70

The transmission of EV-70 is reliant on a variety of factors. Population centers which are overcrowded and share substandard sanitary facilities and common fomites, are at risk for acquiring EV-70 AHC (Reeves et al. 1986). Factors such as high relative humidity, the retention of moisture on fomites and body perspiration on hands are thought to aid virus survival outside of its host (Sattar et al. 1988). It has been suggested that these conditions favouring virus transmission and survival have resulted in the confinement of EV-70 to the AHC belt of the world (Hierholzer and Hatch 1985).

Since 1969, two major pandemics of EV-70 AHC have occurred. The first of these in 1969-1971 and the second from 1980-1981. The start of the 1969-1971 pandemic was coincident with the first recording of EV-70 AHC (Chatterjee et al. 1970). From its origin in Ghana, EV-70 AHC spread through central Africa and along the coastal regions of north Africa. AHC then appeared on the Indian subcontinent, followed by Southeast Asia, Hong Kong and Japan by 1971 (Kono 1975). From 1972 to 1980 sporadic epidemics were reported throughout the eastern hemisphere. By early 1980 it was evident from the increasing number of cases that a second pandemic was developing. This

pandemic involved those regions of the world afflicted in the first pandemic and, additionally, the countries of the Caribbean basin. This represented the first reported incidence of EV-70 AHC in the western hemisphere. Since 1981, only sporadic outbreaks similar to those of the inter-pandemic years of the 1970's have been reported. —

The evolution of EV-70 can be traced back to its first reported cases in 1969. Genetic homology studies have prompted researchers to postulate that the evolution of EV-70 has involved recombination events between human and/or animal picornaviruses. Recombination events involving a copy choice mechanism amongst trivalent oral poliovirus vaccine strains have been noted as well as between vaccine and wild type strains (Stanway 1990). Evidence supporting this evolution includes livestock herds and some poultry flocks found to possess neutralizing antibodies against EV-70 prior to and following both EV-70 pandemics (Kono et al. 1981, Sasagawa et al. 1982). EV-70 has also demonstrated a much wider in vitro host range than any other enterovirus. Replication with or without cytopathic effect (CPE) has been detected in human, rabbit, bovine, mouse, hamster and porcine cell lines (Yoshii et al. 1977). These findings have supported the evolution of EV-70 from other picornaviruses whose susceptible host range is still reflected by that of EV-70.

The evolution of EV-70 has been monitored using isolates obtained throughout the world since 1969. Serological evidence demonstrated that different strains of the virus are associated with either the first or second pandemic. Isolates obtained prior to 1975 were neutralized by antisera against the reference strain J670/71. Those obtained after 1976 were neutralized by antisera against the V1250 strain. A 4- to 16-fold lower neutralizing antibody titre was noted when using the J670/71 reference antisera against post 1976 isolates (Hierholzer et al. 1984). Oligonucleotide mapping of EV-70 isolates has supported a consistent rate of evolution based on a nucleotide substitution rate of  $1.83 \times 10^{-3}$ /base/year (Miyamara et al. 1986). These researchers were able to estimate the time of emergence of EV-70 to 1967<sup>+15</sup> months. The evolution of EV-70 since its emergence has resulted in the development of a population pool. Isolates which develop a selective advantage would emerge from the pool as dominant strains. This would account for the association of EV-70 isolates to specific geographic areas and/or outbreaks.

### **1.3 IMMUNOGLOBULINS**

#### **1.3.1 Immunoglobulin Germline Genes and Diversity**

The Ig molecule is one of the body's most versatile components. Its involvement in homeostasis is manifest by its

roles in intercellular communication, defense against pathogens and the detection and removal of foreign compounds and tissues. Each function of which this molecule is capable is unique to a given structural feature on the Ig. These structures are encoded in the germline repertoire of the heavy and light chain Ig gene pool. The murine heavy chain germline pool consists of upwards of 500 variable heavy ( $V_H$ ) segments, 15 diversity ( $D_H$ ) segments, and 4 junctional heavy ( $J_H$ ) segments followed by 8 constant heavy ( $C_H$ ) region segments responsible for the isotype. The light chain gene pool is made up of lambda ( $\lambda$ ) and kappa ( $\kappa$ ) light chain genes. The Kappa gene pool has approximately 200 or more variable segments ( $V_K$ ), 4 functional junction segments ( $J_K$ ) and one constant segment ( $C_K$ ). The lambda murine germline gene pool consists of 2 variable segments ( $V_\lambda$ ), and four clusters of junctional-constant gene pairs ( $J_{\lambda 1-4} - C_{\lambda 1-4}$ ) (Hunkapiller and Hood 1989). These multiple segments contribute to the overall germline diversity of the Ig. The recombination of gene segments is another factor accounting for diversification of the Ig. A third factor which plays a role in Ig diversity is recombinational inaccuracies at the junction of these segments. It has been noted that although sacrificing recombinational efficiency has permitted additional diversity, it also results in the production of non-productive genes. The

random addition of non-encoded ntd to free DNA ends at the recombination sites, by deoxynucleotide transferase, accounts for this N region diversity (Hunkapiller and Hood 1989). The joining of one of the eight  $C_H$  genes adds a final factor to the diversity of the Ig prior to antigen (Ag) exposure. Somatic mutations in the newly rearranged genes, following Ag exposure, will greatly influence the diversity of the Ig.

### 1.3.2 Immunoglobulin Structure

The Ig molecule is composed of two light and two heavy chains. Within each of these chains are the characteristic Ig subunit domains: two in each light chain and four in each heavy chain. Each domain has approximately 110 amino acids and is maintained by the presence of an intrachain disulphide bond (S-S). Two light chain - heavy chain pairs are held together by numerous S-S bonds. A hinge region between the 1st and 2nd  $C_H$  domains permits a degree of flexibility between the F(ab) and F(c) regions.

Each domain structure is such that 7 anti-parallel polypeptide strands line a barrel-like structure. Three strands line one side and four strands line the other. The strands fold back on themselves and the amino acid backbone is in the  $\beta$  conformation. The interior of each domain is hydrophobic as a result of the amino acid side chains

extending into the pocket. The amino acid sequences connecting these antiparallel  $\beta$  strands form loops extending from the core of each domain. The domains of the  $V_L$  and  $V_H$  chains lie juxtaposed to each other with the back side of their 3-stranded  $\beta$  sheets facing each other. The domains of the  $C_H$  and  $C_L$  chains lie in a similar fashion with their 4-stranded  $\beta$  sheets facing each other. The polypeptide strand connecting the  $V_L$  with the  $C_L$  domain and the  $V_H$  with the  $C_H$  domain is flexible to allow the rotation of one domain relative to the next. The association of the variable region domains is less restricted than that seen for the constant region domains. In the variable region this association forms a hydrophilic pocket which functions as the paratope, F(ab) site. The pairing of the constant region domains results in the formation of a hydrophobic pocket which anchors the F(ab) site (Hasemann and Capra 1989).

Within both the  $V_H$  and  $V_L$  chains, there are three amino acid sequence regions of hypervariability. These three regions, referred to as complementarity determining regions (CDR) I, II and III, are found on the loops connecting the  $\beta$  strands within each domain. The conserved  $\beta$  strands make up the framework sequences of the variable regions. CDR-I and CDR-II of both  $V_H$  and  $V_L$  chains are encoded within the variable gene of the germline gene sequence. The CDR-III region of both

chains is encoded by the nucleotide sequences involved in the splice sites of the germline genes ie;  $V_L-J_L$ ,  $V_H-D$  and  $D-J_H$ .

### 1.3.3 Immunoglobulin Idiotopes

In a surveillance capacity, the Ig molecule has a dual character. It is capable of recognizing molecules via its F(ab) site and of being recognized by other Igs via its own surface markers (Hiernaux 1988). Isotypic markers characterize the F(c) region of each molecule, ie, IgG1, IgA or IgM. A second form of marker, allotypic markers, occurs as variations of the  $C_H$  regions. Allotypic markers are inherited, shared by various members of the species, and can be detected by immune serum from other members of the same species. A third marker found on Igs is the idiotope (Id). Idiotoxes are confined to the variable region of Igs or T-cell receptors. Idiotoxes can be unique to a clone of lymphocytes or shared amongst a selective few clones.

Idiotoxes act as antigenic determinants in the variable region of Igs. They are located in either the framework or paratope and may overlap both regions in the Ig. There are 15-20 Ids within a variable region. Each serves as a phenotypic marker (Kennedy and Chanh 1988) and together they make up the idiootype of the molecule (Hiernaux 1988). The peaks of amino acid sequence hypervariability corresponding to the location

of the CDRs also agree with the location of most Id determinants. It has been noted that more Id determinants are present on an Ig than there are CDRs. This implies the existence of overlapping Ids (Köhler et al. 1989).

The location of idiotopes can be predicted from surface flexibility, hydrophilicity, and accessibility studies. They are usually formed by the association of adjacent heavy and light chain amino acid sequences of CDR and framework residues (Poskitt et al. 1991b). Exceptions have been noted in which Ids were found on either one or the other chain. In the case of Id62, borne on a murine auto anti-thyroglobulin antibody, the Id determinant is expressed on the  $V_H$  chain independent of any association with the  $V_L$  chain (Zanetti and Sollazzo 1990). Idiotoxes have also been located on  $V_H$  chains of isolated human antibodies against hepatitis B surface antigen (HBsAg) (Anderson et al. 1992). A group of unique murine idiotopes from the  $V_{\lambda}21$  family have been shown to be associated only with the light chain (Voss et al. 1992).

Classification of Ids can be based on their association with one or more Ig chains, as described above, or by their uniqueness within a species or according to their location relative to the paratope. Their uniqueness is defined according to an idiotope being private or public.

Private idiotopes (IdI) generally mark sites of somatic

mutation events following Ag stimulation (Greenspan 1989, and Bona 1990). Germline gene products, public idiotopes, are more likely to be conserved throughout and/or between species and to be associated with CDR-I and CDR-II regions rather than the CDR-III region. The source of diversity of CDR-I and CDR-II is restricted to the multiple copy germline repertoire of the  $V_H$  and  $V_L$  genes and to somatic mutation. The source of diversity for the CDR-III region includes the multiple copy germline repertoire of the  $V_H$ ,  $D$ ,  $J_H$ ,  $V_L$ , and  $J_L$  genes, junctional and N region diversity, and somatic mutation (Manser 1989). As a result of these diversity mechanisms being concentrated in this region, quite often no recognizable germline gene segments remain (Hunkapiller and Hood 1989). The CDR-III region is more likely to be of greater influence in determining the idiotype of the Ig (Davie et al. 1986) than the CDR-I, CDR-II and framework regions. Since Ag driven somatic mutation drives affinity maturation, it is reasonable that selection pressures skew the manifestation of expressed mutations and the resulting new Ids towards those associated with increased affinity. Thus, up to and during primary immune responses, public idiotopes (IdXs) will dominate the idiotype. In subsequent memory based responses, IdIs will become increasingly relevant. Somatic evolution produces a mutation frequency ten times greater in the CDR regions than in the

framework region. An estimated mutation rate of  $10^{-3}/V$  region base pair/cell division has been calculated for the CDR regions. It has been shown that antibodies expressing N region alterations have affinities 20 fold greater than those expressing the germline canonical sequences (Manser 1989). As these higher levels of affinity are achieved during maturation, Id changes become less frequent. Changes beyond those following the attainment of maximal affinity will be due to random somatic mutations at locations which do not diminish the level of affinity (Poskitt et al. 1991b).

Public idiotopes are expressed on clones of given individuals of an outbred species or all individuals of an inbred species. This is exemplified in the case of a major cross reactive idiotope associated with the antibody response to *Haemophilus influenzae* b polysaccharide (Lucas and Granoff 1990). Public idiotopes are inherited in Mendelian fashion and can be detected by the sera of individuals from different species (Poskitt et al. 1991b). Considerable Id overlap is known to exist between disparate species. Human anti-idiotypic antibodies are known to readily recognize markers on bovine and murine Igs, demonstrating shared interspecies paratope-associated Ids (Roux and Tankersley 1990). It has been postulated that evolutionary conservation of paratopes and their associated overlapping Ids on protective Igs have in

some cases conferred a survival advantage to different species (Lucas et al. 1991).

Classification of Ids according to their location relative to the paratope is based on the ability of an anti-idiotypic antibody to compete with an Ag for binding to the Ag-specific antibody. If the Id is distal relative to the paratope such that both an anti-idiotypic antibody (Ab2) and Ag can simultaneously bind to the antigen specific antibody (Ab1), then the Ab2 is referred to as Ab2 $\alpha$ . These Ids are generally framework related. If the Ab2 binding to its Id is inhibited by the presence of Ag, then the Ab2 is an Ab2 $\beta$ . Ab2 $\beta$  antibodies are said to possess the internal image of the Ag and thus bind to the paratope of the Ab1 (Jerne et al. 1982). Jerne's classification has been subsequently modified. A new designation, Ab2 $\gamma$ , referred to antibodies whose binding to Ab1 was Ag inhibitable but they did not possess the internal image of the original Ag. Their corresponding Ids lay adjacent to or overlapped with the Ab1 paratope (Bona and Köhler 1984). A fourth, but somewhat obscure designation, Ab2 $\epsilon$  or epibody, is associated with rheumatoid factor and autologous antibodies.

