

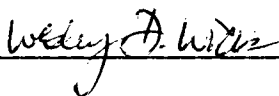
To the Graduate Council:

I am submitting herewith a thesis written by Deidrienne M. Fredin entitled "Studies on cross-reactive monoclonal antibodies to Sindbis and Semliki Forest virus E1 glycoproteins." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.



Carl J. Wust, Major Professor

We have read this thesis
and recommend its acceptance:



Accepted for the Council:



Lew Minkal
Vice Provost
and Dean of the Graduate School

STUDIES ON CROSS-REACTIVE MONOCLONAL ANTIBODIES
TO SINDBIS AND SEMLIKI FOREST VIRUS E1 GLYCOPROTEINS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Deidrienne M. Fredin

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DEDICATION

This thesis is lovingly dedicated to my fiancée, Monty Hatcher, without whose love, help and support, this would never have been finished, and to my brother, Cort Davin, whose patience, support and common sense helped more than he knows.

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ABSTRACT

Mouse MAbs were made against Sindbis (SIN) and Semliki Forest viruses (SFV), members of two different alphavirus subgroups. Some MAbs were found to react with the E1 glycoprotein of both viruses using the immunoblot technique. These MAbs can lyse SIN- or SFV-infected L929 cells in the antibody-dependent, complement-mediated cytotoxicity assay to high titer. Of four selected MAbs studied in depth, one is IgM, two are IgG2a, and one is IgG2b, and none neutralize either virus. The IgG MAbs were found to protect mice from lethal SFV infection, but the IgM MAb did not. Digestion of SFV virions with either chymotrypsin, Staphylococcal V8 protease or cyanogen bromide treatment generated some fragments, but no fragments smaller than Mr = 19,000 reacted with the MAbs. These data, suggesting that the protective epitope(s) defined by these MAbs is (are) probably dependent on conformation, are discussed.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
II. SURVEY OF THE LITERATURE	3
Description of Togaviruses	3
Alphaviruses	4
Replication of Alphaviruses	8
Cross-protection Across Alphavirus Subgroups	12
Monoclonal Antibodies (MAbs) Conferring Protection	14
Alphaviruses: Sindbis and Semliki Forest.	14
Other Alphaviruses: VEE and WEE	17
Flaviviruses	18
Other Viruses	19
III. MATERIALS AND METHODS	22
Cell Cultures	22
CEF	22
L929	23
Preparation of Virus Stocks	23
Crude virus stock	23
Purified virus stock	24
Injection stock of SFV	24
Plaque Assay Procedure	25
Production of Extended Hyperimmune Antisera	25
Gamma Globulin Precipitation	26

CHAPTER	PAGE
III. (Continued)	
Production of Monoclonal Antibodies	26
Antibody-Dependent, Complement-Mediated Cytolysis	29
Neutralization Assay	30
Passive Protection	31
Enzyme-Linked Immunosorbent Assay (ELISA)	31
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)	32
Immunoblotting	33
Digestion of El	34
IV. RESULTS	36
Antibody-Dependent, Complement-Mediated Cytolysis (ADCMC)	36
Enzyme-Linked Immunosorbent Assay (ELISA)	46
Neutralization of Virus Infectivity	54
Passive Protection	61
Summary of Serological Reactivities	63
Prediction of Cross-Reactive Epitopes	63
Immunoblots of Digested SFV	70
Immunoblots of Cyanogen Bromide (CNBr)-Treated SFV El .	73
V. DISCUSSION	79
LIST OF REFERENCES	85
VITA	93

LIST OF TABLES

TABLE	PAGE
1. Classification of alphaviruses including associated diseases .	6
2. Summary of MAb information	28
3. Summary of ADCMC assays	49
4. Summary of ELISAs	59
5. Antiviral neutralizing activity of MAbs	60
6. Passive protection of mice against intraperitoneal SFV challenge	62
7. Summary of MAb reactivities	64
8. Amino acid sequences of probable antigenic determinants of SIN and SFV E1 glycoproteins	68

LIST OF FIGURES

FIGURE	PAGE
1. Titration of cross-reactive Ab by ADCMC with extensive hyperimmune antiSIN serum on SIN-infected, SFV-infected, L929 cells or mock-infected L929 cells	37
2. Titration of cross-reactive Ab by ADCMC with extensive hyperimmune antiSFV serum on SIN-infected, SFV-infected or mock-infected L929 cells	38
3. Titration of cross-reactive MAb by ADCMC with MAb 32-15-1-45 on SIN-infected, SFV-infected or mock-infected L929 cells	40
4. Titration of cross-reactive MAb by ADCMC with MAb 37-113-2-3 on SIN-infected, SFV-infected or mock-infected L929 cells	41
5. Titration of cross-reactive MAb by ADCMC with MAb 37-114-2-20 on SIN-infected, SFV-infected or mock-infected L929 cells	42
6. Titration of cross-reactive MAb by ADCMC with MAb 37-139-2 on SIN-infected, SFV-infected or mock-infected L929 cells	43
7. Titration of cross-reactive MAb by ADCMC with MAb 28-95-1 on SIN-infected, SFV-infected or mock-infected L929 cells	44

FIGURE	PAGE
8. Titration of cross-reactive MAb by ADCMC with MAb 28-91-1 on SIN-infected, SFV-infected or mock-infected L929 cells	45
9. Titration of cross-reactive MAb by ADCMC with MAb 37-145-1-1 on SIN-infected, SFV-infected or mock-infected L929 cells	47
10. Titration of cross-reactive MAb by ADCMC with negative control antiRSV ascites on SIN-infected, SFV-infected or mock-infected L929 cells	48
11. Recognition of purified virions by ELISA with extensive hyperimmune antiSIN serum on SIN or SFV virions	51
12. Recognition of purified virions by ELISA with extensive hyperimmune antiSFV serum on SIN or SFV virions	52
13. Recognition of purified virions by ELISA with MAb 32-15-1-45 on SIN or SFV virions	53
14. Recognition of purified virions by ELISA with MAb 37-113-2-3 on SIN or SFV virions	55
15. Recognition of purified virions by ELISA with MAb 37-114-2-20 on SIN or SFV virions	56
16. Recognition of purified virions by ELISA with MAb 37-139-2 on SIN or SFV virions	57
17. Recognition of purified virions by ELISA with negative control antiRSV ascites on SIN and SFV virions	58

18.	Hydrophilicity and loop probabilities of Sindbis E1 glycoprotein and homology with Semliki Forest virus E1 glycoprotein	65
19.	Hydrophilicity and loop probabilities of Semliki Forest virus E1 glycoprotein and homology with Sindbis virus E1 glycoprotein	66
20.	Immunoblot reactions of chymotrypsin-digested SFV proteins with polyclonal antisera and MAbs	71
21.	Immunoblot reactions of Staphylococcal V8 protease-digested SFV proteins with polyclonal antisera and MAbs	74
22.	Immunoblot reactions of CNBr-treated SFV E1 with polyclonal antisera and MAbs	76

CHAPTER I

INTRODUCTION

Alphaviruses are small, enveloped, positive-stranded RNA viruses that can infect animals and humans causing a variety of disease symptoms ranging from fever and hemorrhage to fatal encephalitis. They are transmitted by arthropod vectors, i.e., mosquitoes and ticks. The viruses are classified into subgroups on the basis of serological neutralization assays but there can be extensive cross-reactivity between subgroups in hemagglutination-inhibition (HAI) and complement fixation testing. Although there is no serological cross-neutralization between subgroups, immunization with a member of one subgroup can lead to cross-protection against the lethal challenge of members of a second subgroup (Casals, 1963).

The two viruses used in this study, Sindbis (SIN) and Semliki Forest virus (SFV), are representatives of two different alphavirus subgroups. As prototypes of cross-protection among the alphaviruses, immunization with SIN will protect mice against a lethal dose of SFV with no detectable cross-neutralizing antibodies (Casals, 1963).

SIN has two membrane-bound glycoproteins, E1 and E2, while SFV has three glycoproteins, E1, E2 and E3, embedded in the lipid bilayer envelope. The E2 glycoprotein contains the epitopes responsible for neutralization, whereas hemagglutination as well as cross-protective epitopes are found on the E1 glycoprotein (Dalrymple, 1976; Wolcott,

1982; Wolcott, 1985). Studies have shown that the conformation of the E1 on the infected cell surface may be different from that of E1 on virions (Wolcott, 1985).

Monoclonal antibodies (MAbs) have been used extensively to determine protective epitopes on a number of different viral proteins (Dix, 1981; Balachandran, 1982; Chanas, 1982; Mathews, 1982; Roehrig, 1982; Schmaljohn, 1982; Boere, 1983, Boere, 1984, Mathews, 1984; Boere, 1985a, Hunt, 1985; LeFrancois, 1985; Boere, 1986; Stanley, 1986). The summation of these studies indicates that there is no correlation between the immunoglobulin isotype and the possession of an in vitro biological function, such as HAI or neutralization, and in vivo protection against lethal virus challenge (Balanchandran, 1982; Chanas, 1982; Mathews, 1982; Roehrig, 1982; Schmaljohn, 1982; Boere, 1984; Boere, 1985; Hunt, 1985; Roehrig, 1985; Boere, 1986; Stanley, 1986); however, there is a positive correlation between the ability of the MAb to lyse infected cells (in the presence of complement) in vitro and its ability to protect in vivo (Schmaljohn, 1982; Mathews, 1985; Boere, 1986; Stanley, 1986).

In the present study, cross-reactive MAb to E1 glycoprotein of SIN and SF were found to lyse SIN- and SF-infected cells in vitro and the IgG isotypes protected mice from SF challenge. In addition, several digestions by proteolytic enzymes and CNBr produced fragments that were unreactive with the MAbs by immunoblotting. These data suggest that there are protective, non-neutralizing epitopes on E1 glycoprotein which are conformation-dependent.

CHAPTER II

SURVEY OF THE LITERATURE

Description of Togaviruses

The family Togaviridae contains small positive-stranded RNA viruses, in which there are four genera: arterivirus, rubivirus (Rubella), pestiviruses and alphaviruses (Westaway, 1985). The viruses of each genus are related serologically, but there is no serological relationship between genera nor is there any significant RNA homology between genera.

Most togaviruses that cause disease are transmitted by arthropods (e.g., mosquitos and ticks). The virus replicates in the arthropod, first infecting the gut and then passing the gut barrier and traveling to the salivary glands. The arthropod never becomes sick. Transmission of the virus is nearly always by the bite of an infected arthropod. While the majority of vertebrate infections are subclinical, in some cases, symptoms such as arthritis, rash, encephalitis and hemorrhage can occur. Viremia often precedes these symptoms, and thus, the virus can be transmitted from the host to potential vectors, where the transmission cycle is continued. However, most humans and domestic animals are "dead-end" hosts in that they do not develop a sufficiently high enough viremia to infect biting arthropods. There are other unusual methods of transmitting togaviruses: aerosolization during laboratory manipulations, by blood

transfusions or from contact with the blood or milk of infected livestock (Shope, 1985a).

Alphaviruses

Alphaviruses have remarkably similar morphology. They are spherical particles 60-65 nm in diameter as seen by negative staining in the electron microscope. Surface filaments are 6-10 nm in length and project through a bilayer envelope that surrounds an icosahedral nucleocapsid which is 30-35 nm in diameter (Shope, 1985).

Alphaviruses contain single-stranded RNA which is positive sense, capped at the 5' end, polyadenylated and infectious. This genome is 42S RNA with a molecular weight (MW) of 4.4×10^6 daltons (4400K). It is bound closely to the nonglycosylated capsid protein (C) (MW = 30K), and the nucleocapsid complex is enveloped by a lipid bilayer derived from the host cell plasma membrane upon budding of the virus from the surface of the cell. Glycoproteins span the envelope, embedded in the membrane via hydrophobic peptide segments and being anchored to the nucleocapsid at the carboxy-terminal end of the glycoprotein spike by electrostatic associations. The sugar moieties on the glycoproteins are variable depending on the host cell that is infected. There are several non-structural proteins synthesized during the replication cycle that have several functions including the enzyme, an RNA-dependent RNA polymerase (Shope, 1985).

The two alphaviruses used in this study were Sindbis (SIN) and Semliki Forest virus (SFV). SIN has two surface glycoproteins, E1

(52K) and E2 (50K), that complex to form a heterodimer spike arranged in a trimer cluster. SFV has three surface glycoproteins, E1 (49K), E2 (52K) and E3 (10K), that complex to form a heterotrimer spike protein arranged in trimer clusters.

The genus alphavirus is subdivided by the serological hemagglutination-inhibition (HAI) test into three complexes: the Venezuelan encephalitis (VE) complex whose members cause encephalitis in both humans and horses, the Western encephalitis (WE) complex of which SIN is a member and the Semliki Forest (SF) complex of which SFV is a member (see Table 1). There is extensive serological cross-reactivity among these subgroups (i.e., HAI and complement fixation, especially with the capsid protein), but there is no cross-neutralization between subgroups. However, vaccination with one alphavirus can often result in protection against challenge by members of the same subgroup or by members of a different subgroup, even in the absence of cross-neutralizing antibodies. The primary basis for defining the species of alphaviruses is the plaque-reduction neutralization assay.

The group-specific reactivity is found in the C protein which is highly conserved. The complex-specific (e.g., WE, VE, SF) antigen is found on the E1 glycoprotein (hemagglutination) while the species-specific antigen is on the E2 glycoprotein (neutralization) (Shope, 1985b).

Alphaviruses can be grown in nearly all vertebrate cells, where they produce cytopathic effect (CPE) and plaques in cell culture

Table 1. Classification of alphaviruses including associated diseases (adapted from Shope, 1985).

Complex	Virus	Equine Disease	Human Disease
Venezuelan encephalitis	Venezuelan encephalitis (VEE) Everglades Mucambo Piyuna Cabasson	Encephalitis	Fever, encephalitis Encephalitis Fever
Western encephalitis	Western encephalitis (WEE) Y62-33 (USSR) Highlands J Fort Morgan Sindbis Aura	Encephalitis	Encephalitis Fever, rash, arthritis
Semliki Forest	Semliki Forest Chikungunya O'nyong-nyong Getah Ross River Mayaro	Fever, rash, edema	Encephalitis Fever, rash, arthritis Fever, rash, arthritis Fever, rash, arthritis Fever, rash, arthritis
Others	Eastern encephalitis (EEE) Ndumu Middleburg Barmah Forest		

monolayers. They also replicate well in mosquito (invertebrate) cell lines, indicating the broad host range of these viruses; however, they do not cause CPE in these cells and appear to develop persistent infections.

Encephalitis caused by alphaviruses (VE and WE complexes mostly; SF complex only rarely) is an acute, necrotizing disease of the central nervous system (CNS) accompanied by lysis of the neurons, destruction of neurons by phagocytic cells, and gliosis. The damage to neurons can be irreversible and the disease fatal. Alphaviruses can also cause acute arthritis and rash (WE and SF complexes), but these conditions are not fatal.

The level of viremia is critical for the virus to reach its target organ (e.g., the CNS), and young animals are more susceptible than older ones. Both antibody and cell-mediated immunity (CMI) are important defense mechanisms in alphavirus infections.

Alphavirus-caused encephalitis has been a major problem in horse and human populations worldwide. Since the disease is fatal in some cases (Western Equine Encephalomyelitis [WEE] virus is 50% fatal; Venezuelan Equine Encephalomyelitis [VEE] virus is 80% fatal), the demand for a good vaccine is high. A vaccine of inactivated virus is currently being used for WEE, but it is not yet licensed. There is no treatment for VEE or Eastern Equine Encephalomyelitis (EEE) virus. These three viruses have been responsible for the deaths of over 200,000 horses and 20,000 humans in various outbreaks and epidemics (Shope, 1985b). The vaccines containing inactivated virus achieve only

variable amounts of homologous antibody titers and little to no cross-reactive antibody titers, emphasizing the need for better vaccines (Roehrig, personal communication).

Replication of Alphaviruses

The replication of an alphavirus begins with the binding of the virus to a susceptible host-cell surface in which the specific host-cell receptor is still unknown (Oldstone, 1980). Once bound to the host-cell surface, the virus can enter the cell via an adsorptive endocytosis pathway, taking advantage of the cellular mechanism for internalizing receptor-ligand complexes (March, 1980). In this pathway, the viruses bound to the cell surface accumulate in clathrin-coated pits which are endocytosed as receptosomes or endosomes. These endosomes then fuse with lysosomes and become acidified which appears to trigger a conformational change in the viral glycoproteins allowing for fusion of the viral membrane with the vesicle membrane (Edwards, 1983; Kielan, 1985).

The mechanism for transferring the RNA genome to the cytoplasm in order to begin replication is still unclear. It is assumed, however, that the nucleocapsid is flexible enough to allow the RNA to dissociate when it enters the cytoplasm. For SFV, it has been shown that exposure to low pH causes an irreversible 15% contraction in the nucleocapsid (Soderlund, 1972) which could release the RNA (Schlesinger, 1985).

The virion genome is capped with a 5' 7-methyl-guanosine and is polyadenylated at the 3' end, so it functions as messenger RNA (mRNA)

once it is released from the capsid. Ribosomes bind at a start codon that is 59 nucleotides from the cap (Cancedda, 1975; Glanville, 1976). Translation proceeds for 2/3 the length of the 42S RNA, and a large precursor polyprotein is produced which is subsequently cleaved into four nonstructural polypeptides, NS60, NS89, NS76, and NS72 (Schlesinger, 1985). The stop codon for this precursor resides just upstream from the 5' end of a subgenomic 26S RNA. This latter RNA species contains all the necessary activities and recognition signals needed for the translation and processing of the virus structural proteins (Rice, 1985).

The nonstructural proteins catalyze replication and transcription of the virus genome, which appears to take place on host-cell membranes. A viral-encoded RNA-dependent RNA polymerase polymerizes full-length minus-strand RNAs from the genomic template and two mRNAs, a 42S and a 26S, are transcribed off the minus strand. The other nonstructural proteins are responsible for capping these messages as well as providing protease activity to cleave the nonstructural proteins from the precursor polyprotein. Little information is available on the precise functions of these proteins, and there are probably some host cell proteins involved in the process of replication. Togaviruses are known to shut off host-cell macromolecular synthesis shortly after infection. The nonstructural proteins, however, are mostly made early during the infection before the host cell machinery is shut off, and are difficult to detect after three to four hours p.i. (Schlesinger, 1985).

Once translation of the 42S RNA is underway, several other pathways emerge and compete. The negative strand 42S RNA species can be transcribed in full from positive-stranded 42S RNA or from an internal initiation codon to produce plus-stranded 26S RNA. The larger RNA can bind to ribosomes and be translated to produce nonstructural proteins, or it can serve as a template for negative-strand synthesis. Later as capsid protein accumulates, the positive-stranded 42S RNA binds to it and is packaged into the nucleocapsid. Regulation of these three events is presently unknown, but capsid appears to play an important role (Schlesinger, 1985).

After minus-strand synthesis ceases, the synthesis of plus-strand 42S and 26S continues at a constant rate. Most of the 26S complexes are found bound to polysomes for the translation of the structural proteins. This is observed as early as two to four hours p.i. One large polyprotein, 130K, is translated from the 26S RNA since there is only one initiation site for translation. The coding sequence on the 26S RNA is capsid-precursor for E3-E2(p62)-E1 (Clegg, 1975). The first co-translational cleavage releases the capsid protein, probably due to an autoprotease present in the capsid protein (Alpert, 1978; Hahn, 1985).

Translation proceeds after the capsid protein has been cleaved. A hydrophobic signal sequence interacting with the host-cell signal recognition particle (SRP) system directs the transmembrane translocation of the p62 protein into the lumen of the endoplasmic reticulum (ER) (Bonatti, 1984). The transfer of p62 is halted after a

hydrophobic region is translated which anchors the p62 end in the membrane (Cutler, 1986). As translation of the remainder of the 26S RNA is continued, it produces a 6K protein which contains the signal sequence that directs the E1 into the ER lumen using the same SRP system as p62 (Hashimoto, 1981; Melancon, 1986). E1 is translated vectorially into the ER lumen until a hydrophobic sequence, following the coding sequence for E1, stops transport. The p62 is then released from the polyprotein by a protease. Oligosaccharides are added by host-cell enzymes to both p62 and E1 in the ER as protein synthesis proceeds (Sefton, 1977; Schlesinger, 1985). Two co-translational cleavages, one at the p62-6K junction and the other at the 6K-E1 junction, release the 6K protein.

Shortly after glycosylation, p62 and E1 complex to form a heterodimer. The heterodimer is moved to the Golgi stack, probably through endosomes (Saraste, 1984). Further processing, such as the conversion of simple oligosaccharides to complex ones, fatty acid acylation and the trimming of the terminal carbohydrates, occurs in the Golgi complex. All of this activity is done by host-cell enzymes. The orientation of the glycoproteins at this point is such that the spike portion which eventually will be protruding from the viral membranes is in the internal Golgi compartment (Jones, 1977; Schmidt, 1980; Simons, 1982). p62 is cleaved into E2 and E3 either in the trans-Golgi or on the post-Golgi pathway to the cell surface and is essential for virus maturation (Simons, 1984; Schlesinger, 1985). In SFV, the E3 remains

associated with the glycoproteins, but in SIN, the E3 is lost into the medium.

The final steps in alphavirus replication involve the assembly of the nucleocapsids and the glycoproteins within the cell membrane. The glycoproteins are transported to the plasma membrane by the host-cell pathway for cell-surface receptors where small patches of glycoproteins on the cell surface attract nucleocapsids which recognize the carboxy-terminus of E2 and bind by electrostatic interactions. The binding of the nucleocapsids freezes the glycoproteins in place, causing the membrane to fold around the nucleocapsid. When it is completely enclosed by the membrane, it appears to pinch off and release mature virion (Ziemiecki, 1980; Simons, 1984, Schlesinger, 1985).

Cross-protection Across Alphavirus Subgroups

Infection or immunization with one alphavirus has been shown to protect against later infection or challenge with some heterologous alphaviruses in the absence of cross-neutralizing antibodies (Abs) (Casals, 1963). Work done to elucidate the in vivo mechanism of this cross-protection showed that cell-mediated immunity (CMI) is involved (Peck, 1975). In adoptive transfer experiments where donor animals were immunized with SIN, protection against challenge with SFV was conferred by T-cell enriched populations only.

Cross-reactive antibodies were also found using an antibody-dependent, complement-mediated cytotoxicity (ADCC) assay on

virus-infected cells with heterologous antisera produced after extensive immunization (King, 1977). Thus, extensive hyperimmune antiserum raised against one virus was able to recognize heterologous viral antigens on infected cells. These antisera contained no cross-neutralizing Abs.

Further work with these cross-reactive, cytolytic antisera suggested that cell-associated viral determinants, rather than virion-associated ones, were involved in the cross-reactivity and, by inference, cross-protection (Gates, 1982). Absorption of antisera with heterologous virus-infected cells completely removed the cross-cytolytic activity of the sera while heterologous virions as well as infected cells did, however, remove cross-HAI Abs. Also, if the antisera were raised against inactivated virus, no cross-cytolytic activity was found, further suggesting a role for a cell-associated viral antigen in cross-reactions between alphaviruses.

Temperature-sensitive mutants of SIN were used to identify the viral protein that is the target for cross-cytolysis (Wolcott, 1982). Only those mutants able to express viral E1 glycoprotein at the cell surface were recognized by heterologous, anti-SFV serum in an ADCMC or by cross-reactive cytotoxic immune cells (Wolcott, 1985).

Evidence for a unique conformation of the E1 on the infected cell surface was found (Wolcott, 1984). E1 from detergent-treated SIN virions was recognized by anti-SFV serum or immunoblots, whereas E1 from detergent-disrupted infected cells was not. However, the latter E1 can be immunoprecipitated from infected cell lysates by

heterologous serum. These findings suggest that the cell-associated E1 is in a different conformation or stage of maturation than the virion E1.

Monoclonal Antibodies (MAbs) Conferring Protection

During a viral infection, a variety of polyclonal Ab populations are elicited to the viral proteins. Some possess hemagglutination-inhibition (HAI) activity; others can neutralize (N) the virus in vitro and protect animals from homologous challenge in vivo, while still others protect but do not neutralize or bind to virions in vitro. Some antibodies can fix complement and are involved in antibody-dependent, complement-mediated cytolysis of infected cells in vitro. These latter Abs have been correlated with passive protection for both homologous and heterologous virus challenge in vivo, although the protection did not depend on the activity of complement. The precise roles or relative importance that each subset in the population plays in mediating protection or recovery in an animal are unknown (Roehrig, 1982; Schmaljohn, 1982; Stanley, 1986).

The use of monoclonal antibodies has simplified the study of virus neutralization and protection by allowing the focus to be on the role of one viral component. The inherent monospecificity of MAbs ensures that only one determinant is studied at a time (East, 1982).

Alphaviruses: Sindbis and Semliki Forest

Schmaljohn, 1982 found that both neutralizing (i.e., anti-E2) and non-neutralizing (i.e., anti-E1 or anti-E2) MAbs were protective

against challenge with a lethal neurovirulent strain of SIN. All protective MAbS, either anti-E1 or anti-E2, were of an IgG isotype, predominantly IgG2a, and could lyse infected cells in the presence of complement.

Chanas, 1982 isolated two anti-SIN E1 MAbS: one that had both N and HAI activities and one that had no biological activity in vitro. The finding that anti-E1 MAbS had N activity disagrees with an earlier report in which it was concluded that N epitopes reside only on the E2 (Dalrymple, 1976). Roehrig, 1982 argued that the neutralizing activity of the anti-E1 MAbS could be due to the close spatial arrangement of the E1 and the N epitope on E2. Both of the anti-E1 MAbS were of the IgG2a isotype and could be used to protect newborn mice from an intracerebral challenge of SIN.

Stanley, 1986 generated a panel of 30 different MAbS to a neurovirulent strain of SIN (NSV). Only MAbS specific for the glycoproteins of SIN were able to protect against homologous challenge in vivo, i.e., no anticapsid MAbS conferred protection. Of the protective MAbS, 23 had some in vitro biological function, such as neutralization, HAI or ADCMC. Several other anti-E1 MAbS were able to neutralize and inhibit HA. Conversely, some anti-E2 MAbS had HAI and/or N activity. In general, anti-E2 MAbS with N activity protected mice better against homologous challenge than did any other MAbS regardless of in vitro biological activity. The majority of protective MAbS were either IgG2a or IgG2b isotypes, although one was IgG1, and some were IgG3. Another interesting feature of this study was that

some MAbS were able to exert their influence after challenge with NSV. This feature distinguishes a role for antibody in prophylaxis as opposed to treatment before onset of infection.

A similar study was done with MAbS to SFV (Boere, 1983; Boere, 1984; Boere, 1985a; Boere, 1985b). Using a panel of MAbS to the glycoproteins of SFV, six epitopes on the E1 and five epitopes on the E2 were identified. Of the six epitopes on E1, three defined N epitopes (E1^a, E1^b, and E1^f), two defined HAI sites (E1^c and E1^d) and one had no biological activity in vitro (E1^e). MAbS to epitopes E1^a, E1^b, E1^c, E1^e, and E1^f protected mice from lethal SFV challenge. The non-protective, HAI-positive MAb (anti-E1^d) was an IgG₁ isotype and had a relatively low avidity for SFV as demonstrated with ELISA. The protective anti-E1 MAbS were either IgM or IgG2a isotypes. Of the five MAb-defined epitopes on E2, two defined HAI sites (E2^b and E2^e), two defined neutralization sites (E2^c and E2^d) and one had no in vitro biological functions (E2^a). MAbS to epitopes E2^a, E2^c, and E2^d but not to E2^b and E2^e were protective for in vivo challenge with SFV. Protective anti-E2 MAbS were either IgG₁, IgG2a, or IgG2b. A positive correlation between in vitro ADCMC and in vivo passive protection to homologous challenge was found (Boere, 1986).

Two important points can be made from these studies. Firstly, there is no correlation between in vitro biological functions, such as N or HAI, and passive protection since MAbS with no N or HAI activity protected mice in vivo and HI-positive MAbS did not. Secondly, these

findings strongly support the hypothesis that antigens on infected that antigens on infected cells are the target antigens involved in protection and not those on viruses (i.e., neutralization or HAI epitopes), because a positive correlation between ADCMC and in vivo protection was a consistent feature.

From the anti-E2 MAbs that protected, one was chosen for further study because of its high N titer (anti-E2^d) (Boere, 1985a). This MAb appeared to identify a "critical" N site. Such a "critical" N site was also noted in studies with MAb to VEE (Mathews, 1982; Roehrig, 1985). MAbs to the N site on SFV were successfully used for long-term prophylaxis, e.g., the MAbs could be given four weeks before challenge, and for short-term therapy against SFV challenge (24 hours prior).

Other Alphaviruses: VEE and WEE

VEE has two surface glycoproteins designated E1 and E2. Unlike SIN and SFV, both HAI and N activities are attributed to sites on the E2 glycoprotein. Immunization against VEE strain TC83 elicited MAbs to both E1 and E2 (Mathews, 1982; Roehrig, 1982; Roehrig, 1985). The in vitro neutralization functions were primarily attributed to one site on the E2 (E2^c). MAbs to this site, and other MAbs with high avidities to sites on E1, were shown to passively protect weanling mice from lethal VEE challenge. All the MAbs used protected mice, but those to the "critical" N site protected better, i.e., lesser amounts of MAb were required for passive protection. The N site was later shown to be resistant to treatment with SDS and beta-mercaptoethanol and appeared

to be present on several other subtypes of VEE. A positive correlation between in vitro ADCMC and passive protection in vivo was noted. These latter findings also support the hypothesis that infected-cell antigens are the target antigens involved in protection.

WEE is an alphavirus with two surface glycoproteins, E1 and E2. There is some ambiguity regarding the functions attributable to these glycoproteins (Yamamoto, 1985). Purified E1 has HA activity, and some anti-E1 MAbs possess HAI activity, but anti-E2 MAbs also inhibit HA by WEE. Hunt, 1985 made a panel of MAbs to the E1 of WEE and used them to passively protect mice from WEE challenge. Using infected mouse brain antigens in an ELISA, eight determinants were defined by this set of MAbs. Some were specific for WEE, while others reacted with other alphaviruses from other complexes (i.e., VEE, EEE, and SFV) as well as viruses from the WEE complex, e.g., SIN and Highlands J (cf. Table 1). Three epitopes, an alphavirus group-reactive HA site, a WE-complex reactive HA site and a WEE-virus specific HA site, were identified. No neutralizing activity was associated with any of these anti-E1 MAbs. MAbs protective against WEE were confined to those that specified the alphavirus group-reactive site. MAbs to the complex-specific site were only able to protect half the mice challenged with WEE.

Flaviviruses

Protective MAbs to the one structural glycoprotein of Saint Louis encephalitis (SLE) virus, a flavivirus, have also been studied

(Mathews, 1984). One MAb with high N and HAI activity was able to passively protect weanling mice from SLE challenge given 24 hours later. Mixtures of MAbs with different epitope specificities were found to enhance the protection.

Tick-borne encephalitis (TBE) virus is also a flavivirus with only one glycoprotein, E, that possesses HA, N and protective epitopes. Heinz, 1984 digested TBE E glycoprotein with trypsin, chymotrypsin and CNBr and found fragments from each digestion that reacted with neutralizing MAbs. Furthermore, immunization with these fragments (Mr = 9K for trypsin or chymotrypsin, and 19K for CNBr) elicited Abs reactive with the peptide, denatured glycoprotein and native glycoprotein. Abs to fragments also showed N and HAI activities to the whole virus. The fragments may represent useful reagents for a synthetic vaccine, but no passive protection experiments were done in these studies.

Other Viruses

Monoclonal antibodies to herpes simplex virus type 1 (HSV-1) glycoproteins have been shown to be protective against acute neurological disease (Dix, 1981). MAbs to the gC and gD glycoproteins were able to protect against HSV-1 passively transferred to mice. Both of these MAbs neutralized HSV-1 in vitro, but only the MAb to gD could neutralize HSV-2, another herpesvirus in vitro. Likewise, only MAbs to gD protected against HSV-2 challenge in mice.

Balachandran, 1982 found that MAbs to all five surface glycoproteins on HSV-2 were protective in vivo. From a panel of MAbs of either IgG2a or IgG, isotypes, anti-gAB, anti-gD, anti-gE, and anti-gF and all but one anti-gC protected BALB/c mice from lethal doses of HSV-2. None of these MAbs showed any in vitro neutralizing activity. These same MAbs also protected complement-deficient mice and showed a good correlation between their in vitro antibody-dependent, cellular cytotoxicity (ADCC) activity and in vivo protection.

Neutralizing and non-neutralizing MAbs to the glycoprotein of vesicular stomatitis virus (VSV), a rhabdovirus, were able to protect mice (LeFrancois, 1984). Some of these MAbs protected against other serotypes of VSV in the absence of cross-neutralization. On the other hand, neutralizing MAbs were better in protecting mice from homologous challenge than were non-neutralizing ones. Protection from VSV challenge did require that the MAbs be given before the challenge, i.e., detectable levels of circulating Abs were necessary at the time of challenge.