#### **1.3.4 Network Theory**

The duality of the Ig molecule is illustrated by its role

in the network theory in the regulation of the immune system (Jerne 1974). In this theory, the immune system is viewed as a complex web of paratopes that recognize sets of Ids and vice versa. The theory states that for every exogenously derived antigenic epitope, there exists within the immune system an endogenously derived replica. While the system is at rest, homeostasis is maintained by an equilibrium balance of suppressive and stimulatory factors communicating via paratope - idiotope interactions. The entry of an exogenous epitope into the system upsets the homeostatic balance to favour the production of stimulatory factors triggering the proliferation of antigen specific clones and their products. This response is seen as the system's means of restoring a level of homeostasis. As the system returns to equilibrium, it must adapt to the presence and concentration of antigen in the system and to the various idiotypes elicited due to the presence of antigenic variants. The homeostatic level must also permit the maintenance of a memory cell population following termination of the response (Roitt et al. 1981).

Control of the immune response via Id markers must account for the appearance of new or disappearance of old IdI and IdX as Ig affinity evolves with the antigen. Partial or complete Id change while maintaining antigen specificity (Ag', Id'), allows a clone to temporarily escape network regulation until

the system can adapt to the new idiootype (Poskitt et al. 1991b). Likewise, production of an unrelated Ig still sharing Ids (Ag<sup>-</sup>, Id<sup>+</sup>), following paratope alterations during affinity maturation, will continue until it is detected. The network system also allows for the replacement of a suppressed clone by another sharing antigen specificity but not idiootype (Ag<sup>-</sup>, Id<sup>-</sup>) (Roitt et al. 1981). A corollary of this is the presence of multi-specific antibodies, as in the neonatal repertoire which has yet to develop a network of high affinity Igs (Köhler et al. 1989). These multi-specific Ig populations diminish with time as specificity and affinity mature with antigen exposure.

The use of anti-idiotypes as internal images of foreign antigens has suggested to some that the immune system has evolved from a primordial mechanism used in the control of macromolecular and cellular homeostasis (Bona 1990). Idiotoxes utilized in such a manner should also be able to mimic self antigens to regulate the presence of non-self markers, ie; tumour antigens, markers on degenerating cells. In view of the potential size of the Id repertoire, it is difficult for the immune system to avoid self-recognition (Urbain 1990). However, auto-aggressive antibodies responding to normal self-Ids are generally absent from the immune system. A regulatory role within the network system involving idiotypic markers on

T-cell antigen receptors for the induction of suppression and/or amplification signals directed to antigen specific B cell clones has been postulated (Poskitt et al. 1991b, Kennedy 1991). Such a role may account for the absence of auto-aggressive B cell clones. A global or open concept network system can be interpreted to involve regulation of self-Ids on T and B lymphocyte receptors. At early stages of B and T cell ontogeny, negative signals targeted to B cells bearing self-Ids would result in clonal anergy (Urbain 1990). Development of the idiotypic network following these events would require a degree of adaptation to accommodate the internal image Ids of all subsequently encountered exogenous Ags and yet continue to suppress the activation of auto anti-idiotypic antibody production. The mechanism by which this regulation is maintained and its role at times of failure still remains ambiguous (Monroe and Greene 1986).

Evidence has recently been presented which supports the regulation of B cell activity via B-B cellular interactions. These interactions are mediated by direct idio-anti-idio receptor interactions in association with major histocompatibility complex (MHC) II molecules. It has been postulated from these results that B cells are capable, in a manner similar to that of T cells, to adaptively differentiate self from non-self markers (Bitoh et al. 1989). Further to

this, this B-B cellular interaction was shown to take place with membrane bound idiotype and anti-idiotype receptors. However, the precise molecular association of the Id receptors with the MHC II is still unclear (Bitoh et al. 1990).

### **1.3.5 Immunization with Ab2s.**

Application of the network theory pertaining to idiotypic regulation has shown that individuals can be immunized against an Ag without prior exposure to it. In limited instances, Ab2s which do not bear the internal image of the Ag have been used to prime the immune system for subsequent Ag challenge. These Ab2s are thought to recognize regulatory Ids associated with antigen specific clones. The induction of specific responses in unrelated individuals using non-internal image Ab2s is dependent on IdXs being associated with receptors on B cell clones. The induction of Ag specific immune responses by these Ab2s is more the exception than the rule. In general, these Ab2s are unable to cause maturation of the B cell clones (Ertl and Bona 1988). Their priming of the immune system manipulates the selection of specific B cell clones. Subsequent Ag exposure is needed for clonal maturation and the production of antibodies. The priming of an immune response using these Ab2s has, in some cases, lead to the expression of Ag specific B cell clones which do not share the same idiotype as those

clones elicited during a normal Ab1 response. These B cell clones have been referred to as silent clones. Under normal circumstances they are thought to; i) possibly be under active idiotype suppression which is not relieved upon Ag stimulation or, ii) represent rare B cell clones whose V region genes are not selected in Ab1 responses (Kennedy 1991).

Silent clone induction is typified by the murine response to bacterial levan, a bacterial cell wall polysaccharide. Antigen exposure at one month of age elicits a response devoid of A48 Id<sup>+</sup> Igs. Immunization of 1 day old mice with low doses of anti-A48 Ab2 followed by Ag exposure 1 month later resulted in a significant portion of the response being composed of A48 Id<sup>+</sup> Igs. This activation was specific for A48 Id<sup>+</sup> Igs as administration of unrelated antibodies or Ag did not elicit A48 Id<sup>+</sup> clones (Hiernaux et al. 1981). It has been speculated that by 1 month of age the dominance of alternative Ids was established and the A48 Id was effectively suppressed. Further investigation of this silent clone response showed that minute amounts of A48 Id<sup>+</sup> Ig administered at birth could also elicit A48 Id<sup>+</sup> clones following Ag exposure. This activation of A48 Id<sup>+</sup> clones was linked to the expansion of A48 Id specific helper T cells as shown by the requirement for T cells in the anti-levan response following their transfer into lethally irradiated mice. Helper T cells are thought to fine tune Id

expression via the selection of specific B cell precursors for expansion (Rubinstein et al. 1982). Recent speculation on the role of helper T cells in the activation of unresponsive clones was made regarding the melanoma associated antigen MPG. The anti-MPG Ab1 did not recognize the Ag in situ. However, an Ab2 made from anti-MPG Ab1 induced anti-anti-idiotypic antibodies (Ab3) which did recognize the Ag in situ. The different epitope specificities of the Ab1 and Ab3 could possibly be attributed to the induction of T cell help by a network antigen (Chattopadhyay et al. 1992). Induction of silent clones has been shown to cross the species barrier. A murine Ab3 response to a rabbit MAb2 made against rabbit anti-tobacco mosaic virus (TMV) Ab1 produced Ab3 idiotypes identical to that seen for the rabbit Ab1 response but not normally seen in the Ab1 murine anti-TMV response (Francotte and Urbain 1984).

### **1.3.6 Ab2 $\beta$ Properties**

The exploitation of the network theory is best exemplified by the use of the internal image bearing Ab2 $\beta$  antibody in the induction of an Ag specific immune response. A variety of criteria establishing the properties of Ab2 $\beta$ s have been proposed. The ability to inhibit the binding of Ag to Ab1 is an initial indication of an Ab2 $\beta$ . This indication is

reinforced in cases where the Ab1 and Ab2 $\beta$  are derived from alternate species. In theory, Ab2 $\beta$ s should also recognize any accessible receptors used by the original antigen (Nisonoff 1991). By recognizing the receptor, the Ab2 $\beta$  should also be able to inhibit attachment of the antigen to the receptor.

Structural criteria are presented in terms of amino acid sequence homology, three dimensional spatial homology and the disposition of critical contact points common to both the Ag and the Ab2 $\beta$ . Amino acid sequence homology is limited to protein Ags only. Such homology has been demonstrated in the reovirus type 3 model and in the copolymer antigen Glu-Ala-Tyr model (Bruck et al. 1986, Ollier et al. 1985). Three dimensional homology and the resulting number and disposition of critical contact points are far more relevant in assessing internal image criteria despite the possible dissimilarity of antigen epitope and idiotope composition, ie; protein or carbohydrate or lipid epitopes vs protein idiotopes. The Ab2 $\beta$  contact points consist of amino acid residues or relevant portions of their side chains. The presentation of the charge patterns of these contact residues result in the formation of ionic, hydrogen, and Van der Waals type bonding at the paratope-idiotope interface (Bentley et al. 1990). These charge patterns mimic those presented by the 'energetic epitope subset' of the original epitope (Laver et al. 1990)

in both spatial and functional properties. The multivalent nature of the bonding contributes to the binding affinity and subsequent stimulation of an immune response. Optimizing the number and disposition of these contacts is accomplished by the spatial arrangement of the hypervariable loops within the paratope associated idiotope of the Ab2 $\beta$ . These factors significantly influence the structural and functional mimicry of an epitope by an Ab2 $\beta$  internal image Id (Roitt et al. 1985).

The functional criteria upon which an Ab2 $\beta$  is considered to possess an internal image is its ability to mimic the immunological properties of the original Ag. The induction of an Ag specific immune response in a xenogeneic animal model, in addition to satisfying structural criteria, provides strong evidence that the Ab2 possesses a true internal image Id (Nisonoff and Lamoyi 1981). This assumes that a xenogeneic species will respond to the Ab2 $\beta$  and that the response will be similar to that seen against the original Ag. The generation of a xenogeneic Ag specific response is based on cross-species Ids associated with critical Ag specific paratopes of different genetic origin (Davie et al. 1986). In a xenogeneic model the entire surface of the Ig is immunogenic. Thus only a small portion of the Ab3 response is directed against the Id of interest (Poskitt et al. 1991a). While detection of an

Ag specific response following anti-idiotypic immunization is preferable, it is not a universal criterion. A variety of criteria must be evaluated in the classification of an AB2 $\beta$  (Ertl and Bona 1988). These criteria may include: i) inhibition of Ag:Ab1 binding, ii) recognition of Ag specific xenogeneic Ab1, iii) recognition of cellular receptor and inhibition of Ag binding to it, iv) mimicry of the structure and biological activity of the Ag, and v) elicitation of an Ag specific response in a xenogeneic model.

A recently proposed revision of the network theory suggests the abandonment of the Ab2 $\alpha$ , Ab2 $\gamma$  and Ab2 $\beta$  classification as well as the concept of the three dimensional internal image Id (Köhler et al. 1989). In its place, Köhler proposes a network antigen concept in which no reference to conformational homology between antigenic epitope vs network idiotope is made and the inhibition of Ab1-Ab2 $\beta$  binding by Ag is not a condition. In the absence of these criteria, total reliance on the results of biological in vivo response experiments are the only means of assessing the efficacy of anti-idiotypic antibodies in immune therapy.

### **1.3.7 Ab2 $\beta$ as Surrogate Vaccines**

The use of anti-idiotypic antibodies as surrogates for a variety of molecules is based on the internal image idiotope

concept. The application of interest is that of a surrogate vaccine. The principle of vaccination has been applied successfully in several experimental models involving viral, bacterial, and parasitic infections. The induction of an anti-HBsAg immune response in chimpanzees using a rabbit anti-idiotypic vaccine (Kennedy et al. 1986) is of particular interest since chimpanzees and humans are the only species susceptible to hepatitis B virus. In this system, the vaccine induced long term protection against a hepatitis B virus challenge, produced an Ab3 response that serologically mimics the human anti-HBsAg Ab1 response, and crossed species lines (Kennedy and Attanasio 1990).

The mimicry of selective epitopes by anti-idiotypic vaccines is of benefit in the following cases: i) if the epitope in question is difficult to obtain, ie; the culture of fastidious pathogens - protozoa and leprosy, ii) where a characterized protective antigen is not available (Pohl et al. 1992), iii) mimicry of a protective epitope for the induction of an immune response without risking the elicitation of a cross-reactive auto-immune response resulting from the presence of additional epitopes (Dreesman and Kennedy 1985), iv) in cases which place an individual at risk, ie; reversion of avirulent vaccine strains, the presence of residual pathogens following inactivation, and v) situations where

handling of lethal toxins or pathogens has precluded vaccine development, ie; anti-idiotypic vaccine to trichothecene mycotoxin T2 (Chanh et al. 1991).

This vaccine approach has definite advantages over other alternative approaches being investigated. Synthetic peptide and recombinant DNA vaccines are able to mimic linear peptide epitopes. However, mimicry of conformational epitopes presents problems for these two approaches. Anti-idiotypic vaccines are able to conform to the three dimensional configuration of linear and conformational epitopes. In addition, most protective epitopes on bacteria and various tumour associated antigens involve non-protein moieties which these other approaches are incapable of accommodating. The composition of the antigenic epitope is immaterial in the case of anti-idiotypic vaccines.