In conclusion, MAbs with either virus-neutralizing or non-neutralizing properties have been shown to protect mice from lethal challenge with homologous and closely related (same genus) viruses (Dix, 1981; Balachandran, 1982; Chanas, 1982; Mathews, 1982; Roehrig, 1982; Schmaljohn, 1982; Boere, 1983; Boere, 1984; Mathews, 1984; Boere, 1985a; Hunt, 1985; LeFrancois, 1985; Boere, 1986; Stanley, 1986). Generally, however, neutralizing MAbs protected better (Dix, 1981; Mathews, 1982; Schmaljohn, 1982; Boere, 1983; Hunt, 1985; LeFrancois,

1985; Stanley, 1986). Most protective MAbS were IgG2a isotypes, but other isotypes could also protect in some cases. Therefore, there is no correlation between immunoglobulin isotype and protection (Balachandran, 1982; Chanas, 1982; Mathews, 1982, Schmaljohn, 1982; Boere, 1983; Boere, 1984; Boere, 1986; Stanley, 1986). Some studies suggested a correlation between ADCMC and protection (Schmaljohn, 1982; Mathews, 1985; Boere, 1986; Stanley, 1986) while others did not (Heinz, 1984). In all cases, it is obvious that there is no correlation between the possession of in vitro biological activity such as HAI or N and protection, since some HAI-positive MAbS failed to protect while MAbS lacking these functions were protective (Balachandran, 1982; Chanas, 1982; Mathews, 1982; Roehrig, 1982; Schmaljohn, 1982; Boere, 1984; Boere, 1985; Hunt, 1985; Roehrig, 1985; Boere, 1986; Stanley, 1986). In at least two cases, the protective epitope defined by the MAbS was resistant to enzymatic cleavage (Heinz, 1984; Roehrig, 1985).

The work in this thesis was designed to test the reactivity of certain MAbS that were made available to me. We asked if MAb cross-reactive to El glycoprotein of SIN and SF by ELISA could also react by ADCMC and immunoblot, and if any would passively protect from SF challenge in vivo. We further asked if El glycoprotein could be degraded by a number of different treatments to yield a small fragment that would still react with the MAb in the hope that the cross-reactive epitope could be defined.

CHAPTER III

MATERIALS AND METHODS

Cell Cultures

CEF. Primary chicken embryo fibroblasts (CEF) were prepared from ten- to eleven-day old embryos from pathogen-free hen's eggs (Truslow Farms, Maryland). The eggs were first sprayed with 70% ethanol, then swabbed with 2% (w/v) iodine in 70% ethanol. The embryos were removed, decapitated and eviscerated, then washed five times in Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY) before being forced through a 20 ml syringe into Saline A (0.8% NaCl, 0.1% dextrose, 0.04% KCl, 0.4 ml of a 0.5% phenol red solution, pH 7.4) containing 0.025% trypsin (Gibco). The cells were trypsinized for one hour at 37°C with constant stirring. The resulting cell suspension was filtered through sterile gauze and pelleted by centrifugation at 800 x g for 15 minutes. The pellet was suspended in Medium-199 (M-199) (Gibco) supplemented with 0.075% sodium bicarbonate (Sigma, St. Louis, MO), 25 mM N-2 hydroxyethylpiperazine-N-2' ethane sulfonic acid (HEPES) (Sigma), 10 mM glutamine (Gibco), 5% fetal bovine serum (FBS) (Gibco) and 100 U/ml penicillin/streptomycin (Pen-Strep) (Gibco). After appropriately diluting, the cells were seeded into tissue culture flasks or roller bottles (Corning Glass Works, Corning, NY) and grown at 37°C in 5% carbon dioxide (CO₂) for up to one week.

L929 cells. L929 cells were also used in this study.

The L929 cell line was established in 1948 by K. Sanford, W. Earle, and G. Likely as a clone of a mouse connective tissue cell line designated Strain L. This latter cell line was derived from normal subcutaneous areolar and adipose tissue of a 100-day old C3H/AN mouse by Earle in 1940. Clone 929 was selected from the ninety-fifth subculture of the parent strain.

A stock of these cells were obtained from the Naval Biosciences Labs at the U. S. Naval Institute and were grown in McCoy's 5A medium (Gibco) supplemented with 25 mM HEPES, 5% FBS and 100 U/ml Pen-Strep in a 5% CO₂, 37°C incubator.

Preparation of Virus Stocks

Crude virus stock. Laboratory stocks of Semliki Forest virus (SFV) and Sindbis virus (SIN) were both used in this study. Virus stocks were prepared by infecting CEF monolayers with plaque-purified virus in roller bottles at a low Multiplicity of Infection (MOI) of 0.01 plaque-forming units (pfu) per cell. Infected monolayers were maintained in M-199 with 2% FBS. For SFV-infected monolayers, supernatant fluids were harvested on ice at 16-18 hours post infection (p.i.). For SIN-infected cells, supernatant fluids were harvested on ice at 22-24 hours p.i. The fluids were clarified by centrifugation at 800 x g for 15 minutes at 4°C. Clarified fluids were stored in aliquots at -70°C. Virus titers were assayed by the plaque assay procedure described later.

Purified virus stock. To purify virus from clarified supernatant fluids, one part of the fluid was mixed with three parts of a 40% polyethylene glycol-8000 (PEG-8000) (Sigma) in 2 M NaCl solution and kept at 4°C with constant stirring. Total protein was pelleted by centrifugation at 5000 x g for one hour. The pellet was suspended and diluted in cold TNE (50 mM Tris, pH 7.4, 0.2 M NaCl, 10 mM disodium ethylene diamine tetraacetate [EDTA]). The diluted proteins were overlayed onto a step gradient of 15% sucrose in TNE over a cushion of 60% sucrose in TNE and ultracentrifuged for 2.5 hours at 25,000 x g. The band at the interface of the two sucrose concentrations was collected and diluted in cold TNE buffer before overlaying onto a continuous sucrose (20-60% in TNE) gradient. This latter gradient was ultracentrifuged 16-18 hours at 25,000 x g. Virus bands were collected and pooled. Virus was pelleted by ultracentrifugation at 25,000 x g for 3 hours and stored at -70°C.

Protein concentration was determined by the Biorad Protein Assay (Biorad Labs, Richmond, CA). To eliminate the possibility of cellular protein contamination, viral protein purity was determined by gel electrophoresis (described later). Neutralization assays (described later) with homologous antiserum were done to ensure the homogeneity of the virus stocks.

Injection stock of SFV. A virulent stock of SFV for injections was also maintained. This stock was passaged one to two times through mouse brains to increase virulence. Brains were harvested just prior

to death, homogenized, filtered, and passaged once on CEF monolayers to expand the stock. Supernatant fluids were harvested and clarified as for crude stocks. A 50% lethal dose (LD₅₀) titration was done in mice to titer the virulence. Aliquots were frozen at -70°C. This virus was used in passive protection experiments.

Plaque Assay Procedure

Virus stocks were titered for pfu/ml on CEF in 6-well plates (Corning). Virus was diluted in cold Brain-Heart Infusion (BHI) (Gibco) in serial ten-fold dilutions on ice. Cell monolayers were washed once in HBSS and then inoculated with 0.2 ml of the various virus dilutions in duplicate. The plates were incubated for one hour at 37°C to allow for adsorption of the virus. The inoculum was removed, and the monolayers were overlaid with Modified Eagle's Medium (MEM) (Gibco) containing 2% FBS, 10 mM glutamine and 100 U/ml Pen-Strep in 0.9% agar. After two days, neutral red (diluted 1/10,000 in distilled water) was added. Four hours later, plaques could be counted.

Production of Extended Hyperimmune Antisera

Purified SIN or SFV (0.25 mg) was emulsified in Freund's Complete Adjuvant (FCA) and injected into the gastrocnemius muscle of a rabbit. The second injection was given intraperitoneally (i.p.) in Freund's Incomplete Adjuvant (FIA) one week later. For extended hyperimmune antisera, further immunizations were given intravenously (i.v.) for a

ten to twelve month period. Serum was collected, aliquoted and stored at -40°C.

Gamma Globulin Precipitation

Gamma globulin (including immunoglobulins) was precipitated in 33% ammonium sulfate and centrifuged at 800 x g for 30 minutes. The proteins were suspended in phosphate-buffered saline, pH 7.4 (PBS) and dialyzed overnight. The gamma globulin fraction was finally resuspended to its original volume and stored at -40°C.

Production of Monoclonal Antibodies (Mab)

Mabs made against SIN or SFV were made and provided by Dr. Judith Nicholas at the Upjohn Co., Kalamazoo, MI. The methodology used is based on standard procedures (de St. Groth, 1980; Lerner, 1981; Kennel, 1982).

Briefly, purified SIN or SFV virions were emulsified in FCA, and a series of injections were given to BALB/c mice at multiple sites subdermally. Ten days later, a heterologous virus boost was given. Six to ten days after the boost, splenocytes were harvested and non-adherent lymphocytes were fused with the myeloma cell line SP2/0 in the presence of PEG. The draining lymph nodes were also a source of immune cells.

Hybrids resulting from the fusion were put in microtiter plates with feeder cells (resident peritoneal macrophages from BALB/c mice) and selective (HAT) medium. Growing hybrids were expanded in medium

conditioned by growth of the parent myeloma cell, then cloned by limiting dilution.

The products of the clones were assayed by an enzyme-linked immunosorbent assay (ELISA) using SIN- and SFV-infected cell lysates and were isotypized by an enzyme-linked immunosorbent assay (ELISA) with rabbit antimurine immunoglobulins isotypes antisera.

Ascites fluid was generated by i.p. injection of 5×10^6 hybridoma cells into BALB/c mice which had been pristane-primed for two to four weeks. Fluid was collected 10 to 14 days later.

Mab specificity was determined by immunoblotting. These immunoblots were provided by Dr. Douglas Dodd and were done according to the same procedure described later. Of eight Mabs tested, four recognized the denatured E1 glycoprotein (E1) of both SIN and SFV virions (designated 32-15-1-45, 37-113-2-3, 37-114-2-20, and 37-139-2), one recognized the E1 of SIN only (28-95-1), one recognized the E2 glycoprotein on SIN only (28-69-1), one recognized the capsid protein (C) of SIN (28-91-1) and one recognized nothing from either virus (37-145-1-1).

Of the four Mabs that recognized denatured E1 from both viruses, one was initially made in mice given SIN with SFV as the boost. The other three were made in mice given SFV first and SIN as the boost. This information is summarized in Table 2.

Table 2. Summary of MAb Information

Mab	Ig Isotype ^a	<u>Specificity^b</u>		First Injection ^d	Boost
		SIN	SFV		
32-15-1-45	IgM	E1	E1	SIN	SFV
37-113-2-3	IgG2a	E1	E1	SFV	SIN
37-114-2-20	IgG2b	E1	E1	SFV	SIN
37-139-2	IgG2a	E1	E1	SFV	SIN
28-95-1	IgM	E1	- ^c	NA ^e	NA
28-69-1	IgG3	E2	-	NA	NA
28-91-1	IgG2b	C	-	NA	NA
37-145-1-1	IgM	-	-	NA	NA

^aDetermined by ELISA with anti-murine Ig isotypes (Dr. Nicholas, Upjohn).

^bDetermined by immunoblotting described later (Dr. Dodd, UT/K).

^cSignifies no recognition.

^dTo generate cross-reactive Mabs, mice were first given one virus and then boosted with a heterologous virus ten days later (Dr. Nicholas, Upjohn).

^eData not available or relevant.

Antibody-Dependent, Complement-Mediated Cytolysis (ADCMC)

Cells (CEF or L929s) were passaged less than 20 hours before use in this assay. The monolayers were washed once in HBSS and infected with virus in HBSS at an MOI of 20. Virus was allowed to adsorb for one hour and the inoculum was removed. The cells were trypsinized and pelleted at 800 x g for five minutes.

The cell pellets were suspended in Na⁵¹Cr (ICN, Irvine, CA), diluted in HBSS with 2% FBS to a concentration that provided 100 uCi per 10⁷ cells. The cells were incubated for 1.5 hours on a rocker panel at 37°C, then washed at least five times. The cells were finally suspended in medium (M-199 or McCoy's 5A) with 2% FBS, counted, diluted to 2 x 10⁵ cells/ml, and 0.1 ml was seeded into wells of a 96-well microtiter plate.

Antisera and ascites were heat-inactivated at 56°C for 30 minutes. Serial two-fold dilutions of antisera and ascites were made in medium with 2% FBS immediately before use. Rabbit complement (Low-Tox-M, Cedarlane Labs, Ontario) was diluted 1:7 in cold HBSS and added in equal volume to serial dilutions of antibody.

At nine hours p.i., 0.1 ml of the antibody-complement solution was added to the infected or mock-infected cells. Control wells received complement in medium with 2% FBS. After one hour, 0.1 ml of the supernatant fluid was harvested from each well. To some wells, 0.1 ml of a 3% Triton X-100 (Sigma) solution was added before harvesting to yield total releaseable counts. Triplicate samples of supernatant

fluid were analyzed for ^{51}Cr release in an LKB 1274 RiaGamma spectrometer. The percent specific release was calculated as:

$$\frac{(\text{cpms released in test well} - \text{cpms released by C alone})}{(\text{total releasable cpms} - \text{cpms released by C alone})}$$

where cpms are counts/minute and C is complement.

Neutralization Assay

Neutralization titers for all ascites fluids were assayed on CEF in 6-well tissue culture plates (Corning). Ascites were diluted in HBSS with 2% FBS and sterilized by filtration through a 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI). Virus was diluted in HBSS to a concentration of 150 pfu/ml. Virus suspensions and dilutions of ascites were mixed 1:1 and incubated at room temperature for one hour. Final ascites dilutions were serial four-fold dilutions from 1/10 to 1/40,960. Virus control consisted of virus mixed with HBSS and 2% FBS only.

CEF were washed once in HBSS and duplicate wells were overlaid with 0.2 ml of each virus-ascites dilution mixture. After one hour to allow for adsorption, the inoculum was removed and an overlay of MEM in 0.9% agar was added. Two days later, neutral red diluted 1/10,000 in distilled water was added and plaques were counted after four hours. The reciprocal of the highest dilution of ascites that gave >80% reduction in pfu was defined as the neutralization titer.

Passive Protection

Protection against a lethal dose of SFV was tested in 5-6 week old female C3H/HEJ mice. The mice were injected with ascites diluted in PBS. Control groups received PBS only. The mice then received one i.p. LD₅₀ (5×10^3 pfu) of SFV in 0.1 ml PBS i.p. 24 hours later. The positive control group received gamma globulin from extended extended hyperimmune anti-SIN serum. Beginning 24 hours after challenge, the mice were examined daily and deaths recorded.

Enzyme-Linked Immunosorbent Assay (ELISA)

Purified virions were diluted in 0.05 M carbonate buffer, pH 9.6. Immulon-2 microtiter plates (Dynatech, Alexandria, VA) were coated with 3 ug virus proteins and left overnight at 4°C. The plates were washed three times in PBS with 0.05% Tween-20 (Sigma) (PBS-T), and all empty sites were blocked with PBS-T and 3% FBS for one hour at 37°C. The plates were washed again, and serial two-fold dilutions of antisera or ascites (in PBS-T with 3% FBS) were added (0.05 ml) to the plates. After at least one hour at 37°C, the plates were washed thoroughly, and horseradish peroxidase (HRP)-conjugated second antibody (Cappel, Malvern, PA) was added. This antibody was either goat antirabbit (GAR) or goat antimouse (GAM) depending upon the origin of the primary antibody and was diluted 1/1000 in PBS-T with 3% FBS. This was allowed to incubate for at least one hour at 37°C. After washing as before, substrate-chromogen solution was added in which hydrogen peroxide (Fisher, Fairlane, NJ) at 0.03% in 0.1 M citrate buffer, pH 6.0 was the

substrate while ortho-phenyldiamine (OPD) (Sigma) at 1 mg/ml in citrate buffer was the chromogen. This solution was added to each well; the plates were covered in aluminum foil for fifteen minutes, and absorbance at 405 nm was read immediately in a EIA autoreader (Bio-Tek, Burlington, VT).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a 0.75 mm or 1.5 mm vertical slab-gel apparatus (Hoefer, San Fransisco, CA). A discontinuous system was used. The stacking gel was 4% acrylamide, 3% N, N',-methylene-bis-acrylamide (BIS) in 0.5 M Tris-HCl, pH 6.8 and was polymerized using a photochemical method with 0.05% riboflavin and N,N,N',N'-tetramethylethylenediamine (TEMED) in buffer. The separating gel was either 12 or 15% acrylamide-BIS in 1.5 M Tris-HCl, pH 8.8. Separating gels were polymerized using ammonium persulfate and TEMED. The separating gels could be cast 24 hours before use, but stacking gels were cast only immediately before use.