Like any vaccine, anti-idiotypic vaccines must induce T and B cell immunity as well as establish an Ag specific memory cell subset. The means by which T cell activation occurs within the idiotope network is vague (Lambert 1986). Coupling of the Ab2 $\beta$  to a carrier such as keyhole limpet hemocyanin or lipopolysaccharide to induce a T cell dependent response has proven advantageous relative to relying on T cell independent responses (Uytdehaag et al. 1986). Recognition by Ab2s of Id markers on B and/or T cell surface receptors, followed by

processing and presentation of the Ab2 with MHC II to antigen specific T helper subsets, results in the necessary amplification signals for the stimulation of B cell clones (Roitt et al. 1981). However, presentation to T suppressor subsets is a possibility which in turn may trigger the suppression of B cell proliferation. Ab2 induction of T cells has been demonstrated in an Ag specific delayed type hypersensitivity (DTH) response using a Sendai virus - murine model (Ertl et al. 1984). In a reovirus - murine model, Ab2 $\beta$  elicited a localized DTH reaction whereas injection of the hybridoma cells bearing the same Ab2 $\beta$ s as surface receptors in association with MHC I elicited a cytotoxic T cell response (Sharpe et al. 1984). In addition, it is possible to recruit T cells via Ab2 recognition of shared T cell idiotopes for the production of antibodies of differing specificity (Lambert 1986). Processed Ab2 peptides mimicking tumour antigens presented on the surface of antigen presenting cells in context with MHC II have induced a cytotoxic T cell response (Kennedy 1991).

For use as a surrogate vaccine, Ab2 $\beta$  must: i) possess a three dimensional arrangement homologous with the Ag epitope so as to mimic the disposition of critical contact points, ii) induce the equivalent T and B cell clones and protective response as induced by the Ag epitope despite the fact that

the Ag and Id will enter the idiotypic network at different locations, iii) have an affinity for the Ab1 equal to that of the antigen, iv) induce a protective immune response in a xenogeneic species, and v) bind the Ab1 of a xenogeneic species (Hiernaux 1988). There are a number of instances in which some of these criteria are not met in spite of other evidence supporting the case of the Ab2 $\beta$  as a potential vaccine. The application of non-internal image Ab2 will be determined solely by the immunological response elicited.

As surrogate vaccines, anti-idiotypic antibodies have numerous beneficial applications. By exploiting regulatory pathways, it is possible to select idiotypic clones known to be highly protective or to select suppressive clones to shut down transplantation/graft rejection. Idiotypic manipulation will also apply to the enhancement of those clones which correlate to disease remission and to the suppression of those clones involved in disease progression.

An additional form of manipulation is the conversion of a normal thymus independent response to a normal thymus dependent response. In infants, the ontogeny of the immune response does not reach a level of maturity sufficient to produce a normal protective T cell independent response to bacterial polysaccharide epitopes until at least 2 years of age. However, T cell dependent responses can be elicited

within the first year of life. By mimicking a polysaccharide epitope with a protein Id thereby eliciting a T cell dependent response, it is possible to induce a protective response to a pathogen prior to maturation of the normal T cell independent response mechanism. This shift to a T cell dependent response also results in the earlier establishment of memory cell subsets than would be seen with a polysaccharide induced T cell independent response (Stein 1991, Roitt et al. 1985). Antibodies raised to capsular polysaccharide antigens would confer earlier immunity to infants against a variety of pathogenic bacteria such as *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Escherichia coli*.

The priming of a protective immune response using either idiotypic or anti-idiotypic immunization was first demonstrated in a neonatal mouse model challenge with *E. coli* K13. Administration of 1 ug of the protective Ab1 within 24 hours of birth or immunization within 12 weeks of birth with 50 ng of the anti-Id primed a protective response against bacterial challenge (Stein and Söderström 1984).

Anti-idiotypic vaccines have recently been successfully implemented in a murine model against the meningococcal group C capsular polysaccharide (MPC) of *N. meningitidis*. Mice immunized with the anti-idiotypic vaccine showed 100% survival

and significantly reduced levels of bacteremia 24 hours following bacteria challenge. The significance of this protection was more evident in the priming and subsequent immunization of neonatal mice with the anti-idiotypic vaccine. These mice showed 100% survival and total clearance of the bacteria 8 hours post challenge (Westerink and Giardina 1992). Anti-idiotypic vaccines have also been effective in murine models against *S. pneumonia* (McNamara et al. 1984) and lately for *Pseudomonas aeruginosa* (Schreiber et al. 1992).

The use of anti-idiotypic vaccines has been applied to a greater extent against viral diseases. In the reovirus type 3 system, an Ab2 $\beta$  recognized the cellular receptor and inhibited binding of the virus to susceptible cell lines (Co et al. 1985). This Ab2 $\beta$  was capable of eliciting a reovirus type 3 specific delayed type hypersensitivity reaction and the activation of specific cytotoxic T cells (Sharpe et al. 1984). The application of this Ab2 $\beta$  as a prophylactic vaccine demonstrated that the protective response elicited was specific and comparable to that seen using inactivated virus. In addition, it was shown that maternal immunization confers neonatal protection in a murine model (Gaulton et al. 1986).

Additional successful applications of anti-idiotypic viral vaccines have included murine coronavirus (Lamarre et al. 1991), human respiratory syncytial virus (Palomo et al. 1990),

poliovirus type II (Uytdehaag and Osterhaus 1985) and rabies virus (Reagan et al. 1983).

Numerous animal and human studies have demonstrated potential benefits in the use of anti-idiotypic vaccines for the treatment of cancers (Herlyn et al. 1990, Dunn et al. 1987). Anti-idiotypic vaccines are capable of targeting specific Ids on the surface of B lymphoid cells (Kwak et al. 1990) thus selecting them for destruction by the complement system or an antibody dependent cellular cytotoxicity response (ADCC) (Monroe and Greene 1986).

In addition to vaccine applications, these antibodies have been extensively used for predicting cellular attachment sites (Marriott et al. 1987), studying the structural features of pathogens (Sacks et al. 1985), and in pharmacology/physiology research (Sege and Peterson 1978, Hasemann et al. 1991, and Zenke et al. 1992).

As with all vaccines, anti-idiotypic immunizations are not without their draw-backs. The duality of these vaccines, ie: their use as surrogate immunogens and their role in immune regulation, is inseparable. The network system into which these vaccines are integrating is a complex communication web and various factors will influence the outcome of the idiotypic manipulation. Some of these factors are: i) the nature of the target idiotope (private or public), ii) the

nature of the anti-Id antibody (monoclonal or polyclonal, syngeneic, allogeneic or xenogeneic), iii) the use of adjuvants and coupling proteins, iv) the dose and regime of immunization, and v) the isotype.

The sources of these vaccines have been primarily animal models such as mice and rabbits. Therapeutic applications will require human derived anti-idiotypic vaccines to avoid inducing a response to foreign species markers. Such a response would diminish the efficacy of the vaccine in humans. The use of chimeric anti-idiotypic antibodies using human F(c) chains spliced to murine F(ab) chains may alleviate some of this problem. Genetic engineering has recently been used in the expression of a large combinatorial library of murine F(ab) fragments using a novel bacteriophage lambda vector system (Huse et al. 1989). The application of this technology to the human Ig repertoire has the potential to alleviate immune responses associated with the use of foreign or partially foreign Ig.

At present, there is more to learn than there is known about these vaccines.

**CHAPTER 2****STATEMENT OF OBJECTIVES**

The principal goal of this research project was the development of a specific enterovirus-70 immune response using anti-idiotypic antibodies. This goal was subdivided into three objectives according to the three generations of antibodies elicited.

The first objective was the development of a panel of enterovirus-70 specific monoclonal antibodies of which one or more were required to possess viral neutralizing capabilities. Characterization of these monoclonal antibodies was to be assessed for their potential use as diagnostic reagents.

The second of these objectives was the production of one or more anti-idiotypic antibodies directed against an enterovirus-70 neutralizing monoclonal antibody. These anti-idiotypic antibodies must inhibit the recognition of the virus by the EV-70 neutralizing monoclonal antibody.

The third objective was the development and assessment of anti EV-70 specific neutralizing sera in an in vitro model using the anti-idiotypic antibodies as surrogate immunogens. In this manner the applicability of anti-idiotypic antibodies as surrogate vaccines would be demonstrated by their ability to elicit a specific immune response without the subject ever having been exposed to the original antigen.

## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1 Cells and Viruses

Rhesus monkey kidney cells, LLC-MK2 derivative, (ATCC CCL 7.1) were used throughout this study for propagation of EV-70 and in the assays pertaining to the development of the antibodies produced. All strains of EV-70 used in this study were obtained from the U.S. Centers for Disease Control (Atlanta, GA.). These strains were KW97, R6, RU3875, 1604, V1250, and the prototype strain J670/71. All other picornaviruses were obtained from the Viral Products Division of the Bureau of Biologics, Health and Welfare Canada, Ottawa, Ontario.

#### 3.2 Virus Purification

T 180 cm<sup>2</sup> flasks (Nunc/Delta, Denmark) of LLC-MK2 cells approaching 100% confluence were infected with EV-70 at a multiplicity of infection of approximately 0.1. Twelve mL of basal medium Eagle (BME) (Gibco Laboratories, Burlington, Ont.) supplemented with 2mM L-glutamine (Sigma Chemical Company, St. Louis, MO) and bovine calf serum (1% (v/v) final concentration) (Hyclone Laboratories Inc., Logan, UT) was added to each flask. The cultures were then incubated at 33°C until +4 cytopathic effect was observed. (Cytopathic effect

was graded from +1 to +4 with +1 indicating initial signs of viral destruction of the cell monolayer to +4 indicating 100% destruction of the cell monolayer). The flasks were then frozen and thawed three times. The medium was pooled, centrifuged at 400 xg for 10 minutes at 4°C in a Damon/IEC CRU5000 centrifuge (IEC, Needham Heights, MA) and again at 12,000 xg for 20 minutes at 4°C in a Sorval SS-34 rotor, RC5C centrifuge (Sorval/Dupont, Wilmington, DE) to remove cellular debris. The supernatant was removed and centrifuged again at 113,000 xg at 4°C using a Beckman SW27 rotor and L8-70 ultracentrifuge (Beckman Instruments, Palo Alto, CA). The virus pellet was resuspended in a 0.5 g/mL cesium chloride (Mallinckrodt, Germany) solution and spun (20°C) for 24 hours at 255,000 xg in a Beckman SW 60Ti rotor (Beckman). Two hundred uL fractions were collected from the tubes, dialyzed against serum free Dulbecco's modified Eagle medium (DMEM) (Gibco) and tested for infectivity by plaque assay. Virus-containing fractions were stored at -70°C for future use.

### **3.3 Electron Microscopy**

Purified virus was dialyzed against distilled water, then dropped onto a formvar coated 400 mesh copper grid. The sample was then stained with 2% (v/v) phosphotungstic acid, pH 6.8, for 5 seconds. A Zeiss 10C electron microscope (Zeiss Canada

Ltd, Don Mills, Ont) was used to view the sample.

### **3.4 Animals**

BALB/c mice were obtained from the Charles River Laboratories (St. Constant, Quebec) at eight weeks of age. The (BALB/c X Swiss Webster) F1 mice used for ascites fluid production were obtained from the Animal Resources Division, Health Protection Branch, Health and Welfare Canada, Ottawa, Ontario.

### **3.5 Immunization of Mice with EV-70**

Pre-immune serum samples were taken from each mouse prior to immunization with purified EV-70. One-hundred uL of Freund's complete adjuvant:virus suspension was injected intraperitoneally. Subsequent injections were given every 3 weeks using Freund's incomplete adjuvant. Trial bleeds were taken one week after each boost and were tested by immunofluorescence and in plaque reduction assays to determine the EV-70 specific antibody titre. When the neutralization titre reached 1/4000, the mouse in question was given a final boost and exsanguinated 4 days later. The spleen was removed for use in a fusion experiment.

### **3.6 Fusion Procedure and Monoclonal Antibody Production**

The fusion procedure used was modified from one previously described (Brodeur et al. 1985). Following removal of the spleen, connective tissue was removed and the organ was teased apart to produce a cellular suspension. Spleen cells and the non-secreting plasmacytoma, SP2/0, were mixed at a ratio of 10:1 and washed in 10 mL of serum-free DMEM. The cell suspension was pelleted by centrifugation at 200 xg for 10 minutes at 20°C (Damon/IEC CRU5000 centrifuge). Two grams of polyethylene glycol (PEG) 1450 (Eastman Kodak, Rochester, NY) was melted at 56°C and added to an equal volume of serum-free DMEM. The pH was adjusted to 7.2, filter sterilized using a 0.22 um filter and placed at 37°C until its use. Following removal of the supernatant from the spleen-SP2/0 cells, the pellet was gently resuspended in approximately 100 uL of serum-free DMEM. One mL of the PEG:DMEM solution was added to the cell suspension over 45 seconds. Subsequent to this, 10 mL and 15 mL aliquots of serum-free DMEM, at 37°C, were added over a 10-minute and 6-minute period, respectively. The cell suspension was then incubated in a 37°C water bath for 20 minutes. The cells were then pelleted at 200 xg for 10 minutes (Damon/IEC CRU5000 centrifuge), resuspended in 10 mL of serum-free DMEM, counted and dispensed into 96-well Nunc tissue culture plates (Nunclon Delta) at  $1.5 \times 10^4$  cells/well. Each

culture plate had been seeded with a feeder layer of macrophages,  $10^3$ /well, at least 24 hours prior to the fusion experiment. The hybridoma and macrophage cell suspensions were dispensed and cultured in DMEM supplemented with 15% (v/v) bovine calf serum (Hyclone), 2mM L-glutamine (Sigma) and 50 ug/mL of gentamicin (Sigma) in the presence of hypoxanthine, aminopterin and thymidine, ( $5 \times 10^{-2}$  mM,  $2 \times 10^{-4}$  mM, and  $8 \times 10^{-2}$  mM final concentrations respectively) (HAT) (Sigma) as selective agents. Fourteen days after the fusion, the aminopterin was removed from the culture medium. Class and subclass determinations of the monoclonal antibodies were carried out by enzyme immunosorbent assay (EIA). The Fisher Biotech class/subclass determination kit was used in accordance with the manufacturer's instructions (Fisher Scientific, Orangeburg, N Y). Selected hybridomas were cloned twice by limiting dilution and then used for ascites production (Brodeur and Tsang 1986). In this study, we also used two EV-70 specific neutralizing monoclonal antibodies, 72-5E and 73-2F, which were kindly donated by L.J. Anderson (Anderson et al. 1984).