All samples were mixed with an equal volume of sample buffer prior to electrophoresis. This buffer contained 0.5 M Tris-HCl, pH 6.8, 20% sucrose in 0.5 M Tris-HCl, pH 6.8, 2% SDS and bromophenol blue to color. For some samples, 2% beta-mercaptoethanol (Sigma) was added to the buffer. After mixing, the samples were boiled for a least three minutes.

For electrophoresis gels were run at 15 mA/side of an 0.75 mm thick slab gel in running buffer (20 mM Tris, 150 mM glycine, 0.1% SDS,

pH 8.8). After the tracking dye had migrated through the stacking gel, the power was increased to 20 mA/0.75 mm side. When the dye front was approximately 1 cm from the bottom of the unit, the run was stopped.

Gels were either stained immediately with Coomassie Brilliant Blue R in 25% methanol and 7% acetic acid or blotted onto nitrocellulose. In some cases, gels were silver-stained after Coomassie staining. This was done using a Biorad Silver Stain kit (Biorad).

Immunoblotting

Immediately after SDS-PAGE, proteins were transferred onto nitrocellulose sheets (0.2 μ m pore size) (Schleicher and Schuell, Keene, NH). The sheets were soaked in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) before use. Transfer was performed in a TE42 Transphor unit (Hoeffer) at 100 V for two to three hours at 4°C.

After transfer, the sheets were soaked in TBS (10 mM Tris, 0.9% NaCl, pH 7.4) with 0.1% Tween-20 (Sigma) for one hour at room temperature on a rocker panel to block all available sites. The blocking solution was removed and antiserum or ascites diluted in TBS with 1.0% gelatin (Sigma) was added. Antibody was allowed to react for one to two hours, and the sheets were washed twice in double-distilled water, then three times in TBS with 0.05% Tween-20 (TTBS) for ten minutes each.

Second antibody was added at 1/1,000 diluted in TBS with 1.0% gelatin. The second antibody was either HRP-conjugated GAR or GAM depending upon the origin of the primary antibody used. This was

incubated for one to two hours followed by washing as before with TTBS.

Substrate-chromogen solution was made on ice immediately before use. Sixty mg of chromogen (4-chloro-1-naphthol) (Biorad) was dissolved in ice-cold methanol and added to 100 ml of cold TBS containing 0.06 ml of 30% hydrogen peroxide (substrate) (Fisher). Color was allowed to develop for 15 minutes when the sheets were washed in water and dried onto filter paper. This chromogen gives a purple color.

In some cases, 3',3'-diaminobenzidine (DAB) (Sigma) was used as the chromogen. DAB gives a dark brown color with some background darkness also. Fifty mg/ml of DAB was dissolved in a small volume of methanol and then diluted to its final concentration in 10mM Tris-HCl, pH 7.6 and 30% hydrogen peroxide at 0.01 ml/ml of buffer was added. Color was allowed to develop for 10 minutes and stopped by washing with water.

Digestion of E1

Staphylococcal V8 protease (Sigma) or chymotrypsin (Sigma) were used to digest viral proteins by a method described by Cleveland, 1970. Whole virus was suspended in 0.25 M Tris-HCl, pH 6.8 with 0.5% SDS and boiled for three minutes to disrupt the virion. After cooling, 20 ug of enzyme was added. Digestion proceeded for specified time intervals and was stopped by adding an equal volume of electrophoresis sample buffer and boiling for five to ten minutes. Samples were stored at

-20°C until they were loaded into the wells of a 15% SDS-PAGE. Control samples received distilled water instead of enzyme.

Cyanogen bromide (CNBr) (Sigma) was also used in digestion according to a procedure outlined by Zingde, 1986. In this case, gel pieces containing only E1 from 12% SDS-PAGE of whole virions were cut out after staining with Coomassie Blue. The gel pieces were lyophilized in 12 x 75 mm borosilicate tubes. Large Coplin jars were prepared with either 15-20 ml of water, 70% formic acid or 0.5 mg/ml CNBr in 70% formic acid and allowed to equilibrate for thirty minutes. The tubes containing the gel pieces were placed in the jars and covered for 2-4 days. The pieces were lyophilized and rehydrated in water at least five times to wash and then stored at -20°C. Before electrophoresis, the pieces were suspended in electrophoresis sample buffer and loaded into wells of 15% 1.5 mm thick SDS-PAGE gels.

CHAPTER IV

RESULTS

Antibody-Dependent, Complement-Mediated Cytolysis (ADCMC)

The ability of complement to lyse infected cells after activation in the presence of each MAb was assayed by ADCMC as described in Materials and Methods. In these experiments, L929 cells were infected with SIN or SFV at an MOI of 20, or mock-infected.

Figures 1 and 2 present results when extensive hyperimmune polyclonal antiSIN (hSIN) (Figure 1) or antiSFV (hSF) (Figure 2) were used. It appears that hSIN is able to recognize L929 cells infected with either virus (Figure 1) in that the homologous 50% lysis titer (hSIN on SIN-infected cells) was $\geq 3,200$ while the heterologous titer (hSIN on SFV-infected cells) was ≥ 800 . hSF did not appear to be as efficient in cross-ADCMC (hSF on SIN-infected cells) (Figure 2) as was the hSIN on SFV-infected cells. The homologous 50% lysis titer was high, $\geq 6,400$, but the heterologous titer was low, ≥ 200 .

The MAbs (cf. Table 2) were used in the ADCMC assay and the results are presented on pages 40 to 48. Owing to a positive correlation that had been shown between ADCMC and in vivo protection (Schmaljohn, 1982; Mathews, 1985; Boere, 1986; Stanely, 1986), the ADCMC assay was considered to be important despite the lack of reactivity of the MAbs on immunoblots (cf. Table 2).

Extensive hyperimmune antiSIN

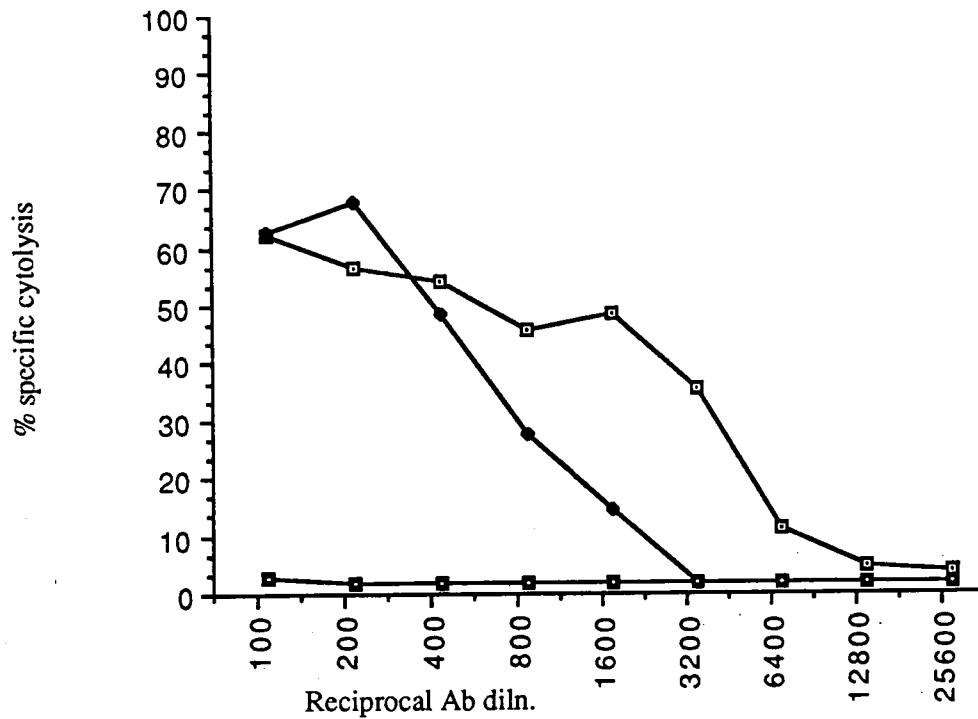


Figure 1. Titration of cross-reactive Ab by ADCMC with extensive hyperimmune antiSIN serum on SIN-infected (□), SFV-infected (◆) L929 cells or mock-infected L929 cells (□). Dilutions of Ab and a constant amount of complement were added to ^{51}Cr -labelled L929 cells at nine hours p.i. Supernatant fluids were harvested after one hour and ^{51}Cr release was counted in a gamma spectrometer.

Extensive hyperimmune antiSFV

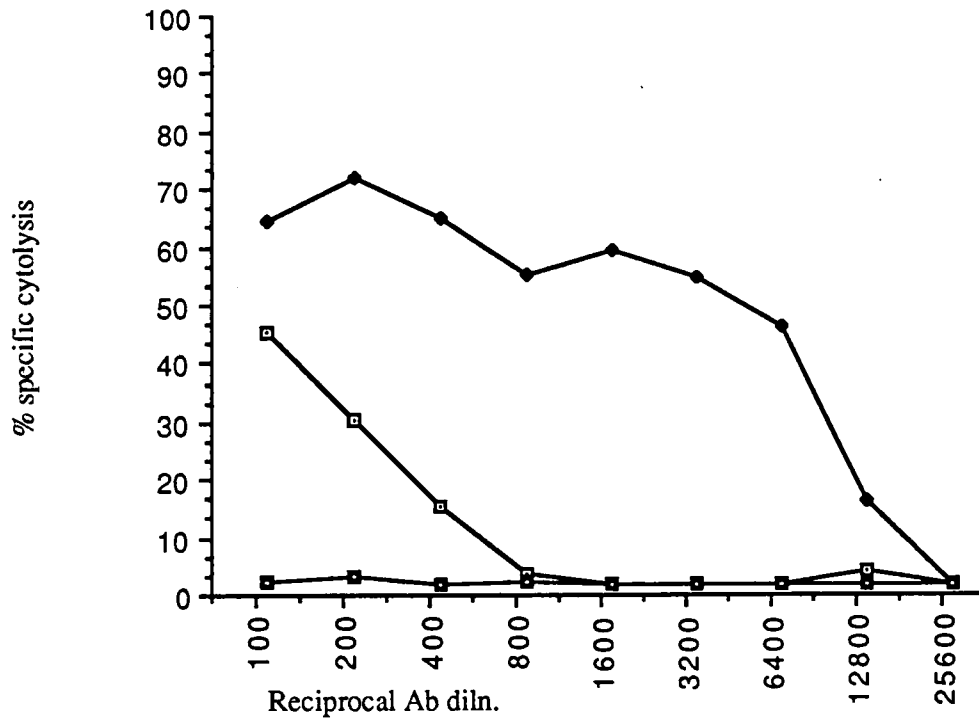


Figure 2. Titration of cross-reactive Ab by ADCMC with extensive hyperimmune antiSFV serum on SIN-infected (□), SFV-infected (◆) or mock-infected L929 cells (■).

The cross-reactive IgM MAb, 32-15-1-45, lysed L929 cells infected with either virus and had the highest 50% lysis titer of any of the MAbs tested, i.e., >50,000 for both SIN- and SFV-infected L929 cells (Figure 3).

The other three cross-reactive IgG MAbs, 37-113-2-3, 37-114-2-20 and 37-139-2, lysed both SIN- and SFV-infected cells (Figures 4, 5, and 6, respectively), but appeared to recognize SIN-infected cells better. This recognition was not due to poor expression of SFV antigens on the infected cell surface, because the hSIN antisera, included as a positive control, lysed SFV-infected cells very efficiently (Figure 1). Titer (i.e., 50% lysis) for the cross-reactive IgG MAbs ranged from $\geq 3,200$ to $\geq 6,400$ on SFV-infected cells.

MAb 28-95-1 recognized only SIN-infected target cells in the ADCMC (Figure 7), which supports the finding that this MAb reacted only with E1 from SIN virions on immunoblots.

MAb 28-91-1 reacted with capsid from SIN virions on immunoblot. Owing to this, the ADCMC assay for this MAb was performed at 12 hours p.i., since it had previously been shown that capsid can be present on the infected cell surface late in infection (Smith, 1985). This MAb did not lyse either SIN- or SFV-infected cells at 12 hours p.i. (Figure 8).

MAb 28-69-1 reacted with E2 from SIN virions on immunoblot. In addition, this MAb was an IgG3 isotype, which does not fix complement. Therefore, after adding serial dilutions of this MAb, the cells were washed and anti-mouse globulin serum was added at a 1/500 dilution for

Mab 32-15-1-45

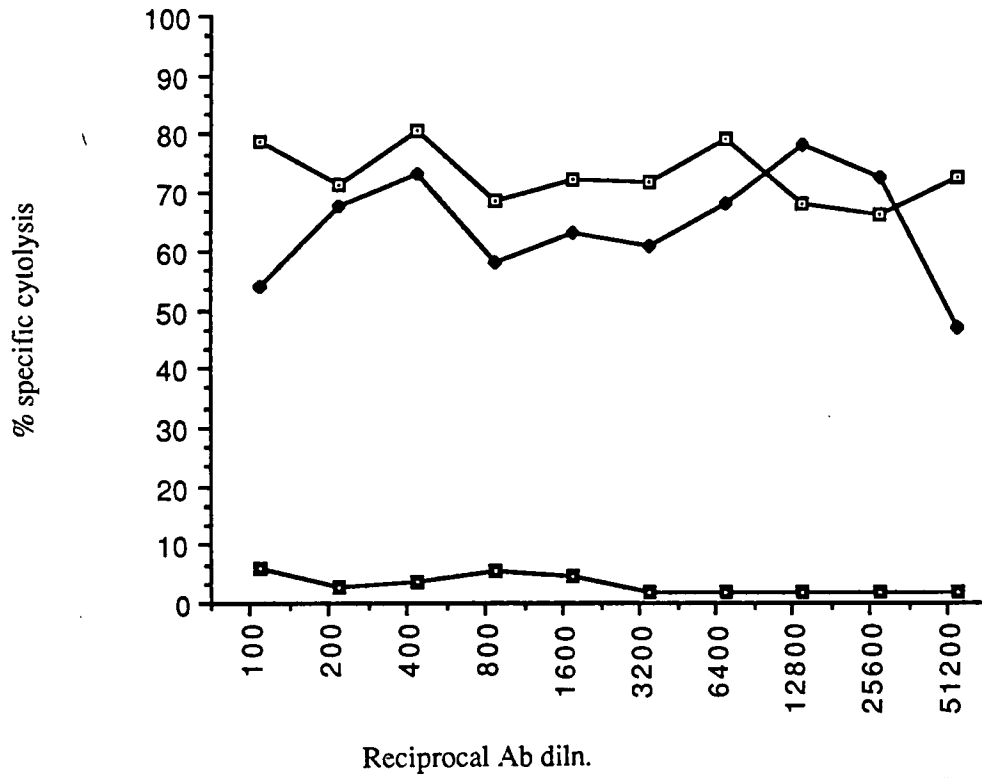


Figure 3. Titration of cross-reactive MAb by ADCMC with MAb 32-15-1-45 on SIN-infected (□), SFV-infected (●) or mock-infected L929 cells (■).