### **3.7 Dot Immunobinding Assay**

Virus was partially purified by removal of cellular debris and then pelleted as described in the purification procedure.

The viral pellet was resuspended in 100 uL of blotting buffer, (10 mM Tris-HCl pH 7.6, 150 mM NaCl), per flask of infected cells and stored at  $-70^{\circ}\text{C}$  until needed. A 1/10 dilution of the virus suspension was made in blotting buffer and aliquoted out at 50 uL per well onto nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Laboratories, Richmond, CA). The virus was allowed to adsorb to the nitrocellulose membrane for one hour at  $37^{\circ}\text{C}$  or overnight at  $4^{\circ}\text{C}$ . The membrane was then washed three times in PBS-Tween 20 (0.02% (v/v) Tween 20) then blocked with 3% (w/v) non-fat dry milk (Carnation Inc., Toronto, Ont.) for one hour at  $37^{\circ}\text{C}$ . Serial two fold dilutions of serum-free hybridoma supernatant were made in PBS. One hundred uL of each dilution was added to each well and allowed to incubate for one hour at  $37^{\circ}\text{C}$ . An unrelated anti-CMV antibody, CMV-B1, (Rossier et al. 1987) was used as a negative control. The wells were then washed three times in PBS-Tween. The membrane was then removed and blocked in 3% (w/v) non-fat dry milk (Carnation) for one hour at  $37^{\circ}\text{C}$ .  $^{125}\text{I}$ -labelled anti-mouse IgG (0.1 uCi/mL) (ICN Biochemicals Canada, Mississauga, Ont.) was added and incubated for another hour at  $37^{\circ}\text{C}$ . The membrane was then washed, dried and exposed to X-ray film (Cronex 4, Dupont, Wilmington, DE) at  $-70^{\circ}\text{C}$  for 18 hours.

### **3.8 Microneutralization Assay**

Initially, the hybridoma clones were screened for the production of neutralizing antibodies using a microneutralization assay. Ninety-six-well tissue culture plates (Nunclon Delta) were seeded with LLC-MK2 cells and allowed to approach 100% confluence. Duplicate samples of 125 uL of hybridoma culture supernatant were removed from the fusion plates and incubated with 5000 plaque forming units (PFU) of EV-70 for 60 minutes at 37°C. Culture supernatant from a hybridoma producing antibody specific for cytomegalovirus, CMV-B1, (Rossier et al. 1987) was tested as a control. LLC-MK2 cell culture fluid was replaced with the virus:hybridoma culture supernatant mixture. Plates were incubated for five days at 33°C. On the third, fourth and fifth day, each well was examined for cytopathic effect. The hybridomas corresponding to those wells which did not display any cytopathic effect relative to controls were retained for further testing.

### **3.9 Plaque Reduction Assay**

For all further characterizations of the antibody neutralizing activity, plaque reduction assays were carried out. The plaque reduction assays were performed in six-well tissue culture plates seeded with LLC-MK2 cells. Hybridoma

culture supernatant or an appropriate dilution of ascites fluid in serum-free BME was incubated with virus (approximately 100 PFU per test well) for 60 minutes at 37°C. This mixture was then added to a LLC-MK2 monolayer which was approaching 100% confluence. Following adsorption for one hour at 33°C, 2 mL of 0.9% (w/v) agar (Oxoid Agar No.#1, Oxoid Ltd., England) in BME medium was added to the cell monolayer. The BME:agar solution was supplemented with 2% (v/v) calf serum (Hyclone), 2mM glutamine (Sigma) and 50 ug/mL gentamicin (Sigma). The plates were then incubated at 33°C for two days. The plaques were counted following fixation in 30% (v/v) glacial acetic acid (BDH Laboratories, Toronto, Ont.) and stained with 1% (w/v) crystal violet (Fisher Scientific, Fair Lawn, N.J.). A modification of this assay was used to assess the inhibition of neutralization by monoclonal anti-idiotypic antibodies. A dilution of neutralizing antibody in serum free BME, neutralizing 90% of the input PFU challenge, was incubated with MAb2 (hybridoma supernatant or ascites fluid at designated dilutions) for 60 minutes at 37°C prior to the addition of the virus (approximately 100 PFU per test well). Following this, the assay was performed as described.

All plaque reduction and inhibition of neutralization assays were done in duplicate and repeated several times. Neutralization activity was considered present if 50% or more

of the input PFU's were neutralized.

### **3.10 Immunofluorescence Assay**

Indirect immunofluorescence assays were carried out using twelve-well multitest tissue culture slides (ICN-Flow Laboratories, Mississauga, Ont.). Wells were seeded with approximately  $1.5 \times 10^4$  LLC-MK2 cells and were allowed to approach 100% confluence. The cell monolayer was infected at a multiplicity of infection of approximately 0.1. The cells were incubated at 33°C until +2 CPE was observed. At this point, the slides were fixed by immersing them in 100% (v/v) acetone (BDH Laboratories) at -20°C for 5 minutes. Following this, the slides could be stored at -70°C until further use. Thirty uL of hybridoma culture supernatant or a dilution of ascites fluid in serum free BME was applied to the wells and incubated for one hour at 37°C. Goat anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (Cappel-Organon Teknika Corp., West Chester, PA) was diluted 1/60 in 0.008% (w/v) Evan's Blue counter stain and applied to the wells for one hour at 37°C. The intensity of the fluorescence was rated from +1 to +4 based on several independent experiments using ascites fluid. Hyperimmune serum was rated at +4 for relative comparison with the ascites fluid. A modification of this assay was used to show inhibition of fluorescence by anti-

idiotypic antibodies. A minimum amount of virus specific antibody giving a strong fluorescence reaction was pre-incubated with dilutions of MAb2 for 60 minutes at 37°C. The mixture was then applied to the wells and the assay was completed as described. All immunofluorescence assays were carried out using a Leitz Ortholux II fluorescence microscope equipped with a BP 450-490 excitation filter (Ernst Leitz Canada Ltd., Midland, Ont).

### **3.11 Radio-Immunoprecipitation Assay**

All radio-immunoprecipitation (RIP) assays were performed as follows. LLC-MK2 monolayers were infected at a multiplicity of infection of approximately 0.1 as they approached 100% confluence. A mock-infected culture served as a control. When infected cells reached +1 CPE, all cultures were incubated in methionine free medium (ICN-Flow) for 90 minutes. Following methionine starvation, 200 uCi of Tran [<sup>35</sup>S]methionine (ICN Biochemicals Canada) was added to each culture. The mock-infected control and one infected culture were then allowed to incubate for 3 hours. A remaining infected culture was allowed to proceed to +4 CPE. Following the 3 hour incubation, cells were scraped from the mock and the EV-70 infected cultures. The cells were washed three times in RIP buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.2) at 4°C and then lysed in

lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, 2% (v/v) Triton X-100) at 4°C. The nuclei were pelleted at 16,000 xg for 60 sec (Eppendorf centrifuge model #5415C) and supernatants were frozen at -20°C for future use. The infected culture which was allowed to proceed to +4 CPE was frozen and thawed three times and the virus was purified by cesium chloride gradient centrifugation. The virus-containing fractions were determined by monitoring radioactivity and assessing the viral infectivity of each fraction collected.

One mL of antibody solution (ascites fluid at 1/1000, serum-free tissue culture fluid at 1/2, or Ab3 serum at 1/10) in serum-free BME was added to each radiolabelled sample containing equal amounts of [<sup>35</sup>S]methionine. This mixture was allowed to incubate for 1 hour at 37°C or overnight at 4°C with constant agitation. Protein A Sepharose beads (Pharmacia, Montreal, Que.) were hydrated in phosphate-buffered saline and incubated in 0.05% (v/v) bovine serum albumin (BSA) for 1 hour at 37°C to block non-specific binding sites. These beads were washed three times in RIP buffer and resuspended in serum-free BME to a final concentration of 30% (v/v). The suspension was then aliquoted out in equal volumes to each of the samples. Samples were incubated for 1 hour at 37°C or overnight at 4°C with constant agitation. The beads were then

pelleted and washed three times in RIP buffer. Following the final wash, dissociation buffer (0.3 mM Tris, pH 6.8, 5% (w/v) sodium dodecyl sulfate, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 10% (v/v)  $\beta$ -mercaptoethanol) was added, the samples were heated for 5 minutes in boiling water and centrifuged at 16,000 xg (Eppendorf centrifuge model #5415C) for 45 seconds. Supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli 1970). The gel was dried and exposed to X-ray film (Dupont) at  $-70^{\circ}\text{C}$  for 18 hours.

### **3.12 Purification of Antibody and Preparation of F(ab)'<sub>2</sub>**

#### **Fraction**

The monoclonal antibodies were purified from ascites fluid by passage through a protein A or protein G - Sepharose column (Pierce Chemicals, Rockford, IL), depending on the isotype of the monoclonal antibody to be purified, and eluted with 0.1 M glycine pH 3.0. Purity of the preparation was assessed by SDS-PAGE and the biological activity was tested by immunofluorescence and neutralization assays.

The F(ab)'<sub>2</sub> preparations were made by pepsin digestion of the purified immunoglobulin fractions (Parham 1983). Briefly, 25 mg of antibody were digested for 3 hours at  $37^{\circ}\text{C}$  in 20 mM sodium acetate buffer, pH 4.1, using 1.0 mg of pepsin

immobilized on Sepharose beads (Pierce Chemicals). The digestion was stopped by the addition of 500 uL of 1.0 M Tris, pH 7.5. Beads were removed by centrifugation and the supernatant was dialysed against PBS, pH 7.2, for 16 hours. The undigested immunoglobulin fraction was separated from the F(ab)'<sub>2</sub> fraction by passage through a protein A - Sepharose column. The purity of the F(ab)'<sub>2</sub> fraction was assessed by SDS-PAGE and the biological activity was confirmed by immunofluorescence. The F(ab)'<sub>2</sub> preparation was titrated for use by EIA.

### **3.13 Immunization with MAbs for Production of Ab2 and Ab3** **Antibodies**

MAbs were coupled to keyhole limpet hemocyanin (Sigma), at a 1/1 (w/w) ratio for 90 minutes at room temperature (RT) with constant agitation in the presence of 0.05% (v/v) glutaraldehyde (final concentration). The sample was then dialysed against PBS, pH 7.2 for 24 hours. Prior to the first injection, a pre-immune serum sample was taken from each mouse. Injections (80 ug of conjugate) were given intraperitoneally with Freund's adjuvant or subcutaneously with Quil A (Cedarlane Laboratories, Toronto, Ont.) (25 ug per mouse) at 4 week intervals. Serum samples were taken 10 days after each injection and assessed for the presence of anti-

idiotypic antibodies by EIA and inhibition of neutralization. In the case of MAb2 production, if a sufficient Ab2 serum titre was detected, a final intravenous boost was given without Quil A. Four days later, the mouse was exsanguinated and the spleen removed for hybridoma production.

### **3.14 EIA Screening for MAb2**

The screening of MAb2 hybridoma supernatant was performed as described elsewhere (Hamel and Brodeur 1990). In brief, EIAs were standardized so that each well was coated with 0.13 ug of the F(ab)'2 fraction of MAb/ev-12 in 100 uL of PBS, pH 7.2 overnight at RT. The plates were washed once in PBS-Tween. Any remaining binding sites were blocked with 200 uL of 1% (w/v) BSA in PBS, pH 7.2 for 1 hour at 37°C followed by three washes with PBS-Tween. One-hundred uL of hybridoma supernatant was added to each well and incubated for 1 hour at 37°C. Following three PBS-Tween washes, 100 uL of alkaline phosphatase-conjugated anti-mouse IgG, Fc-specific antibody (Cappel-Organon Teknika, West Chester, PA) in 3% (w/v) BSA/PBS, pH 7.2 was added to each well. The plates were incubated for 1 hour at 37°C and then washed three times with PBS-Tween. Seventy-five uL of 10% (v/v) diethanolamine, pH 9.8 containing 1 mg/mL of p-nitrophenylphosphate (Sigma) was added. After 30 minutes at RT, the absorbance at 410 nm was

read using a Dynatech MR7000 Microplate Reader.

A modification of this procedure was performed as a competition assay using Ab3 antisera. Inhibition of MAb/ev-12:MAB2 binding was assessed by prior incubation of MAB2 hybridoma supernatant with Ab3 antiserum for 1 hour at 37°C. The mixture was then added to the F(ab)'<sub>2</sub> coated plates for 1 hour at 37°C. Following this, the assay was completed as described.

### **3.15 Inhibition of Binding of MAb/ev-12 to EV-70 by MAB2**

The dot immunobinding inhibition assay used here was a modification of the previously described dot immunobinding assay. Preparation of the virus and adsorption of the virus to the membrane followed by blocking of the membrane were carried according to the previously described assay. The inhibition aspect of this assay was adapted from one previously described (Brodeur et al. 1991). One-hundred uL of MAb/ev-12 hybridoma supernatant and 100 uL of an appropriate dilution of MAB2 hybridoma supernatant were incubated for 1 hour at 37°C. One hundred uL of this antibody mixture was then applied to the membrane and incubated for one hour at 37°C. The membrane was then washed three times with PBS-Tween 20 and removed from the dot-blot apparatus. The assay was then completed as described for the dot immunobinding assay.

MAb2/3C5 was used as an isotype matched unrelated control.

## Chapter 4

### RESULTS

#### 4.1 Characterization of EV-70 Specific MAbs

The electron micrograph, (Figure 1), depicts the purified EV-70 virus preparation used in immunization and radio-immunoprecipitation procedures. The majority of the particles are devoid of their contents (darkened central area of each virion). Those virions which have retained their genomic RNA have not been penetrated by the stain. Any core structures within these particles are still visible. The typical six-sided profile of picornaviruses is easily seen with a number of the virions in this picture.

From four fusion experiments, a panel consisting of twelve EV-70 specific monoclonal antibodies was made. MAb/ev-1 through MAb/ev-11 displayed either low or non-existent neutralization titres against EV-70, whereas MAb/ev-12 showed a high EV-70 neutralization titre when tested by both microneutralization and plaque reduction assays (Table 1). The former group were of the IgG3 isotype whereas MAb/ev-12 was of the IgG2a isotype. Additional neutralizing clones which were obtained were almost entirely of the IgM isotype. These were not selected for further use. All of the selected MABs possessed Kappa light chains. MAb/ev-1 and MAb/ev-6 were taken from the non-neutralizing group and further characterized for

**Figure 1.** Electron micrograph of purified enterovirus-70 using 2% (v/v) phosphotungstic acid stain. Magnification is 190,000 x.

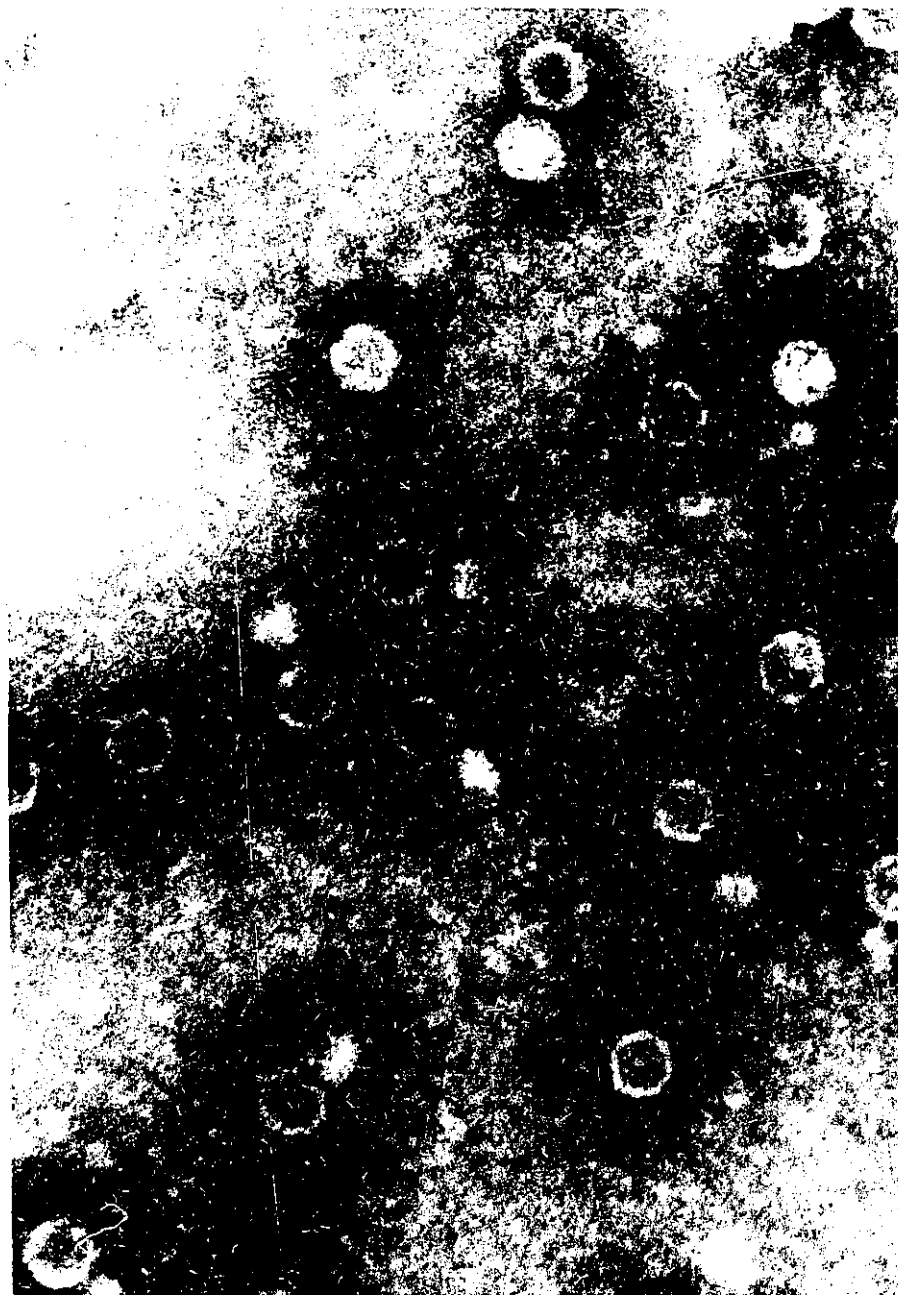


TABLE 1. Characteristics of MABs directed against enterovirus-70.

MAB	IF Titre <sup>a</sup>	Neutralization Titre <sup>b</sup>	Isotype	Specificity <sup>c</sup>
MAB/ev-1	8000	<2	IgG3 K	all strains
MAB/ev-6	8000	50	IgG3 K	all strains
MAB/ev-12	200	16000	IgG2a K	J670/71
72-5E <sup>d</sup>	500	>8000	IgG3	all strains <sup>e</sup>
73-2F <sup>d</sup>	500	>8000	IgG2b	all strains <sup>e</sup>
MAB/P2-4 <sup>f</sup>	<10	<2	IgG2a K	no strains

<sup>a</sup> Reciprocal end point dilution of ascites fluid giving +1 fluorescence.

<sup>b</sup> Reciprocal dilution of ascites giving 50% reduction of 100 PFU/well.

<sup>c</sup> Specificity of MABs for other EV-70 strains tested in this report.

<sup>d</sup> Anti EV-70 MABs obtained from L.J. Anderson (Anderson et al 1984).

<sup>e</sup> Tested by L.J. Anderson (Anderson et al 1984).

<sup>f</sup> MAB/P2-4 is an anti-H<sub>1</sub> influenzae type b MAB (Hamel and Brodeur 1990).

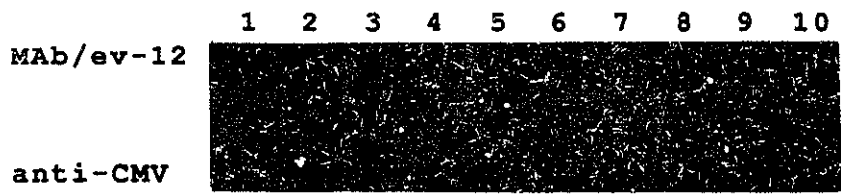
this study. Both of these MAbs demonstrated strong immunofluorescence activity with the prototype EV-70 strain, J670/71, as well as a selection of other EV-70 strains (KW97, R6, RU3875, 1604, V1250). However, MAb/ev-12 showed immunofluorescence activity and neutralizing activity only against the prototype strain. Further evidence that target epitopes for MAb/ev-1 and MAb/ev-6 differed from that of MAb/ev-12 was provided by experiments showing that pre-incubation of the prototype strain with either of the former MAbs (at a dilution of 1/500) did not block neutralization by MAb/ev-12. None of the anti-EV-70 specific MAbs showed immunofluorescence reactivity against a panel of picornaviruses which included coxsackievirus-B1 and B3, echovirus serotypes 1, 9 and 23, poliovirus - Sabin serotypes 1, 2 and 3 and human rhinovirus serotype 14. In addition, no reactivity with uninfected cells was observed. The immunofluorescence pattern presented is typical of that seen with other picornaviruses (Figure 2). The fluorescence is confined to the cytoplasmic region of the cell which reflects the location of viral replication. The dark coloured eccentric nucleus, not being a viral replication site, is pushed to the side due to the abundance of viral antigen in the cytoplasm.

The specificity of MAb/ev-12 for EV-70 is further demonstrated in the dot immunobinding assay (Figure 3). Two

**Figure 2.** Immunofluorescence of enterovirus-70 infected LLC-MK2 monolayer. Magnification is 400 x.



**Figure 3.** Dot immunobinding of MAb/ev-12 to EV-70 using sequential dilutions of MAb/ev-12 or anti-CMV MAb. Hybridoma tissue culture fluids were diluted; 1/2 (lane 1), 1/4 (lane 2), 1/8 (lane 3), 1/16 (lane 4), 1/32 (lane 5), 1/64 (lane 6), 1/128 (lane 7), 1/256 (lane 8), 1/512 (lane 9), and 1/1024 (lane 10).



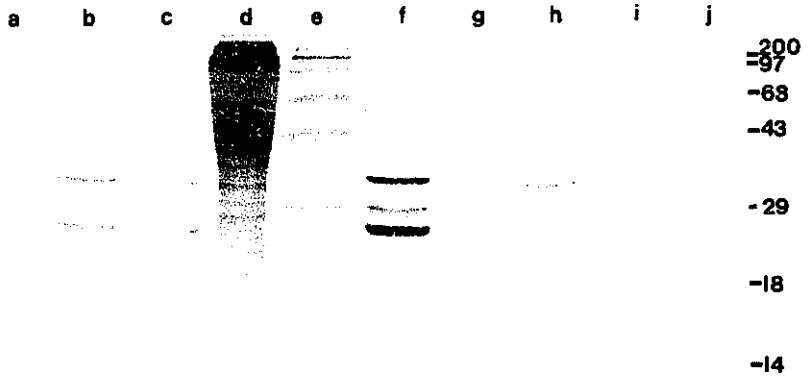
fold titration of MAb/ev-12 resulted in a dose responsive decrease of the radio-label (top row) whereas there is no sign of reactivity with the anti-CMV antibody, CMV-B1, at any dilution (bottom row).

Western immunoblot and radio-immunoprecipitation assays were carried out with each of the EV-70 specific monoclonal antibodies. In western immunoblot assays, no reactivity could be detected (data not shown). In RIP assays, the three major viral proteins were precipitated by all MABs, suggesting the recognition of a conformational/discontinuous viral epitope. Radio-immunoprecipitation assay results representative of those found with all twelve EV-70 specific monoclonal antibodies are presented in Figure 4. No reaction by the MABs was observed with the mock infected cell lysate. The specificity of the MABs for EV-70 was demonstrated by this lack of reactivity against the cellular components and by the corresponding lack of reactivity of an unrelated MAB1 (MAB/P2-4) (Hamel and Brodeur 1990) against the purified virus.

#### **4.2 Characterization of MAb2s**

The production of an Ab3 neutralizing viral response using anti-idiotypic antibodies relies on mimicry of the virion neutralization epitope by the paratope of the MAB2. Since MAb/ev-12 possessed a high neutralization titre, it was chosen

**Figure 4.** Autoradiogram of EV-70 proteins immunoprecipitated by anti-EV-70 MAbs. <sup>35</sup>S-labelled mock-infected cell lysate, EV-70 infected cell lysate or purified EV-70 virus were immunoprecipitated with MAbs and then analyzed by SDS-PAGE as described in Materials and Methods. MAb/ev-1 with mock-infected cell lysate (lane a), with EV-70 infected cell lysate (lane b), and with purified EV-70 virus (lane c); mock-infected cells alone (lane d); molecular weight markers (lane e); purified EV-70 alone (lane f); MAb/ev-12 with purified EV-70 (lane g), with EV-70 infected cell lysate (lane h), and with mock-infected cell lysate (lane i); control MAb/P2-4 with purified EV-70 virus (lane j).



as the antigen for the production of MAb2s. Two fusion experiments yielded nine clones which, by EIA, bound F(ab)'<sub>2</sub> fragments of MAb/ev-12. Five clones, all IgG1, were chosen for further study on the basis of their stability and level of antibody secretion. Purification of MAb1 and each of the MAb2s by affinity chromatography produced the monoclonal antibody preparations seen in Figure 5. Analysis by non-reduced SDS-PAGE demonstrated that the preparations were pure according to Coomassie-Blue detection and thus suitable for use in immunization studies.