Mab 37-113-2-3

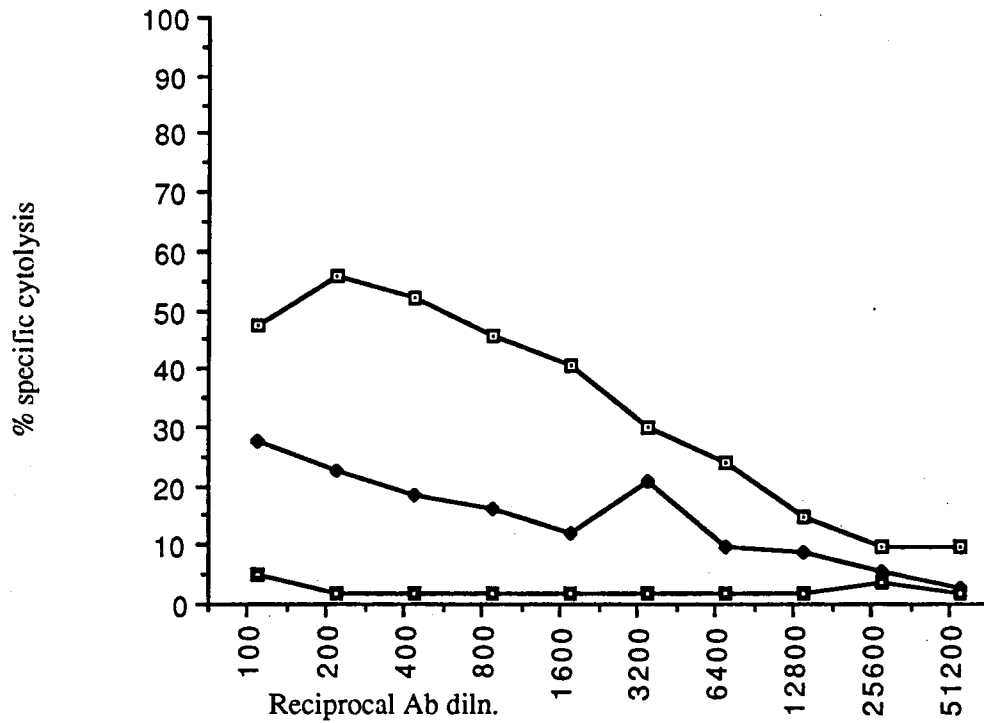


Figure 4. Titration of cross-reactive MAb by ADCMC with MAb 37-113-2-3 on SIN-infected (□), SFV-infected (●) or mock-infected L929 cells (○).

Mab 37-114-2-20

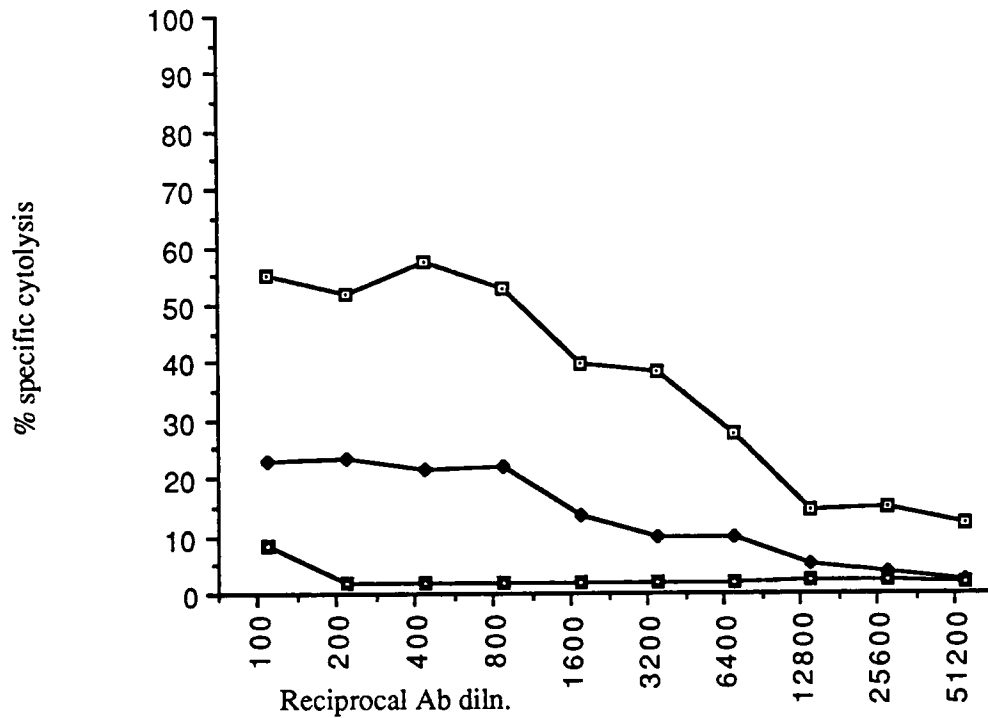


Figure 5. Titration of cross-reactive MAb by ADCMC with MAb 37-114-2-20 on SIN-infected (□), SFV-infected (◆) or mock-infected L929 cells (■).

Mab 37-139-2

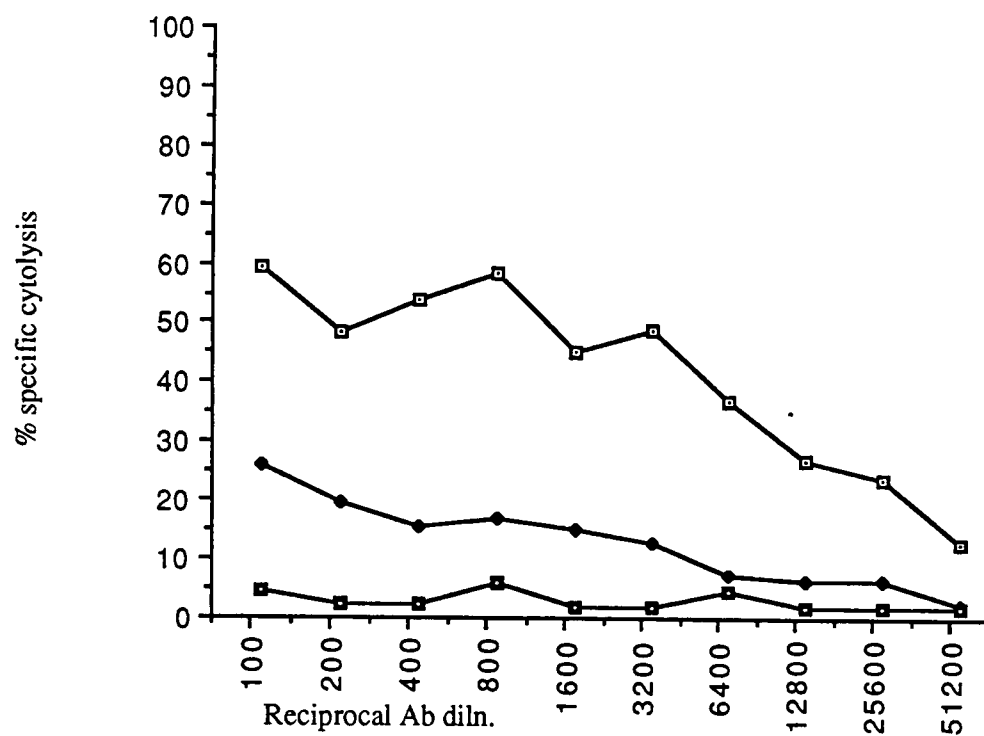


Figure 6. Titration of cross-reactive MAb by ADCMC with MAb 37-139-2 on SIN-infected (□), SFV-infected (◆) or mock-infected L929 cells (■).

Mab 28-95-1

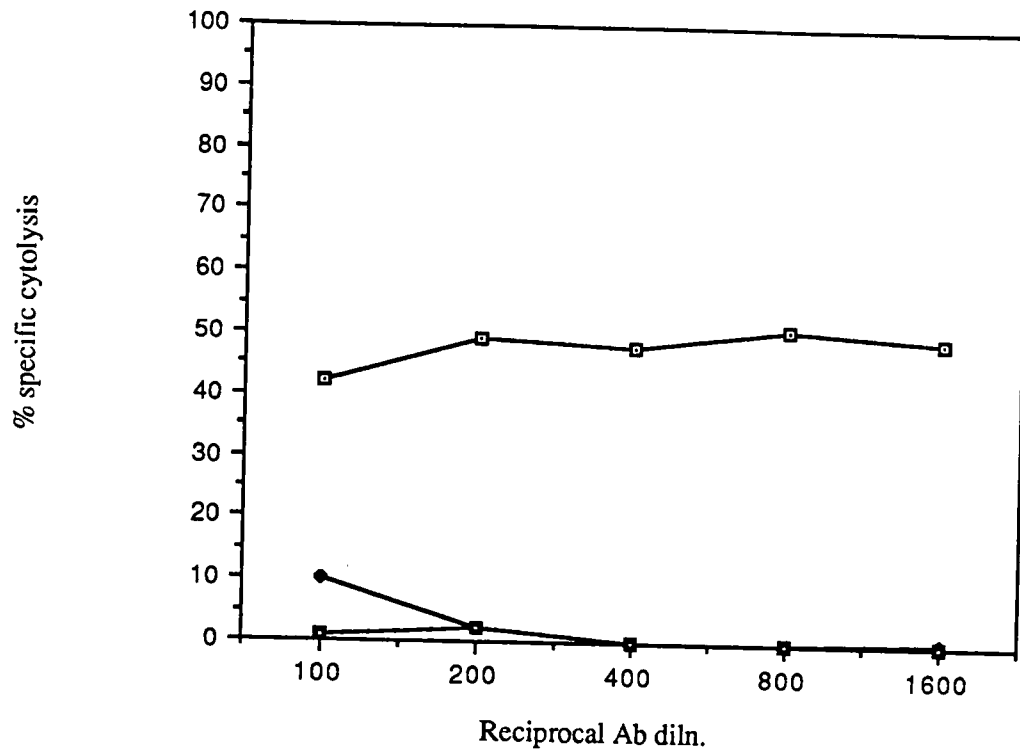


Figure 7. Titration of cross-reactive MAb by ADCMC with MAb 28-95-1 on SIN-infected (□), SFV-infected (●) or mock-infected L929 cells (■).

Mab 28-91-1

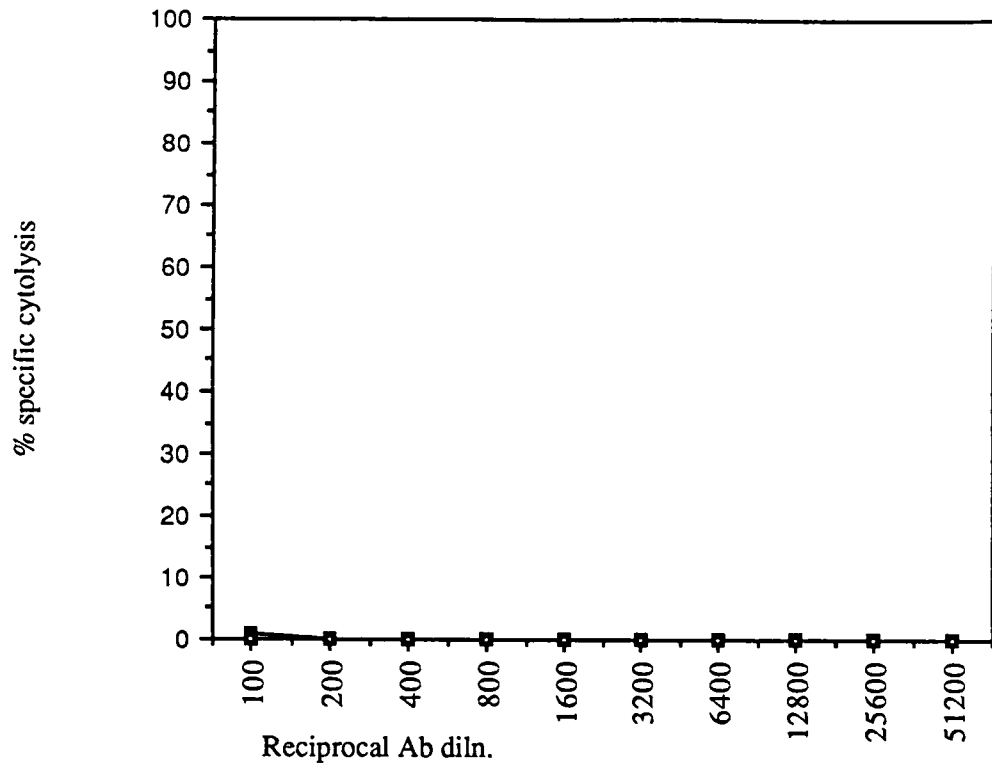


Figure 8. Titration of cross-reactive MAb by ADCMC with MAb 28-91-1 on SIN-infected (□), SFV-infected (◆) or mock-infected L929 cells (□).

one hour. Complement was then added for an additional 30 minutes. In parallel assays, this treatment enhanced the cytolysis mediated by other complement-fixing MAbs (data not shown). Nevertheless, MAb 28-69-1 did not effect lysis of SIN- or SFV-infected cells (data not shown).

MAb 37-145-1-1 did not recognize any viral proteins on immunoblot and did not cause lysis of infected cells (Figure 9).

As a negative control in ADCMC assays, ascites fluid containing MAb against a glycoprotein of respiratory syncytial virus (antiRSV) was used (Figure 10).

The data from all representative ADCMC experiments are summarized in Table 3.

Enzyme-Linked Immunosorbent Assay (ELISA)

Originally the MAbs were screened and selected by ELISA using infected-cell lysates as test antigens. It was of interest, therefore, to determine whether the MAbs recognized purified virions. To test this, only those MAbs that were cross-reactive on immunoblots and that participated in cross-ADCMC were used in these experiments.

Virions (SIN or SFV) purified on sucrose gradients (see Materials and Methods) were diluted in carbonate buffer, pH 9.6 and 3 ug of virus protein was seeded into wells of a microtiter plate. The antigens were allowed to adhere overnight. After blocking all available sites with 3% BSA, dilutions of Ab (i.e., hyperimmune antisera or MAbs) were added, then second antibody was added and allowed to react for one

Mab 37-145-1-1

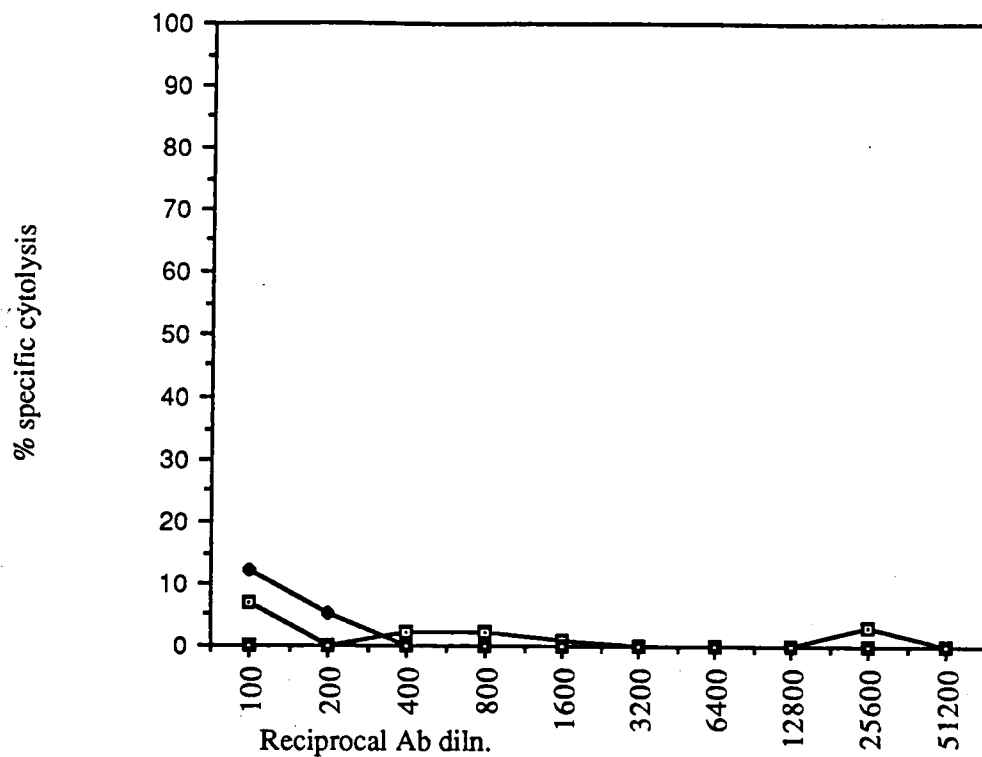


Figure 9. Titration of cross-reactive MAb by ADCMC with MAb 37-145-1-1 on SIN-infected (□), SFV-infected (●) or mock-infected L929 cells (■).

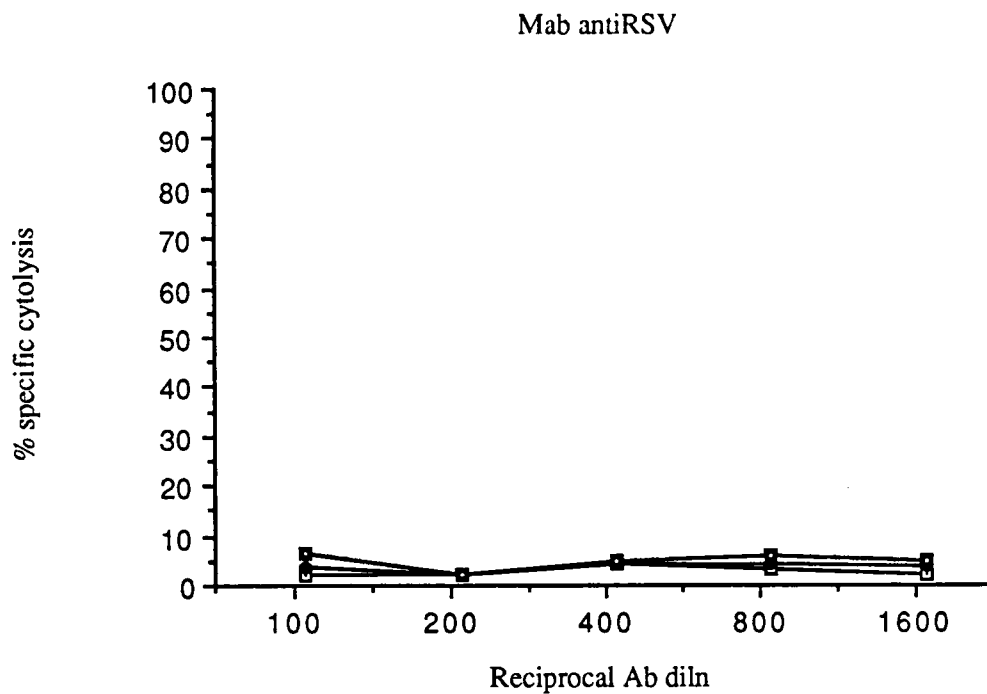


Figure 10. Titration of cross-reactive MAb by ADCMC with negative control antiRSV ascites on SIN-infected (□), SFV-infected (●) or mock-infected L929 cells (○).

Table 3. Summary of ADCMC assays.

Antibody	<u>Maximum lysis^a</u>		<u>ACDMC titer^b</u>	
	SIN	SFV	SIN	SFV
hSIN	60-65	<70	> 3,200	< 800
hSF	<50	<75	> 200	> 6,400
32-15-1-45	80	<70	>50,000	>50,000
37-113-2-3	<60	30	> 3,200	> 1,600
37-114-2-20	55	25	> 6,400	> 1,600
37-139-2	60	25	> 6,400	< 6,400
28-95-1	<50	0	> 1,600	0
28-69-1	0	0	0	0
28-91-1	0	0	0	0
37-145-1-1	0	0	0	0

^aExpressed as % specific cytolysis.

^bReciprocal of the Ab dilution that caused 50% of the maximum lysis.

allowed to react for one hour. Finally, substrate-chromogen solution was added and absorbance at 405 nm was assessed.

Figures 11 and 12 show data from ELISAs using extensive hyperimmune polyclonal antiSIN (hSIN) or antiSFV (hSF) sera. hSIN recognized purified virions very well with a homologous ELISA 50% endpoint titer of 1,600 and a heterologous titer of ≥ 800 (Figure 11). hSF recognized purified SIN and SFV with a homologous ELISA 50% endpoint titer of 3,200 and a heterologous ELISA titer of 1,600 (Figure 12).

MAb 32-15-1-45 did not recognize SIN virions well (maximum OD_{405 nm} reading of 0.35) compared to the recognition of SFV virions (Figure 13). It is possible that this MAb recognizes an epitope present on SIN-infected cell surfaces but cryptic on SIN virions, since it can effect lysis of SIN-infected cells very efficiently (Figure 3). This interpretation has been made in other similar studies in which antiviral MABs do not bind to virions well, but are capable of lysing infected cells in the presence of complement and/or are protective in vivo (Balachandran, 1982; Mathews, 1982; Schmaljohn, 1982; Roehrig, 1983; Boere, 1984; LeFrancois, 1984; Mathews, 1984; Boere, 1985a; Hunt, 1985; Roehrig, 1985; Boere, 1986; Stanley, 1986). It is interesting, though, that this MAb recognizes epitopes on both SFV virions or SFV-infected cells. This phenomenon also points out the basic difference between the ADCMC assay and the ELISA; that is, one is an assay of in vitro biological function while the other is a measure of binding to immobilized antigens.

Extensive hyperimmune antiSIN

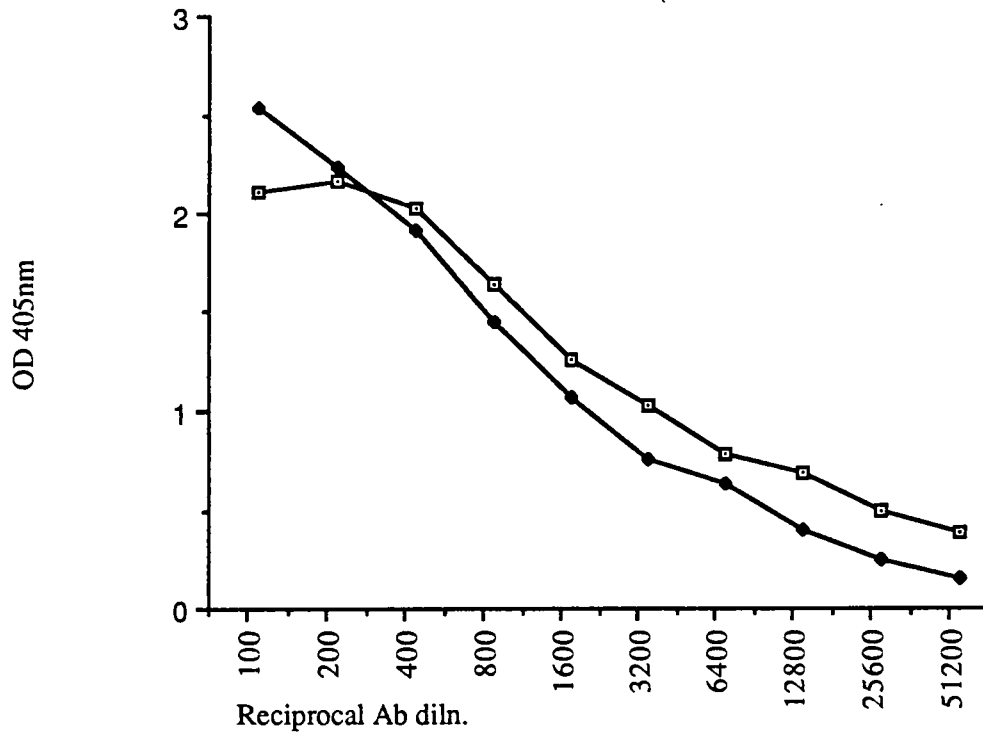


Figure 11. Recognition of purified virions by ELISA with extensive hyperimmune antiSIN serum on SIN (□) or SFV (●) virions. Purified virions (3 ug protein) were adhered to wells of microtiter plates overnight. Ab was added and after blocking available sites, HRP-conjugated antiserum was added next. After washing, substrate-chromogen solution was added and absorbance as optical density (OD) was measured at 405 nm.

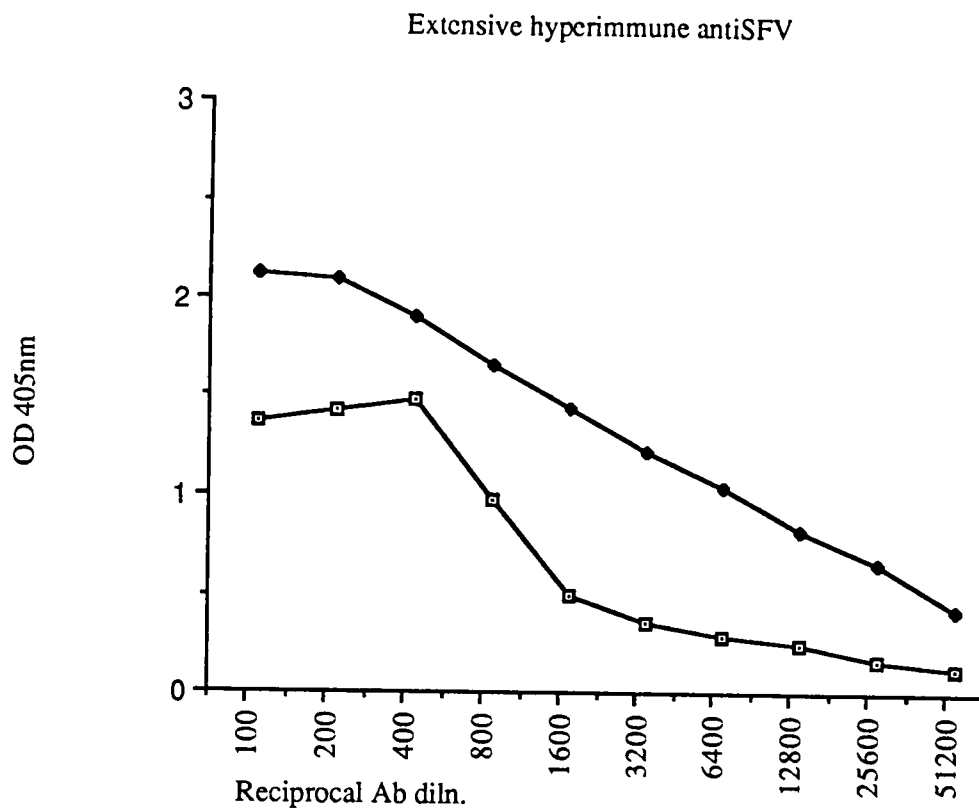


Figure 12. Recognition of purified virions by ELISA with extensive hyperimmune antiSFV serum on SIN (□) or SFV (●) virions.

Mab 32-15-1-45

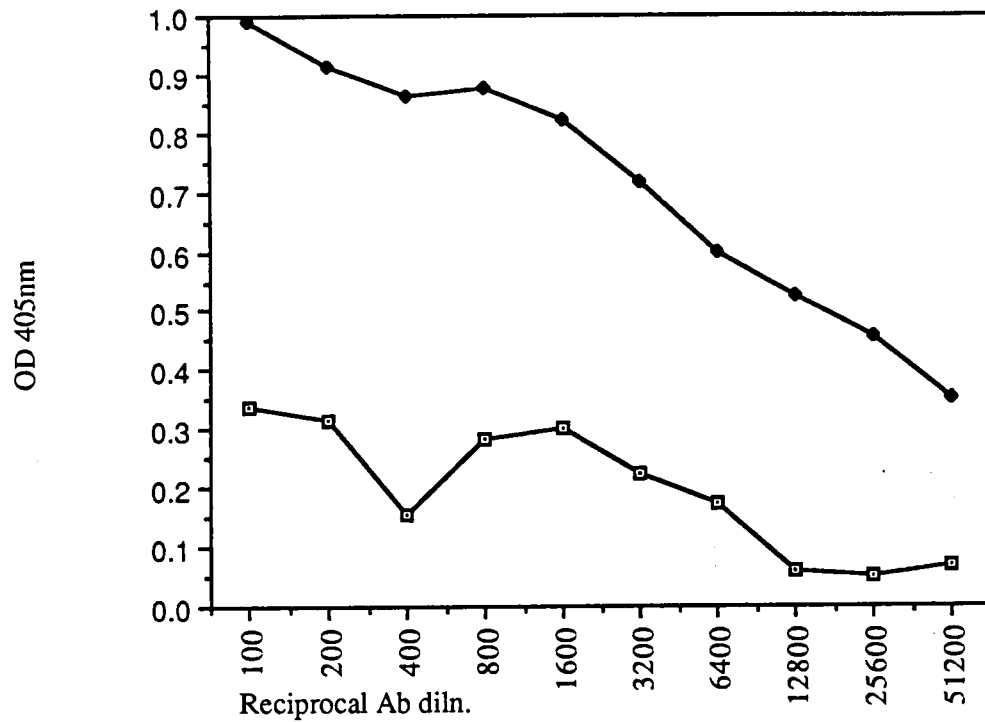


Figure 13. Recognition of purified virions by ELISA with Mab 32-15-1-45 on SIN (□) or SFV (●) virions.

The three IgG cross-reactive MABs, 37-113-2-3, 37-114-2-20 and 37-139-2, recognized purified SIN and SFV virions equally well (Figures 14, 15 and 16, respectively). The SIN and SFV ELISA 50% endpoint titers for MAb 37-113-2-3 were both >50,000 (Figure 14) while the SIN ELISA titer for MAb 37-114-2-20 was >50,000, and for SFV >25,600 (Figure 15). The SIN and SFV ELISA titers for MAb 37-139-2 were both >50,000 (Figure 16).

The antiRSV MAB was included in these experiments as a negative control (Figure 17). These data are summarized in Table 4.

Neutralization of Virus Infectivity

To assay the in vitro neutralizing capabilities of the MABs, serial four-fold dilutions of MAB were incubated with virus dilutions. Aliquots of virus-antibody mixtures were inoculated onto susceptible CEF monolayers, incubated, and the monolayers overlayed with medium in agar. After two days at 37°C, neutral red stain was added, and plaques were counted.

The results from this experiment are presented in Table 5. The polyclonal sera neutralized only homologous virus infectivity, not heterologous virus. None of the MABs were neutralizing in vitro for either SIN or SFV. The antiE2 MAB, 28-69-1, must define a non-neutralizing epitope on E2 as it does not neutralize either virus. Other reports have indicated that some antiE1 MABs can neutralize these viruses in vitro (Chanas, 1982; Mathews, 1982; Clegg, 1983; Schmaljohn, 1983; Boere, 1984; Hunt, 1985; Yamamoto, 1985; Stanley, 1986).

Mab 37-113-2-3

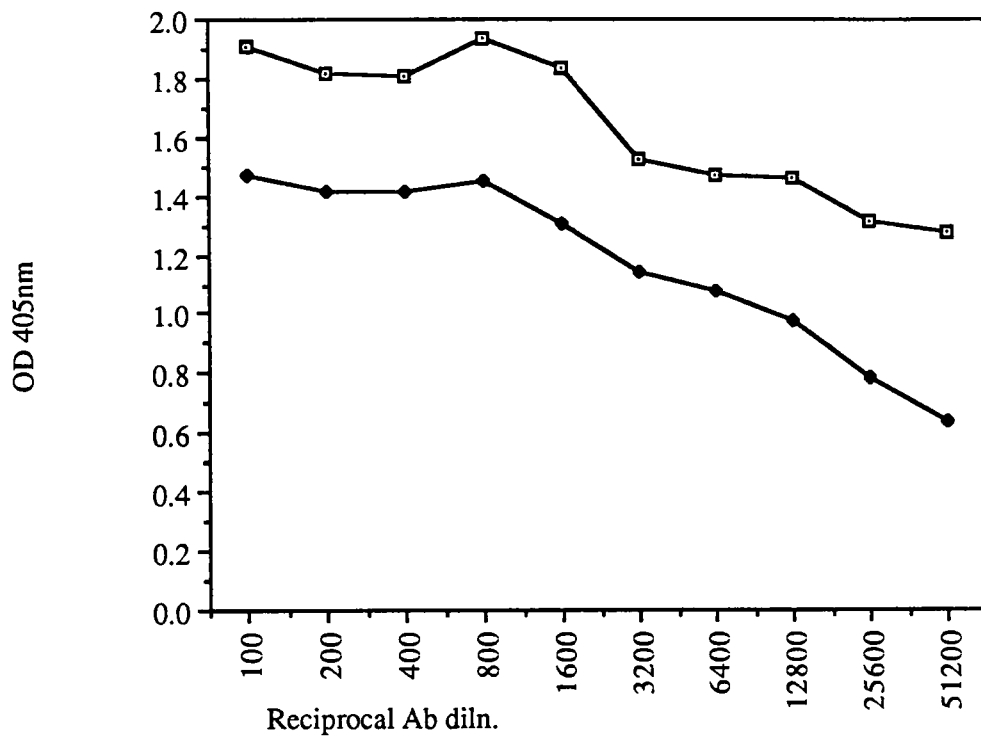


Figure 14. Recognition of purified virions by ELISA with MAb 37-113-2-3 on SIN ($\square$) or SFV ($\blacklozenge$) virions.