In order to determine whether or not the five MAb2s were directed against paratope associated idiotopes of MAb/ev-12, various blocking assays were performed. All of the MAb2s were capable of inhibiting both the immunofluorescence and neutralizing activity of MAb/ev-12, suggesting that each MAb2 was specific for a MAb/ev-12 paratope associated idiotope. Table 2 presents data representative of the ability of each of the MAb2s to inhibit the EV-70 specific neutralization capacity of MAb/ev-12. In the absence of a MAb2, MAb/ev-12 neutralized 90% of the PFU challenge. Prior incubation of MAb/ev-12 with any of the MAb2s inhibited the neutralization of EV-70. The capacity of each MAb2 to inhibit the neutralizing ability of the Ab1 hyperimmune serum was then assayed (Table 3). The Ab1 hyperimmune serum was used at a

**Figure 5.** SDS-PAGE analysis of purified MAb1 and MAb2 preparations under non-reducing conditions. MAb2-2 (lane 1), MAb2-7 (lane 2), MAb2-9 (lane 3), MAb2-13 (lane 4), MAb2-15 (lane 5), MAb/ev-12 (lane 6), molecular weight markers (lane 7).

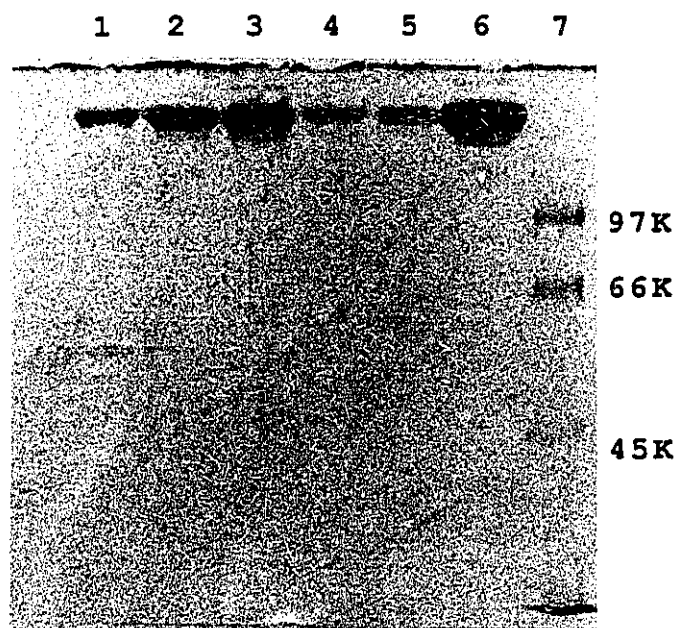


TABLE 2. Inhibition of MAb/ev-12 neutralizing activity by MAb2s.

EV-70 + Antibodies <sup>a</sup>	PFU/Well <sup>b</sup>	% Inhibition of Neutralization
MAb/ev-12 + medium	15, 16	<10
MAb/ev-12 + MAb2-2	150, 130	100
MAb/ev-12 + MAb2-7	160, 150	100
MAb/ev-12 + MAb2-9	120, 140	93
MAb/ev-12 + MAb2-13	126, 123	89
MAb/ev-12 + MAb2-15	150, 150	100
MAb/ev-12 + MAb2/3C5 <sup>c</sup>	16, 16	<10
media + MAb2/3C5	146, 141	100

<sup>a</sup> MAb2 and MAb/ev-12 were preincubated for one hour at 37<sup>0</sup>C. Viral challenge was then added and the mixture was incubated for one hour at the 37<sup>0</sup>C. Plaque assay procedure was then followed.

<sup>b</sup> Residual PFU/well following a 140 PFU/well challenge, tested in duplicate.

<sup>c</sup> MAb2/3C5 is an anti-idiotypic MAb against anti CMV-B1 MAb1 (Rossier et al 1987).

Table 3. Neutralizing Activity of Anti EV-70 Sera in the Presence of MAb2s as Measured by Plaque Reduction Assay.

Treatment	Dilutions of MAb2 Ascites Fluid		
	1:100	1:1000	1:10000
sera <sup>a</sup> + MAb2-2	2 , 3	2 , 4	19 , 16
sera + MAb2-7	5 , 2	6 , 2	5 , 5
sera + MAb2-9	3 , 5	2 , 1	11 , 6
sera + MAb2-13	6 , 6	3 , 1	10 , 1
sera + MAb2-15	3 , 6	3 , 8	10 , 12
sera + MAb2/3C5 <sup>b</sup>	8 , 4	nd <sup>c</sup>	nd
MAb2-2	>100	nd	nd
MAb2-7	>100	nd	nd
MAb2-9	>100	nd	nd
MAb2-13	>100	nd	nd
MAb2-15	>100	nd	nd

<sup>a</sup> Hyperimmune sera obtained from mice immunized with EV-70 and used at 1:16000. Hyperimmune sera alone gave 5 and 12 plaques.

<sup>b</sup> MAb2/3C5 is an unrelated monoclonal anti-idiotypic antibody (Hamel and Brodeur 1990).

<sup>c</sup> not done

titre of 1/16000. This produced a 90% reduction of the PFU challenge. This titre was selected on the basis that it gave a consistent reduction of the PFU challenge and was two fold greater than the titre giving a 50% or less reduction of the PFU challenge in a triplicate series of this assay. Prior incubation of the Ab1 hyperimmune serum with each of the MAb2s at three different dilutions did not result in any inhibition of neutralization. In this assay, each MAb2 was tested against all of the anti EV-70 antibodies in the hyperimmune serum as well as all other antibodies normally present. This is unlike the situation which produced the results depicted in Table 2 in which only one monoclonal antibody was tested against another. In both assays, an unrelated MAb2 had no effect on either the ability of MAb/ev-12 to neutralize EV-70 nor on the viral challenge itself. In addition, none of the MAb2s exhibited any anti EV-70 reactivity nor did they react with non-infected LLC-MK2 cells in immunofluorescence tests (data not shown). It is noteworthy that none of the MAb2s were able to prevent infection of cell monolayers.

The specificity of the MAb2s for the paratope of MAb/ev-12 was also demonstrated by their ability to block MAb/ev-12 binding to EV-70 in dot immunobinding inhibition assays (Figure 6). Two-fold titration of the MAb2s resulted in a dose responsive decrease in this inhibition. The unrelated MAb2/3C5

**Figure 6.** Autoradiogram of dot immunobinding inhibition assay of MAb/ev-12 binding to EV-70 by MAb2s. MAb/ev-12 hybridoma supernatant was incubated with two-fold dilutions of hybridoma supernatant of each MAb2 or an unrelated MAb2 (MAb2/3C5). The antibody preparations were then reacted with the semi-purified EV-70 preparation bound to the nitrocellulose membrane. MAb/ev-12 binding was revealed with  $^{125}\text{I}$ -labelled anti-mouse IgG. Viral material was omitted with control MAb2/3C5 at the 1/32 dilution. MAb/ev-12 alone (lane 1); MAb/ev-12 preincubated with MAb2 diluted 1/2 (lane 2), 1/4 (lane 3), 1/8 (lane 4), 1/16 (lane 5), 1/32 (lane 6), MAb2 alone 1/2 (lane 7).

1 2 3 4 5 6 7

**MAb2-2**

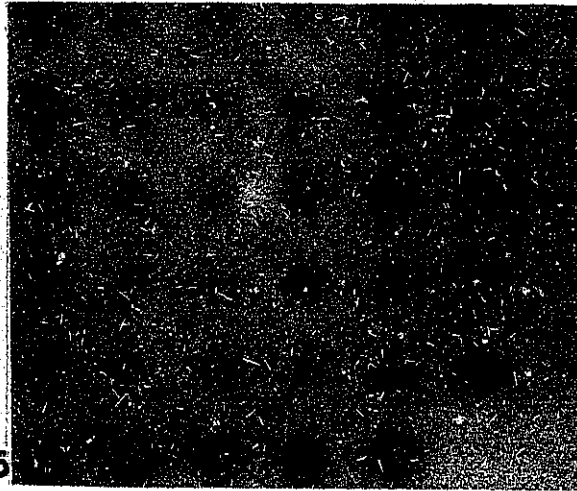
**MAb2-7**

**MAb2-9**

**MAb2-13**

**MAb2-15**

**MAb2/3C5**



did not demonstrate any reactivity with MAb/ev-12 at any of the dilutions tested.

In order to further investigate the specificity of the MAb2s for MAb/ev-12, two EV-70 specific neutralizing MAb1s from another laboratory (Anderson et al. 1984) plus MAb/ev-1 and MAb/ev-6 were tested for their recognition by each of the MAb2s. No recognition of these MAb1s by any of the MAb2s was observed in immunofluorescence inhibition assays. In competition EIAs, titrations of the competing MAb1s at 1/100, 1/1000 and 1/4000 did not result in the inhibition of any MAb2 binding to bound F(ab)'<sub>2</sub> fragments of MAb/ev-12. Only whole MAb/ev-12 demonstrated any inhibition of MAb2 binding to bound F(ab)'<sub>2</sub> fragment of MAb/ev-12. This suggested that the idiotype of each of these four MABs differed from that of MAb/ev-12.

#### **4.3 Characterization of Ab3 Response**

Each of the MAb2s were used as antigen in the immunization of 15 mice for the production of Ab3 antisera. In EIA competition assays, serum obtained following the third injection demonstrated that each mouse had made an Ab3 response to its MAb2 challenge. As shown in Table 4, each homologous MAb2-Ab3 interaction inhibited the MAb/ev-12:MAb2 binding by more than 90% at an Ab3 antiserum dilution of

TABLE 4. Reactivity of Ab3 antibodies with MAb2 as tested by EIA inhibition assay<sup>a</sup>.

Percent inhibition of Mab/ev-12 - MAb2 binding using Ab3 antisera <sup>b</sup>					
MAB2	anti-MAB2-2	anti-MAB2-7	anti-MAB2-9	anti-MAB2-13	anti-MAB2-15
MAB2-2	94	57	71	3	33
MAB2-7	95	92	87	69	86
MAB2-9	88	51	91	66	18
MAB2-13	93	65	82	93	61
MAB2-15	93	87	86	58	96

<sup>a</sup> MAB2 and Ab3 antisera (at 1:500) were preincubated together and the mixture was added to Mab/ev-12 F(ab')<sub>2</sub> coated plates. The inhibition of MAB2 binding relative to controls was then determined by the addition of anti-mouse Fc-specific conjugate.

<sup>b</sup> Percentage values are mean of six values - two tests per mouse, three mice per MAB2 group.

1/500. This level of inhibition gradually subsided to 50% or less at 1/10,000 (data not shown). Negligible MAb/ev-12-/Ab3 binding was observed in the controls. The inhibition levels observed in the heterologous MAb2-Ab3 competitions varied over a wide range which implied the cross-reactivity of each Ab3 sera with most of the other MAb2s. Two exceptions to this were, i) Ab3 sera raised to MAb2-13 did not recognize MAb2-2 and, ii) Ab3 sera raised to MAb2-15 did not recognize MAb2-2 and MAb2-9.

Since each MAb2 had clearly stimulated an immune response in these mice, it was decided to determine whether the Ab3 antisera could neutralize the infectivity of EV-70. As illustrated in Table 5, the immunization of syngeneic mice with MAb2s elicited a neutralizing immune response specific for EV-70. The mice which received MAb2-2 produced the best neutralizing response. Sera from the three mice in this group showed greater than 50% neutralization activity at an Ab3 sera dilution of 1/25. In fact one mouse from this group still retained 50% neutralization activity at a serum titration of 1/50. Of the mice which received MAb2-9 and MAb2-15, two out of the three in each group displayed a 50% or greater neutralization activity at this serum dilution. Of the mice which received MAb2-7 and MAb2-13, only one mouse from each group displayed a 50% neutralization activity at an Ab3

TABLE 5. Neutralizing activity of Ab3 antiserum against enterovirus-70

Ab3 antisera	% Neutralization <sup>a</sup>	
	Ab3 sera dilution	
	1:25	1:50
pre-immune	0	nd <sup>b</sup>
anti-MAb2-2	86, 77, 67 <sup>c</sup>	54, 45, 14
anti-MAb2-7	59, 34, 18	12, 12, <10
anti-MAb2-9	70, 61, 27	14, <10, <10
anti-MAb2-13	54, 40, 12	15, <10, <10
anti-MAb2-15	82, 54, 33	35, <10, <10

<sup>a</sup> Results are % neutralization relative to pre-immune sera of 15 mice following 110 PFU/well challenge.

<sup>b</sup> Not done.

<sup>c</sup> Data presented for individual animals. Tests were done in duplicate. Three mice were immunized per MAb2 clone.

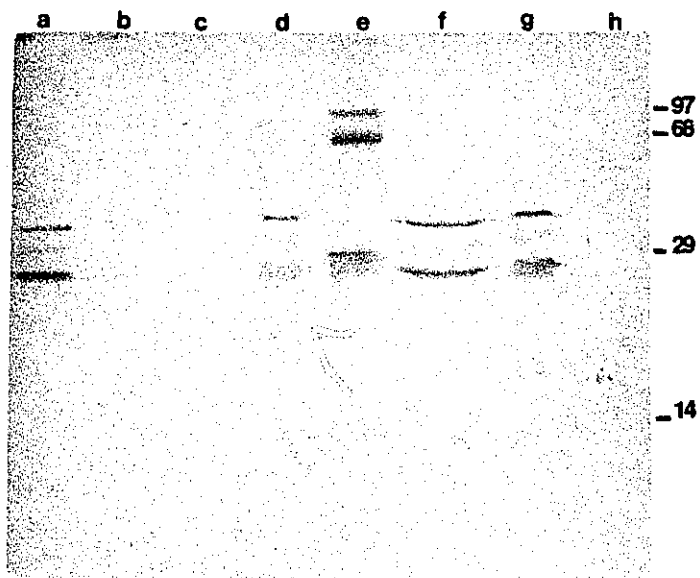
dilution of 1/25.

In an effort to assess whether higher neutralization titres could be generated, a group of mice were immunized with either, MAb2-2 or MAb2-15 using either Freund's adjuvant or Quil A. However, no difference in the neutralization titres were observed following four injections over a 12 week immunization regime. The highest neutralization titres were from a mouse which received MAb2-15 with Freund's adjuvant (1/200) and from a mouse which received MAb2-2 with Quil A (1/100) (data not shown).

Serum from the mouse which produced the highest Ab3 neutralization titre was tested against the same heterologous EV-70 strains as previously done with MAb/ev-12. Neither antibody preparation demonstrated any neutralizing activity against these heterologous EV-70 strains. This suggested that the specificity of both preparations was identical. The MAb/ev-12 - like specificity of the Ab3 antibodies for EV-70 proteins was further assessed by radio-immunoprecipitation. Serum from a mouse immunized with MAb2-15 in Freund's adjuvant and serum from a mouse immunized with MAb2-2 in Quil A were tested. As seen in Figure 7, the same viral bands were precipitated with anti-MAb2-15 antiserum (lane d) and anti-MAb2-2 antiserum (lane g) as were observed with MAb/ev-12 (lane a). The reactivity of the Ab3 antiserum for EV-70 was

**Figure 7.** Autoradiogram of EV-70 proteins immunoprecipitated by Ab3 antiserum. <sup>35</sup>S-labelled mock-infected cell lysate or purified EV-70 virus were immunoprecipitated with Ab3 antiserum and then analyzed by SDS-PAGE as described in Materials and Methods. MAb/ev-12 with purified EV-70 virus (lane a), unrelated Ab3 antiserum with purified EV-70 virus (lane b); anti-MAb2-2 antiserum with mock-infected cell lysate (lane c) and with purified EV-70 (lane d). Labelled molecular weight markers (lane e), purified EV-70 virus alone (lane f); anti-MAb2-15 antiserum with purified EV-70 (lane g) and with mock-infected cell lysate (lane h).

**RADIO-IMMUNOPRECIPITATION USING AB3 SERA.**



supported by the absence of these viral bands when an unrelated Ab3 antiserum was used (lane b) and by the fact that no cellular material reacted with either of the two antisera (lanes c and h).

Evidence was presented earlier that each of the MAb2s were capable of inhibiting the neutralizing activity of MAb/ev-12 (Table 2). In a similar manner, the ability to inhibit the Ab3 neutralization activity using the homologous MAb2 was demonstrated (Table 6). The degree of inhibition reflected the titre of both the Ab3 antiserum and MAb2. Complete inhibition of neutralization was observed at low dilutions of the homologous MAb2. As the MAb2 was further diluted, the neutralizing activity of the Ab3 serum became evident.

TABLE 6. INHIBITION OF AB3 NEUTRALIZING ACTIVITY BY HOMOLOGOUS MAb2.

Ab3 anti-sera	no inhibitor	MAb2 inhibitor dilutions <sup>a</sup>					
		1:50000	1:5000	1:1000	1:500	1:100	1:10
anti-MAb2-2	0, 8 <sup>b</sup>	0, 0	0, 0	0, 0	0, 1	>100, >100	102, 87
anti-MAb2-9	0, 2	1, 2	2, 4	n. d. <sup>c</sup>	40, 68	n. d.	n. d.
anti-MAb2-15	0, 1	0, 0	1, 3	>100, >100	>100, >100	>100, >100	>100, >100

<sup>a</sup> Ab3 antisera diluted 1:25 were preincubated with various dilutions of MAb2 ascites fluid prior to virus neutralization assay.

<sup>b</sup> Results are reported in residual pfu/well from duplicate tests using a 140 PFU challenge of EV-70.

<sup>c</sup> Not done.

**Chapter 5****DISCUSSION**

In order to investigate the effectiveness of anti-idiotypic antibodies as potential surrogate vaccines against enterovirus-70 infection, it was first necessary to produce a specific neutralizing monoclonal antibody. In this project, a panel of twelve anti EV-70 specific monoclonal antibodies was produced. None of these monoclonal antibodies was found to react with any other picornaviruses tested. From this panel, MAb/ev-1 to MAb/ev-11 inclusive were found to react with six different serotypes of EV-70 in immunofluorescence assay. MAb/ev-12 was specific only for the prototype strain J670/71. The reactivity of these first eleven monoclonal antibodies with different serotypes of EV-70 would have beneficial applications in diagnostic testing, notably in conjunction with other procedures as a means of providing confirmatory evidence for EV-70 AHC. Such an application would have been relevant during the Singapore epidemic of 1970-71 in which two clinically indistinguishable picornaviruses causing AHC were in circulation simultaneously, those being enterovirus-70 and coxsackievirus A24 (Kono et al. 1975a, Natori et al. 1984). The long-term supply of these specific murine anti EV-70 monoclonal antibodies would resolve the non-

specific staining problems encountered in rapid immunofluorescence diagnostic assays which used human or monkey serum (Pal et al. 1983). In addition, the simplicity and rapidity of an immunofluorescence test using these specific anti EV-70 monoclonal antibodies would negate the necessity of paired sera required for other proposed tests (Wulff et al. 1987) and the extended time frame required for their completion.

MAB/ev-12, which displayed a restricted specificity for J670/71, was also the only monoclonal antibody capable of neutralizing the virus. From the fusion experiment that produced MAB/ev-12, it was noted that almost all of the isolated neutralizing clones against EV-70 were of the IgM isotype. This observation was surprising in view of the fact that at least three inoculations of purified virus were administered over a twelve week period. This should have been an adequate time frame to allow IgM isotypes associated with primary immune responses to be converted to IgG isotypes which characterize secondary immune responses. Of greater interest, was the fact that this result was in agreement with the prevalent antibody response to EV-70 and other picornaviruses found in a variety of domestic animals. The dearth of alternate immunoglobulin isotypes was hypothesized to be the result of low level, transient exposure to EV-70 as opposed

to repeated exposures (Sasagawa et al. 1982).

The recognition of a variety of EV-70 serotypes by the non-neutralizing group implies that the corresponding epitope(s) would in all probability be conserved and not likely involved in viral binding nor antigenic variability. On the other hand, the specificity of MAb/ev-12 for J670/71 and the fact that it is capable of neutralization, suggests the presence of a second non-conserved epitope unique to the prototype strain. Neutralization of the J670/71 strain by MAb/ev-12 was possible in the presence of any of the non-neutralizing monoclonal antibodies. This neutralization epitope does not seem to overlap with the non-neutralization epitope common amongst the EV-70 serotypes. Based on mapping studies of other picornaviruses (Sherry et al. 1986, Pfaff et al. 1988, Minor et al. 1986) it may be postulated that the neutralization epitope recognized by MAb/ev-12 may be one of a few different neutralization sites on the virion surface.

The immunofluorescence pattern exhibited by EV-70 infected cells displayed the viral cytopathic effects typical of picornavirus infections. In the early stages of the infection, the darkened nucleus lies in a central position in the cell surrounded by a band of cytoplasm displaying a low level of fluorescence indicative of the small amount of viral antigen present. As viral cytopathic effect progresses, the nucleus

becomes eccentric due to the increase in size of the cytoplasmic inclusion. Those cells displaying intense green fluorescence are in the late stage of the viral infection cycle. Throughout the infection cycle, all of the viral replication is confined to the cytoplasm, thus accounting for the lack of nuclear fluorescence. A number of infected cells at various stages of the viral infection cycle are depicted in Figure 1.

The reactivity of the EV-70 specific monoclonal antibodies in western immunoblot and radio-immunoprecipitation assays suggested that the viral epitopes recognized by both the non-neutralizing and neutralizing MAbs are conformational /discontinuous epitopes. The topography of such epitopes is more reliant on the tertiary and quaternary protein structures and interactions than on the primary amino acid sequences of the constituent proteins (M.V.H. Van Regenmortel and G.D. de Marcillac, 1988). Dissociation of the virions prior to exposure to the MAbs, as in western immunoblot assays, prevented antibody binding. This is analogous to the failure of picornaviruses to recognize their cellular receptors following modification of their capsids (Colonno 1987). Conversely, the MAbs were able to recognize virions prior to denaturation, as depicted in Figure 4 which is representative of radio-immunoprecipitation assay results seen for all twelve

EV-70 specific MAbs. Although the data favour the presence of conformational/discontinuous epitopes, it cannot be concluded from the evidence presented here as to which proteins contribute amino acid residues to the structure of the epitopes. Conformational epitopes of other picornaviruses generally consist of amino acid residues from two structural proteins which are brought into juxtaposition by the interactions of their respective polypeptides (Sherry et al. 1986, Minor et al. 1986, and Pfaff et al. 1988). The presence of additional proteins in the RIP assay results may be accounted for by the interactions with one or both of the epitope component proteins or by the possibility that it may also contribute amino acid residues to the epitope structure. Mapping studies using neutralization escape mutants and genomic sequencing of these escape mutants would be required to ascertain the component amino acid residues of any such epitope.

A principal objective in the application of surrogate vaccines is the induction of a protective Ab1-like response. In this case, the purpose was to mimic MAb/ev-12 in its recognition of and neutralizing activity against enterovirus-70. Application of the network theory regarding idiotypic regulation within the immune system states that there exists an endogenously derived idiotope that mimics the exogenous EV-

70 epitope recognized by MAb/ev-12. This endogenous idiotope should therefore be able to recognize or be recognized by MAb/ev-12. The stimulation of this specific anti-idiotypic clone bearing an anti MAb/ev-12 receptor and the production of monoclonal antibodies from it are crucial steps in the development of an anti-idiotypic vaccine for this model. For this reason, MAb/ev-12 was the choice immunogen for the development of the second generation anti-idiotypic antibody.

Purity of the immunogens used in the production of both the Ab2 and Ab3 responses was of concern. The antigenic determinant of interest on MAb/ev-12 and on each of the MAb2s was one paratope associated idiotope amongst a multitude of other determinants. The removal of superfluous determinants from the immunogen preparation would enhance the concentration of the idiotope of interest. Although both of the Ab1 and Ab2 preparations used still retained most of the non-specific IgG fraction of the harvested ascites fluid, the major portion of each preparation should consist of immunoglobulins specific for the immunizing antibody. The purity of each preparation used in this project was considered sufficient for their intended use, (Figure 5).

The screening of the MAb2 containing immune serum prior to the fusion was done by neutralization inhibition of MAb/ev-12 activity. The immunization procedure used proved to be

successful as inhibition titres of 1/2000 were achieved. Selection of the five anti-idiotypic clones was done on the basis of stability and level of production as well as on the neutralization inhibition capabilities of each MAb2 clone obtained.

The specificity of each anti-idiotypic clone for MAb/ev-12 was tested by a variety of means. In competition EIA assays, the non-neutralizing MAb/ev-1 did not compete with the plate bound F(ab)'<sub>2</sub> fragment of MAb/ev-12 for binding to any of the MAb2s. In addition, neither of the two EV-70 specific neutralizing monoclonal antibodies received from another laboratory (Anderson et al. 1984) were able to compete for any of the anti-idiotypic antibodies. This suggested that the MAb/ev-12 idiotope recognized by each of the five anti-idiotypic antibodies was a private idiotope.

It was already known from the previous dot immunobinding assay, (Figure 3), that MAb/ev-12 was specific for the virus. The use of two-fold sequential dilutions of each MAb2 to compete with EV-70 for MAb/ev-12 demonstrated that a MAb2 titre dependent inhibition of MAb/ev-12:EV-70 binding could be established. The results of the dot immunobinding inhibition assay concurred with those from the neutralization inhibition assay (Table 2). The binding of MAb/ev-12 to MAb2 was not displaced by the presence of the virus in this assay.

This suggested that the binding affinity of MAb/ev-12:MAB2 may be greater than that for MAb/ev-12:EV-70. Such a characteristic may be of significance in the elicitation of an Ab1-like response using an Ab2 immunogen. In immunofluorescence inhibition assays, each of the anti-idiotypic antibodies were able to block the recognition of EV-70 by MAb/ev-12 but not by MAb/ev-1 nor MAb/ev-6. The results of the various competition and inhibition assays suggested that the MAb2s were specifically directed against a private paratope associated idiotope on MAb/ev-12.