Mab 37-114-2-20

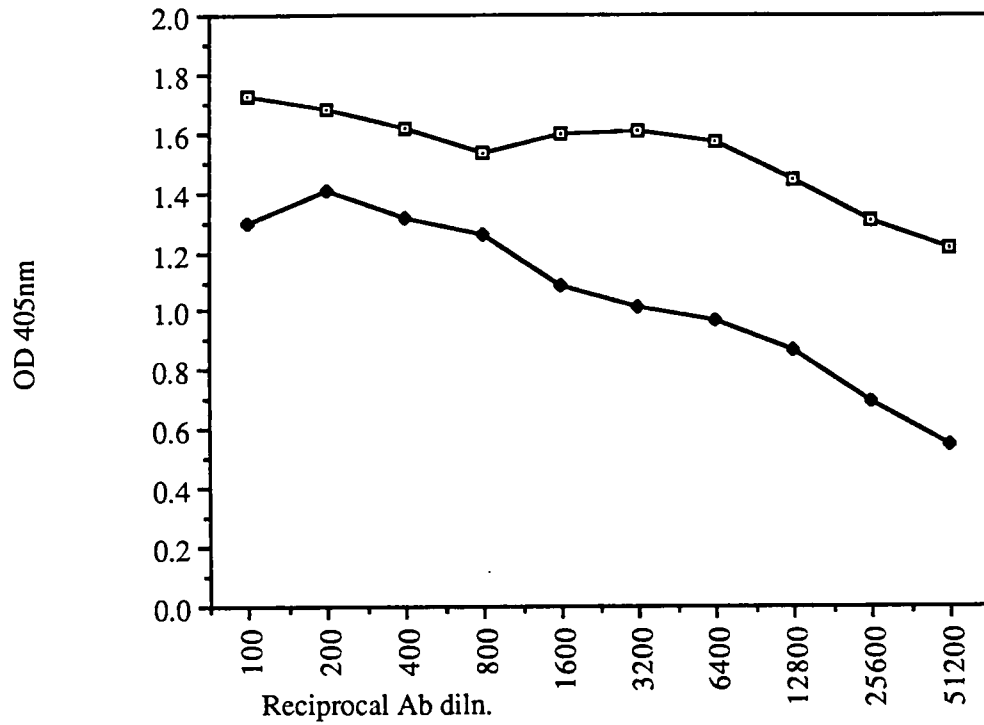


Figure 15. Recognition of purified virions by ELISA with MAb 37-114-2-20 on SIN (—□—) or SFV (—●—) virions.

Mab 37-139-2

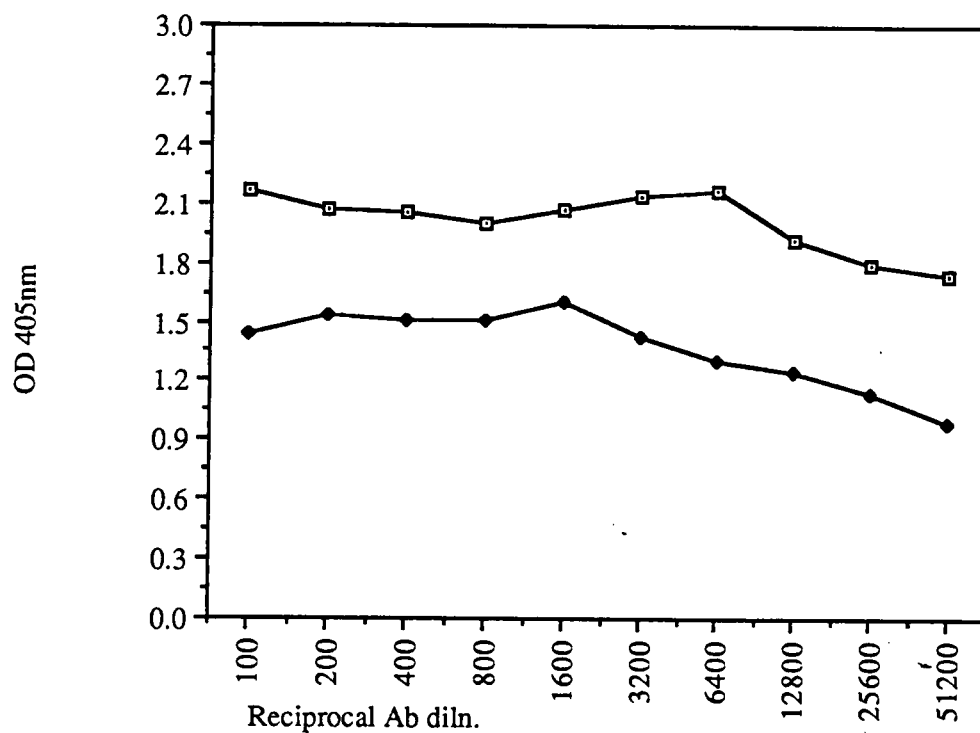


Figure 16. Recognition of purified virions by ELISA with Mab 37-139-2 on SIN (□) or SFV (◆) virions.

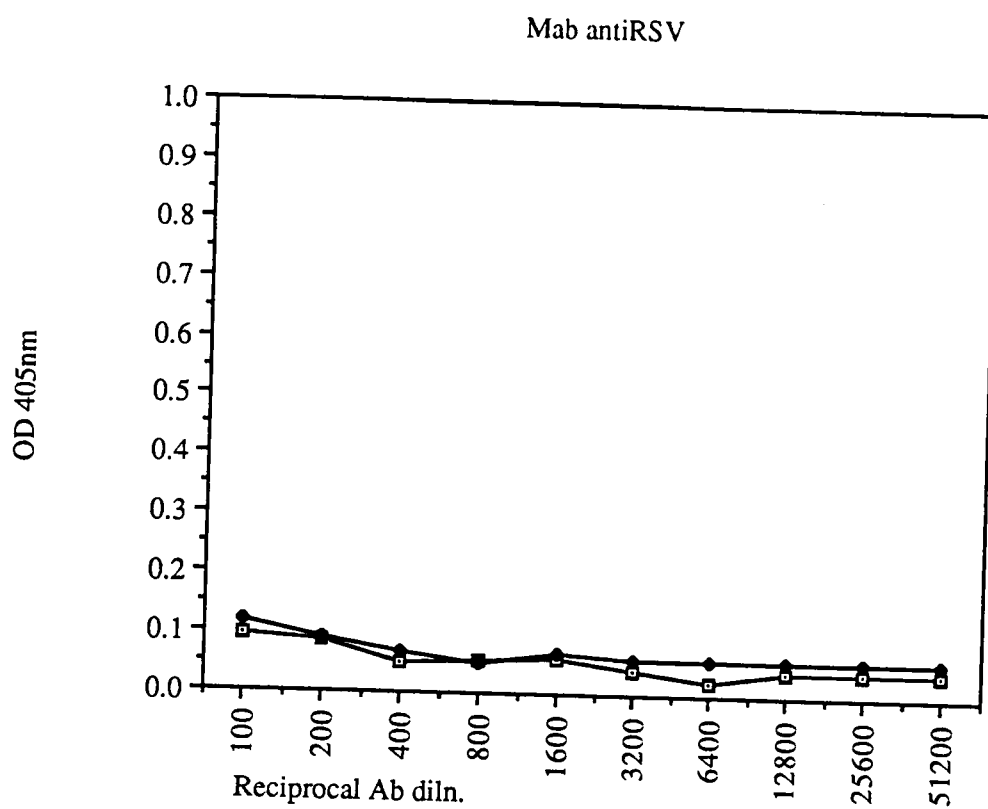


Figure 17. Recognition of purified virions by ELISA with negative control antiRSV ascites on SIN (□) and SFV (◆) virions.

Table 4. Summary of ELISAs.

Antibody	<u>Maximum lysis^a</u>		<u>ELISA titer^b</u>	
	SIN	SFV	SIN	SFV
hSIN	>2.0	>2.5	1,600	> 800
hSF	1-1.5	>2.0	1,600	3,200
32-15-1-45	0.35	>1.0	> 3,200	12,800
37-113-2-3	>1.9	>1.5	>50,000	>50,000
37-114-2-20	>1.7	1.3	>50,000	>25,600
37-139-2	2.15	>1.4	>50,000	>50,000

^aMaximum absorbance as optical density at 405 nm.

^bReciprocal of the Ab dilution that gave 50% of the maximum OD₄₀₅ nm reading.

Table 5. Antiviral neutralizing activity of MAbs.^a

Antibody	Neutralizing Titer ^b	
	SIN	SFV
hSF ^c	<10	>10 ^{5e}
hSIN ^d	>10 ^{5e}	<10
32-15-1-45	<10	<10
37-113-2-3	<10	<10
37-114-2-20	<10	<10
37-139-2	<10	<10
28-69-1	<10	<10

^aDilutions of antibody were mixed with an equal volume of a constant virus dilution for one hour at room temperature. Aliquots of each sample were plated on CEF for plaque assays.

^bThe reciprocal of the highest dilution that gave >80% reduction in pfu was defined as the neutralization titer.

^cExtended hyperimmune anti-SF.

^dExtended hyperimmune anti-SIN.

^eData from Wust, 1987.

Passive Protection

To test whether the MABs were protective in vivo against lethal virus challenge, ascites fluid was diluted in PBS and given i.p. to six week old female C3H/HeJ mice. One day later, one LD₅₀ or 100 LD₅₀ of SFV was given i.p. Control mice received no treatment before virus challenge. Mice were examined twice daily for well-being and deaths.

Laboratory stocks of SIN produce abortive immunizing infections in adult mice, thus only the lethal SFV was used in these passive protection experiments.

Table 6 presents the results from two separate experiments. In the first experiment, MAB 32-15-1-45 (an IgM isotype) was used to protect mice against 100 LD₅₀ of SFV given i.p. This MAB did not protect mice from SFV challenge. That this MAB is an IgM isotype and did not passively protect mice from this high level of SFV challenge supports a report from this laboratory that passive cross-protection to high level challenge by hyperimmune antisera is mediated by ADCC, which could occur only with IgG isotypes, since the cells involved express only Fc-gamma receptors (Wust, 1987). All control mice died in this experiment.

In the second experiment, the three cross-reactive IgG MABs (37-113-2-3, 37-114-2-20, and 37-139-2) were used to passively protect against one LD₅₀ (low level challenge) of SFV given i.p., and each protected. Even though the high ADCMC titer of MAB 32-15-1-45 (Figure 3) suggests that there could be a large excess of MAB available to

Table 6. Passive protection of mice against intraperitoneal SFV challenge.^a

	Group	Avg. Day of Death	Dead/Total	% Survival
Expt. 1 ^b	Virus only (100 LD ₅₀)	7	6/6	0
	hSIN	0	0/6	100
	32-15-1-45	7.5	10/10	0
Expt. 2 ^c	Virus only (1 LD ₅₀)	8.5	5/10	50
	hSIN	0	0/10	100
	37-113-2-3	0	0/10	100
	37-114-2-20	0	0/10	100
	37-139-2	0	0/10	100
	28-69-1	8.5	4/10	60

^aSix-week old female C3H/HeJ mice received 0.1 ml ascites fluid in 0.4 ml PBS or 0.5 ml IgG precipitated from hSIN intraperitoneally 24 hours before challenge with SFV ip. Controls include the hSIN group and groups that received SFV only.

^bMice were challenged with 100 LD₅₀ of SFV in Experiment 1.

^cMice were challenged with one LD₅₀ of SFV in Experiment 2.

protect the mice in experiment 1, further experiments are needed to demonstrate unequivocally that the IgM MAb is not protective.

Summary of Serological Reactivities

Of eight MAbs tested, four were cross-reactive with the E1 glycoprotein of SIN or SFV as presented on immunoblots and on virus-infected cell surfaces or on purified virions. Furthermore, all antiE1 MAbs were non-neutralizing in vitro but only three (IgG isotypes) were protective in vivo. This summary information is presented in Table 7.

Prediction of Cross-Reactive Epitopes

Owing to the various serological reactivities, SFV proteins were digested with proteases in an attempt to identify peptides of E1 with which the MAbs would react.

In order to predict the most likely region of cross-reactivity on E1 between Sindbis (SIN) and Semliki Forest (SFV) viruses, several parameters of protein structure can be considered.

The most obvious parameter is amino acid sequence homology between the two viruses (Simons, 1984b). In Figures 18 and 19, the relative homology is represented by the horizontal bars. Perfect matches and conservative substitutions were determined for each six residue segment (moving average) and scored according to the number of matches, e.g., zero out of six, one out of six, etc., up to six out of six. These determinations were then plotted.

Table 7. Summary of MAb reactivities.

Mab	Isotype ^a	<u>Specificity^b</u>		<u>ADCMC^d</u>		<u>ELISA^e</u>		<u>Neutralization^g</u>		Protective ^h
		SIN	SFV	SIN	SFV	SIN	SFV	SIN	SFV	
32-15-1-45	IgM	E1	E1	80	<70	0.35	>1.0	<10	<10	- ⁱ
37-113-2-3	IgG2a	E1	E1	60	30	>1.9	>1.5	<10	<10	+
37-114-2-20	IgG2b	E1	E1	>55	25	>1.7	1.3	<10	<10	+
37-139-2	IgG2a	E1	E1	60	25	>2.0	>1.4	<10	<10	+
28-95-1	IgM	E1	- ^c	<50	0	ND	ND	ND	ND	ND
28-69-1	IgG3	E2	-	0	0	ND	ND	<10	<10	-
28-91-1	IgG2b	C	-	0	0	ND	ND	ND	ND	ND
37-145-1-1	IgM	-	-	0	0	ND	ND	ND	ND	ND

^aDetermined by ELISA.

^bDetermined by immunoblot.

^cSignifies no recognition.

^dMaximum % specific cytolysis of a 1/100 dilution of ascites.

^eMaximum absorbance as optical density at 405 nm of a 1/100 dilution of ascites.

^fExperiment not done.

^gReciprocal of the MAb dilution giving 80% reduction in pfu.

^hProtected mice against 1 LD₅₀ of SFV.

ⁱSignifies that MAb did not protect.

SINDBIS E1

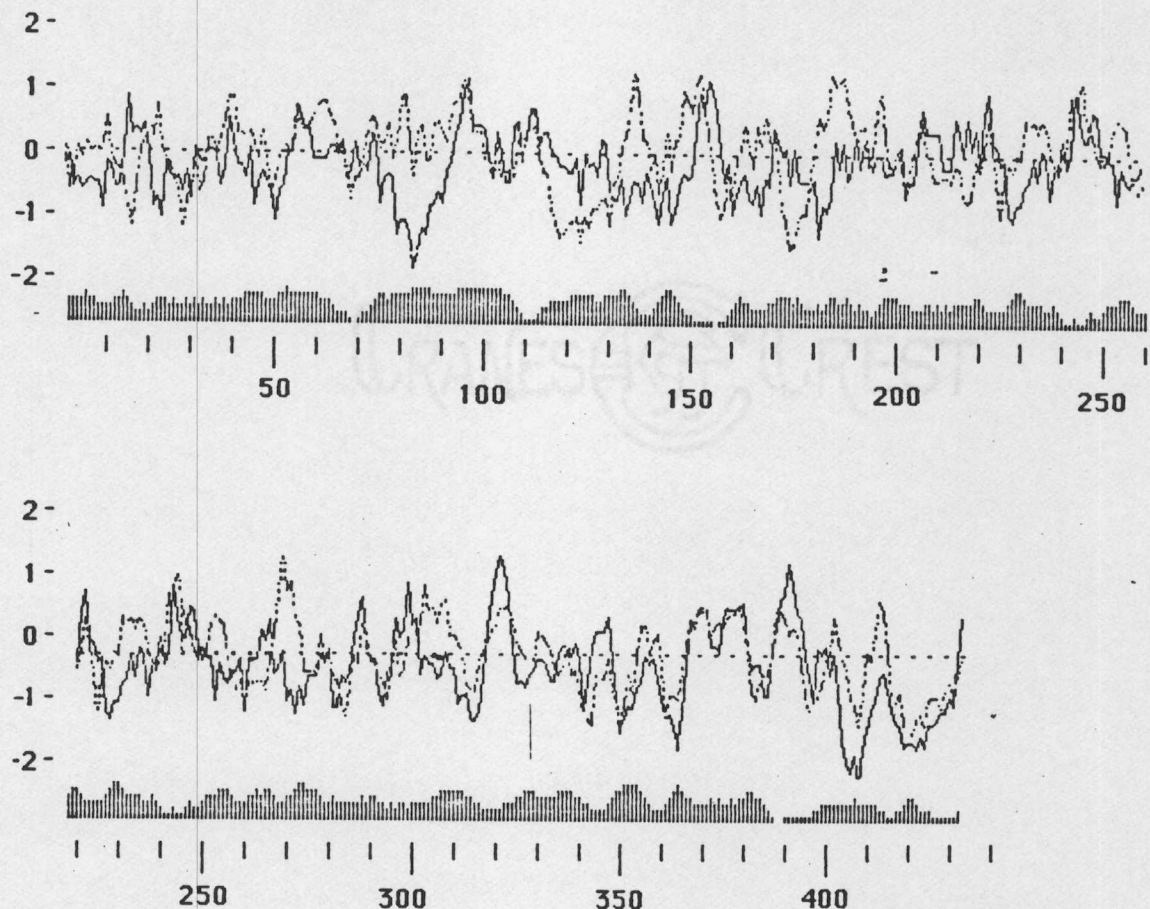


Figure 18. Hydrophilicity and loop probabilities of Sindbis E1 glycoprotein and homology with Semliki Forest virus E1 glycoprotein. Each line represents a moving average of six amino acid residues. The solid line indicates local average hydrophilicity. Regions of hydrophilicity are shown above the dotted line while hydrophobic regions are below. The dotted line represents the probability of the indicated region being in a loop conformation. Positive probabilities are above the dotted line. The horizontal lines represent areas of homology between the E1 of SIN and the E1 of SFV. See text for references and more detailed explanations.