Following the neutralization of MAb/ev-12 activity, the ability of the MAb2s to inhibit the EV-70 neutralizing activity of the Ab1 hyperimmune serum was assessed. Previous titrations of the hyperimmune serum had shown that a dilution of 1/16000 consistently gave a 90% neutralization of the input PFU challenge. At a titre of 1/32000, the degree of neutralization ranged between 40%-60%. Thus the 1/16000 titre was selected as the working titre for this assay. It was already known that the MAb2s were capable of inhibiting the MAb/ev-12 neutralizing activity and that MAb/ev-12 was specific for one EV-70 neutralization epitope. The objective of this inhibition assay was to determine if additional neutralization epitopes on the EV-70 virion existed and were reflected by the presence of corresponding neutralizing

antibodies in the serum. By specifically inhibiting the activity of the MAb/ev-12 fraction of the hyperimmune serum, any further neutralization of the input viral challenge could be attributed to the presence of neutralizing antibodies which do not recognize the MAb/ev-12 specific epitope. Each ascites fluid was used at concentrations 160x's, 16x's and 1.6x's greater than the final concentration of the hyperimmune serum. These quantities should provide an excess of specific MAb2s relative to the MAb/ev-12 fraction present in the serum. These MAb2s did not show any sign of interfering with viral infectivity in the control assays. Using an input viral challenge of approximately 100 PFUs, the hyperimmune serum neutralized 90% of the virus at 1/16000. Of great interest was the observation that even at the lowest MAb2 dilution, the same degree of virus neutralization was recorded. Considering the titre of the Ab1 hyperimmune serum relative to the amount of specific anti MAb/ev-12 antibody present, it could be concluded that additional neutralization epitopes are present on the virion. These epitopes were reflected by the presence of specific antibodies in the hyperimmune serum which are not recognized by the anti MAb/ev-12 anti-idiotypic antibodies. Considering the predominance of the neutralizing IgM fraction in the Ab1 response, it may be postulated that the IgM antibodies were directed against the additional neutralization

epitopes. The suggestion here that additional neutralization epitopes are present on the surface of the EV-70 virion is in compliance with the fact that other picornaviruses are known to possess more than one neutralization epitope (Page et al. 1988, Barnett et al. 1989, Sherry et al. 1986).

In establishing the control tests for the neutralization assays, it was noted that the MAb2s did not reduce the degree of viral infectivity. Immunofluorescence assays using non-infected LLC-MK2 cells with each of the anti-idiotypic antibodies demonstrated no recognition of cellular structures by the MAb2s. This finding is contrary to the evidence which thus far has met the criteria for the Ab2 $\beta$  classification of the MAb2s. This evidence thus far being the binding to MAb/ev-12 at a paratope associated idiotope, the inhibition of MAb/ev-12 anti EV-70 activity and the ability to elicit a specific protective anti EV-70 Ab1-like response. The means by which these MAb2s were generated brings to question the method by which MAb/ev-12 neutralizes the virus and the location of the MAb/ev-12 recognized epitope relative to the viral attachment site on the virion surface. If the neutralization epitope and the viral attachment site are two different sites, then the resulting MAb2s will not possess the internal image of the viral attachment site and thus not recognize any cellular structures. In this situation, virus

neutralization by inhibition of viral attachment will only occur if these two sites are proximal to one another. In the case of picornaviruses, the targets of neutralizing MAbs are the epitopes found along the rim of the canyon structure. The viral attachment sites which are targeted by cellular receptors are located on the floor of the canyon structure (Colonno et al. 1988, Pevear et al. 1989). The narrowing of the canyon structure prevents penetration of the F(ab) arm of the antibody to the canyon floor which in turn shields the viral attachment site on the floor. In this structure, the neutralization epitope and viral attachment site are two independent locations. However, they are close enough that recognition of the epitope will block access to the attachment site on the canyon floor. This type of structure may explain the inability of our MAb2s to recognize the corresponding cellular structures in a manner similar to that postulated for a coxsackievirus B4 anti-idiotypic model (McClintock et al. 1986). The existence of such a structure on EV-70 can only be hypothesized based on the structural homology known to exist amongst other picornaviruses. Confirmation of such a feature will require X-ray crystallography studies.

Functional differentiation of the anti-idiotypic antibodies can be achieved by analyzing the Ab3 immune response elicited following immunization with the MAb2s. The characteristics of

this immune response will dictate the practicality of the anti-idiotypic antibody as a surrogate vaccine. The immunization regime used in this study for the production of Ab3 antiserum resulted in the elicitation of high anti-anti-idiotypic titres. These titres were measured in competition assays using Ab3 antisera to inhibit MAb/ev-12:MAb2 binding as depicted in Table 4. The greatest degree of inhibition of this binding was found with the homologous Ab3:MAb2 competitions. This was indicative of the greater degree of specificity of each Ab3 antiserum for the homologous MAb2 rather than for the heterologous MAb2. This also suggests that despite the presence of shared paratope associated idiotopes, differences do exist between each of the MAb2s. The wide range of inhibition levels recorded in the heterologous Ab3:MAb2 competitions implies that the Ab3 antibodies elicited by each of the MAb2s reflect these differences and thus are distinct. This distinctiveness applies not only to the physical structure of the antibody, but also to the regulatory role that each of the resulting Ab3 antibodies may play in the immune system. The anti MAb2-2 antiserum produced the highest and most consistent degree of inhibition of the heterologous competitions. The range of inhibition recorded for the anti MAb2-9 antiserum was also high and relatively consistent. The levels of inhibition recorded with the remaining three

antisera demonstrated a wider range of variability. Of interest were the heterologous levels of inhibition which were responsible for the widest variations. The reciprocal inhibition levels for the anti-idiotypic and the antiserum in question do not match. This is exemplified by; i) MAb2-13 vs anti MAb2-2 antiserum and MAb2-2 vs anti MAb2-13 antiserum, ii) MAb2-15 vs anti MAb2-2 antiserum and MAb2-2 vs anti MAb2-15 antiserum, and iii) MAb2-15 vs anti MAb2-9 antiserum and MAb2-9 vs anti MAb2-15 antiserum. The lack of inhibition seen in one of the competitions within each group suggests the possibility that i) a shared paratope associated idiotope on one of the MAb2s has a lower affinity for the heterologous Ab3 which is therefore easily displaced from the MAb2 in the presence of the bound F(ab)'<sub>2</sub> of MAb/ev-12 or, ii) that the homologous and heterologous MAb2s share idiotopes possessing a degree of cross-reactivity, but the idiotope on the heterologous MAb2s is that of a framework idiotope which does not completely inhibit with the MAb2:MAb/ev-12 binding.

The evidence from Table 4 clearly supports the induction of an anti-anti-idiotypic response following immunization with each of the MAb2s. Of critical importance at this point was the confirmation of the Ab1-like properties of the Ab3 antiserum. This confirmation was crucial to the legitimacy of these MAb2s as potential surrogate immunogens. Numerous other

anti-idiotypic models have successfully induced a specific neutralizing antiviral response (Anders et al. 1989, Suñé et al. 1991, Uytdehaag et al. 1986). In this study, the induction of a specific neutralizing anti EV-70 response was recorded in nine of fifteen mice immunized. All three mice immunized with the MAb2-2 neutralized 50% or more of the input virus at an Ab3 serum titre of 1/25. This anti-idiotypic clone was also coincidentally the most reactive clone in the inhibition of the heterologous competition assays. Of the mice in the MAb2-9 and MAb2-15 group, two out of three neutralized 50% or more of the virus challenge at an Ab3 serum titre of 1/25. Relative to the heterologous competition assay, the same correlation may be drawn between these two assay results regarding the MAb2-9 group. The wide range of inhibition levels seen with the mice receiving MAb2-15 does not lend itself to a similar conclusion. Within the remaining two groups, only one mouse in each neutralized more than 50% of the input virus at an Ab3 serum titre of 1/25.

The mice immunized in this series all received Quil A as the accompanying adjuvant. As adjuvants, Quil A and others such as Freund's complete adjuvant and a Syntex adjuvant formulation vehicle (SAF) have been thought to improve antibody expression by enhancing the proliferation of immunologically competent cells rather than through the

recruitment of new antigen specific clones (Flebbe and Bradley-Mullen 1986, and Poskitt et al. 1991c). A comparative study of different adjuvants demonstrated that various characteristics of the antibody response to antigen could be influenced by the adjuvant used (Kenney et al. 1989). In our model system, the used of Freund's adjuvant or Quil A with MAb2-2 and MAb2-15 did not produce any difference in the neutralization titres elicited from a group of eight mice tested. Other variables such as dosage and frequency of immunization may prove to be more influential in immune responses to anti-idiotypic vaccines especially since the immunogen is a functional component of the immune system and capable of interacting with the regulatory pathways within it.

In view of the means by which the idiotype immunogens were made and the type of anti-idiotypic responses that were generated, it can be concluded that the level of fidelity with which the anti-idiotypic network produced an image of the viral epitope at the MAb2 paratope was sufficient to elicit Ab1-like antibodies. MAb/ev-12 was found to be specific only for the prototype strain when tested. The elicited Ab3 antibodies did not neutralize any of the alternate EV-70 strains, just as MAb/ev-12 did not. This maintenance of idiotope fidelity and the resulting preservation of strain specificity has been previously demonstrated with the reovirus

type 3 hemagglutinin model (Gaulton et al. 1986, Sharpe et al. 1984). However, a lack of fidelity resulting in the subsequent loss of restricted strain specificity is also possible as seen with influenza and human respiratory syncytial virus anti-idiotypic models (Anders et al. 1989, Palomo et al. 1990). The specificity of the Ab3 response was also demonstrated by radio-immunoprecipitation assay. Ab3 sera produced the same viral precipitation pattern as that seen with MAb/ev-12. A further indication of the Ab1-like nature of the Ab3 antibodies was the ability of the MAb2 to inhibit the Ab3 neutralization of EV-70 in the same manner that MAb/ev-12 neutralization of the virus was inhibited. In this case, three of the MAb2 clones were used to target the specific anti EV-70 antibody fraction of the Ab3 antiserum in three separate assays. Each of the assays was done using homologous Ab3 sera and MAb2 ascites fluids. The titre at which neutralization first becomes evident is unique to each homologous pair. This titre is dependent on the Ab3 anti EV-70 titre and the anti MAb/ev-12 titre of the ascites fluid.

Together, these results clearly establish that neutralizing antibodies for enterovirus-70 can be induced in mice by immunization with syngeneic monoclonal anti-idiotypic antibodies. Analysis of the antiviral antibodies suggested that both MAb/ev-12 and Ab3 antibodies have exactly the same

specificity.

**Chapter 6****CONCLUSIONS**

In conclusion, it can be stated that achievement of each secondary objective lead to the attainment of the principal goal of this research project as set out in the statement of objectives. The application of the panel of EV-70 specific monoclonal antibodies as diagnostic reagents would provide rapid and efficient confirmatory evidence of EV-70 acute hemorrhagic conjunctivitis. The production of the anti-idiotypic monoclonal antibodies used in this model was accomplished with an EV-70 strain restricted neutralizing MAb1 - MAb/ev-12. All of the monoclonal anti-idiotypic antibodies were capable of blocking the recognition of EV-70 by both MAb/ev-12 and EV-70 specific Ab3 antisera. It was of interest however that none of these MAb2s were able to inhibit the neutralizing ability of the anti EV-70 hyperimmune serum. This suggested the possible existence of additional neutralizing antibodies in the hyperimmune serum which would reflect the presence of alternative neutralization epitopes on the virion. The use of these monoclonal anti-idiotypic antibodies as surrogate vaccines proved to be successful, with some MAb2s being more successful than others. The maintenance of the restricted strain specificity of the Ab3 antisera implied the

existence of a higher level of fidelity in the idiotype-anti-idiotype network of this model than seen for other models. Although these MAb2s have yet to be differentiated as Ab2 $\beta$  or Ab2 $\gamma$  (Bona and Köhler 1984), they do meet the criteria of a network antigen (Köhler et al. 1989) since they induce the production of Ab1-like antibodies. Further refinement of the immunization regime used here may augment the level of in vitro protection produced by the Ab3 sera seen in this study.

The research work completed in this study lends itself very well to the continued pursuit of numerous tangential experiments. It would be of practical significance if additional IgG monoclonal antibodies directed against EV-70 neutralization epitopes shared amongst the various viral strains could be isolated and utilized in the production of anti-idiotypic EV-70 vaccines. The success of this syngeneic model encourages the application of these MAb2s in a xenogeneic model. The additional immune responses directed at strain or species specific markers will allow for the study of the hypothesized self-adjuvanting and self-carrier effects associated with xenogeneic immunizations.

**Chapter 7****SCIENTIFIC PUBLICATIONS AND ABSTRACTS****7.1 Publications**

- 1) WILEY, J.A., HAMEL, J. and BRODEUR, B.R. 1992. Monoclonal anti-idiotypes induce neutralizing antibodies to enterovirus - 70 conformational epitopes. J. Virol. (in press).
- 2) WILEY, J.A., BRODEUR, B.R., DIMOCK, K.D., and SATTAR, S. 1990. Neutralizing monoclonal antibody against enterovirus 70 reacts with viral proteins 1C and 1D. Viral Immunology, 3: 137-146.

**7.2 Abstracts**

- 1) WILEY, J.A., BRODEUR, B.R., DIMOCK, K.D. and SATTAR, S. Characterization of an enterovirus - 70 neutralizing epitope using murine monoclonal antibodies. AMERICAN SOCIETY FOR VIROLOGY. University of Western Ontario, London, Ontario, Canada. July, 1989.
- 2) WILEY, J.A., BRODEUR, B.R., DIMOCK, K.D., and SATTAR, S. Analysis of enterovirus - 70 epitopes using monoclonal antibodies. CONJOINT MEETING ON INFECTIOUS DISEASES. Ottawa, Ontario, Canada. November 1987.

- 3) WILEY, J.A., BRODEUR, B.R., DIMOCK, K.D. and SATTAR, S.  
Production d'anticorps monoclonal dirigés contre les  
protéines virales de l'enterovirus - 70. ASSOCIATION  
CANADIENNE-FRANCAISE POUR L'AVANCEMENT DES SCIENCES.  
Université d'Ottawa. Ottawa, Ontario, Canada. Mai, 1987.

## Chapter 8

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