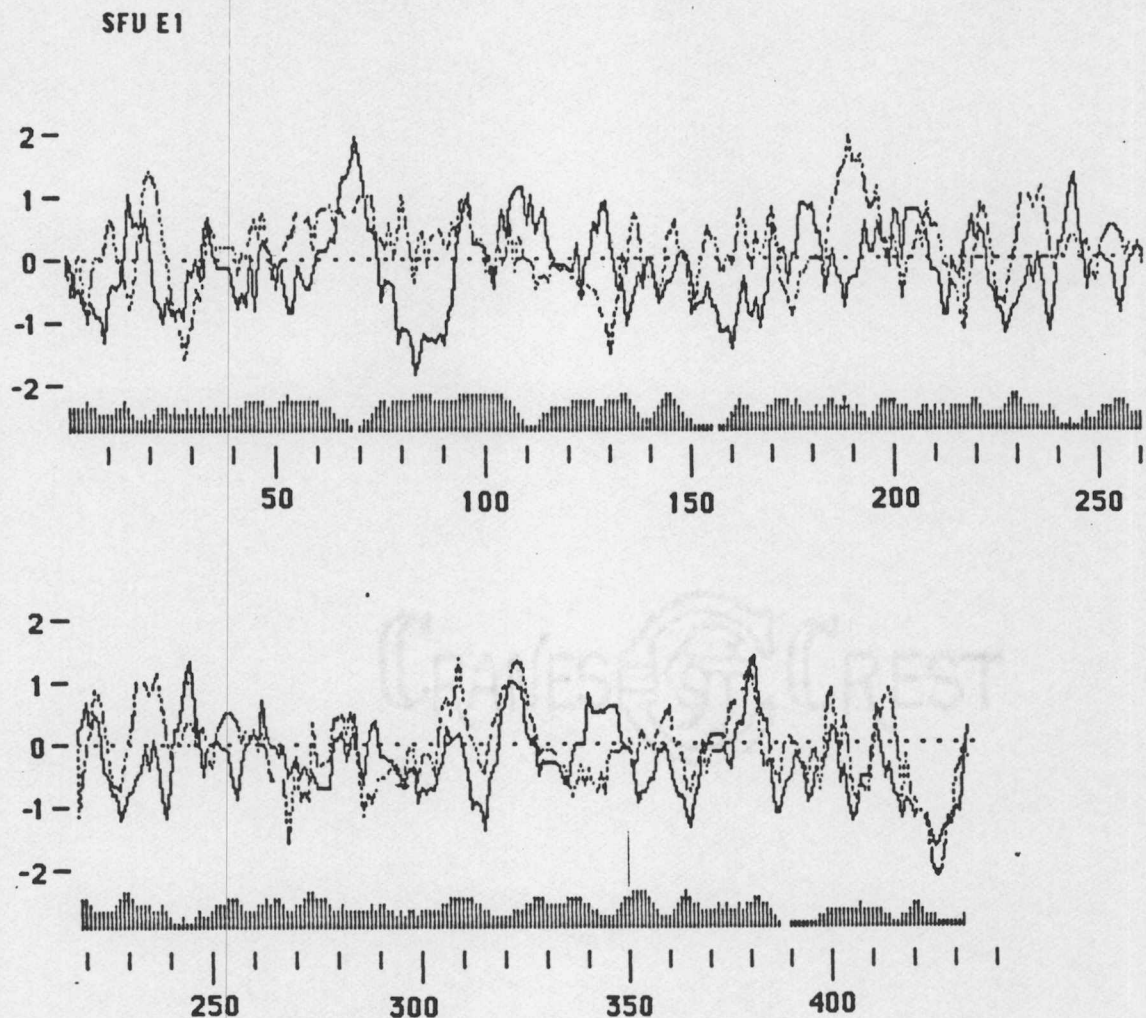


Figure 19. Hydrophilicity and loop probabilities of Semliki Forest virus E1 glycoprotein and homology with Sindbis virus E1 glycoprotein. Each line represents a moving average of six amino acid residues. The solid line indicates local average hydrophilicity. Regions of hydrophilicity are shown above the dotted line while hydrophobic regions are below. The dotted line represents the probability of the indicated region being in a loop conformation. Positive probabilities are above the dotted line. The horizontal lines represent areas of homology between the E1 of SIN and the E1 of SFV. See text for references and more detailed explanations.

A second parameter to consider is the relative hydrophilicity of the glycoproteins, since areas of high hydrophilicity are generally on the surface and highly accessible to antibody binding (Hopp and Woods, 1981). Each amino acid was assigned an hydrophilic value (Hopp and Woods, 1981) and a moving average of six residues was calculated for the E1 glycoproteins of SIN and SF. These averages are presented as the solid line in Figures 18 and 19. Sequences of residues with hydrophilicity are assigned positive values in the display (Hopp and Woods, 1981).

A third parameter to consider is the frequency of loop configurations in these glycoproteins. This is based on the interpretation that loop conformations are always conspicuous surface structures, usually flexible, that are easily accessible to antibody binding (Leszczynski, 1986). In this determination, each amino acid is assigned a numerical value based on the probability that it is in a loop. These values were determined in a moving average of six amino acid residues and plotted as the hashed line in Figures 18 and 19. Areas having a probability of being in a loop configuration are found in the upper portion of the figure (i.e., greater than zero).

Combining these three parameters, allows a visual display of the most likely segments of the glycoproteins for immunological cross-reactivity. These segments are to be residues 92-108, 317-335, and 373-391. The amino acid sequences of these sites are given in Table 8.

Table 8. Amino acid sequences of probable antigenic determinants of SIN and SFV E1 glycoproteins.

1. Residues 92-108	SIN	ala-tyr-cys-phe-cys-asp-ser-glu-asn-ser-gln-met-ser-glu-ala-tyr-val
	SFV	ala-tyr-cys-phe-cys-asp-ser-glu-asn-thr-gln-leu-ser-glu-ala-tyr-val ●
2. Residues 317-335	SIN	thr-leu-gln-tyr-val-ser-asp-arg-glu-gly-gln-cys-pro-val-his-ser-his-ser-ser
	SFV	thr-leu-thr-tyr-lys-thr-asn-lys-asn-gly-asp-cys-ser-val-his-ser-his-ser-asn ●
3. Residues 373-391	SIN	lys-thr-thr-cys-asn-ala-glu-cys-lys-pro-pro-ala-asp-his-ile-val-ser-thr-pro
	SFV	arg-ala-thr-cys-ser-ala-ser-cys-glu-pro-pro-lys-asp-his-ile-val-pro-tyr-pro ●

Note: A dot (●) under an amino acid indicates a conservative substitution.

Of special interest was segment 3 (residues 373-391), because it is close to the carboxy-terminus of the glycoprotein on both viruses. It has been reported that amino- and carboxy-termini usually possess higher than average antigenic activity, presumably owing to their relatively higher flexibility and/or surface location (Westhof, 1984; van Regenmortel, 1986). This segment also contains more proline residues than the other two segments. Proline residues are characteristic of "bends" in the secondary structure of a protein and therefore, correlate with the loop-probability determination (Leszczynski, 1986).

In order to cleave these segments from the intact protein, several proteases with different specificities were investigated. The ideal enzyme would cleave the glycoprotein and leave these three epitopes intact.

Both glycoproteins contain large amounts of lysine and arginine residues, but a limited digestion with trypsin (which has a specificity for lysinyl and arginyl residues) might yield the intact fragments in Table 8. Likewise, Staphylococcal V8 protease has a specificity for glutaminyl and asparaginyl residues at pH 7.8 (glutamyl residues only at pH 4.0), but these residues are also present in large amounts (Croft, 1980). All four amino acids mentioned are charged and more likely to be found in hydrophilic areas preferentially. Therefore, there is a strong possibility that these enzymes could destroy the antigenic sites.

Chymotrypsin is less specific than trypsin or Staphylococcal V8 (Croft, 1980). Although it cleaves preferentially on the carboxy side of bulky hydrophobic amino acids, i.e., tyrosine, tryptophan, phenylalanine, methionine, and leucine, these residues are less likely to be near antigenic (hydrophilic) sites and cleavage should not occur within the three suspected epitopic fragments.

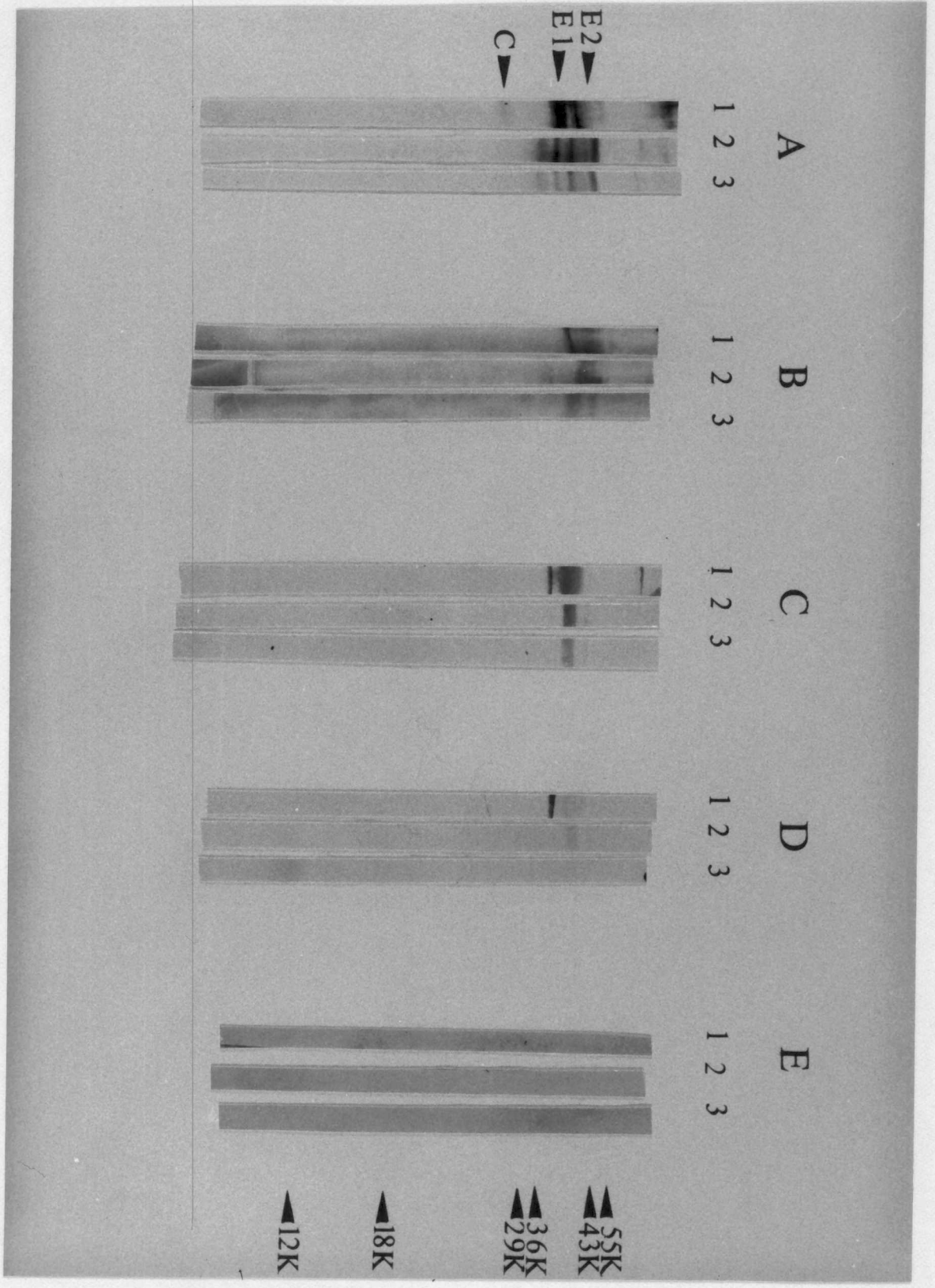
One likely cleaving reagent to test was found to be cyanogen bromide (CNBr), which cleaves at methionine residues. Methionine residues are rare enough in these glycoproteins to leave all these desired fragments intact.

Immunoblots of Digested SFV

Clarified supernatant fluid from SFV-infected CEF was diluted in buffer, and the fluid was treated by boiling in SDS. Chymotrypsin (CT) or Staphylococcal V8 protease (V8) were added at a protein to enzyme ratio of 20:1 and the digestion was stopped by adding electrophoresis sample buffer without beta-mercaptoethanol. Samples were separated on 15% SDS-PAGE and transferred to nitrocellulose. Proteins were detected by amido black staining or by immunoblotting.

CT digested the viral proteins quickly (Figure 20). No small fragments were detectable after amido black staining (data not shown). Immunoblot staining with homologous hSF did not reveal reactive protein fragments (Figure 20, Panel A, lanes 1-3), but did show that the amount of protein decreases with longer periods of digestion. From this

Figure 20. Immunoblot reactions of chymotrypsin-digested SFV proteins with polyclonal antisera and MAb. Protein to enzyme ratio was 20:1 in 0.25 M Tris-HCl, pH 6.8 with 0.5% SDS. Panel A, SFV proteins stained with extensive hyperimmune antiSFV serum; Panel B, SFV proteins stained with extensive hyperimmune antiSIN serum; Panel C, SFV proteins stained with MAb 32-15-1-45; Panel D, SFV proteins stained with MAb 37-139-2; and Panel E, SFV proteins stained with antiRSV ascites. Lane 1 in all panels represents virus control. Lane 2 in all panels shows results after three hour digestion, and lane 3 in all panels shows results after a 24 hours digestion.



decrease, one can deduce that digestion did occur. Immunoblot staining with heterologous hSIN (Figure 20, Panel B, lanes 1-3), MAb 32-15-1-45 (Figure 20, Panel C, lanes 1-3) or MAb 37-139-2 (Figure 20, Panel D, lanes 1-3) did not reveal any small reactive fragments. This finding might suggest that some secondary structure is involved in the epitope defined by these MAbs, since the specificity of CT is for bulky hydrophilic amino acids known to be involved in protein folding.

V8 digestion yielded several digestion products seen by amido black staining (data not shown) or immunoblot staining with homologous hSF (Figure 21, Panel A, lanes 1-3). All bands present in the total protein amido black stain were also present in the hSF stain. Some of these same bands were recognized by heterologous hSIN (Figure 21, Panel B, lanes 1-3). Only one band ($M_r = 20,000$) was recognized by MAb 32-15-1-45 (Figure 21, Panel C, lanes 1-3) and MAb 37-139-2 (Figure 21, Panel D, lanes 1-3).

Immunoblots of Cyanogen Bromide (CNBr)-Treated SFV E1

Gel pieces containing E1 from SFV were cut from the gel of a 12% SDS-PAGE and treated as described in Materials and Methods. No beta-mercaptoethanol was included in these experiments.

Staining with amido black or immunoblotting with homologous hSF or heterologous hSIN revealed two main fragments, $M_r = 30,000$ and $19,000$ (Figure 22, Panels A and B). Only the 30K fragment reacted with the MAbs (Figure 22, Panels C and D).

Figure 21. Immunoblot reactions of Staphylococcal V8 protease-digested SFV proteins with polyclonal antisera and MAbs. Protein to enzyme ratio was 20:1 in 0.25 M Tris-HCl, pH 6.8 with 0.5% SDS. Panel A, SFV proteins stained with extensive hyperimmune antiSFV serum; Panel B, SFV proteins stained with extensive hyperimmune antiSIN serum; Panel C, SFV proteins stained with MAb 32-15-1-45; Panel D, SFV proteins stained with MAb 37-139-2; and Panel E, SFV proteins stained with antiRSV ascites. Lane 1 in all panels represents virus control. Lane 2 in all panels shows results after three hour digestion, and lane 3 in all panels shows results after a 24 hours digestion.

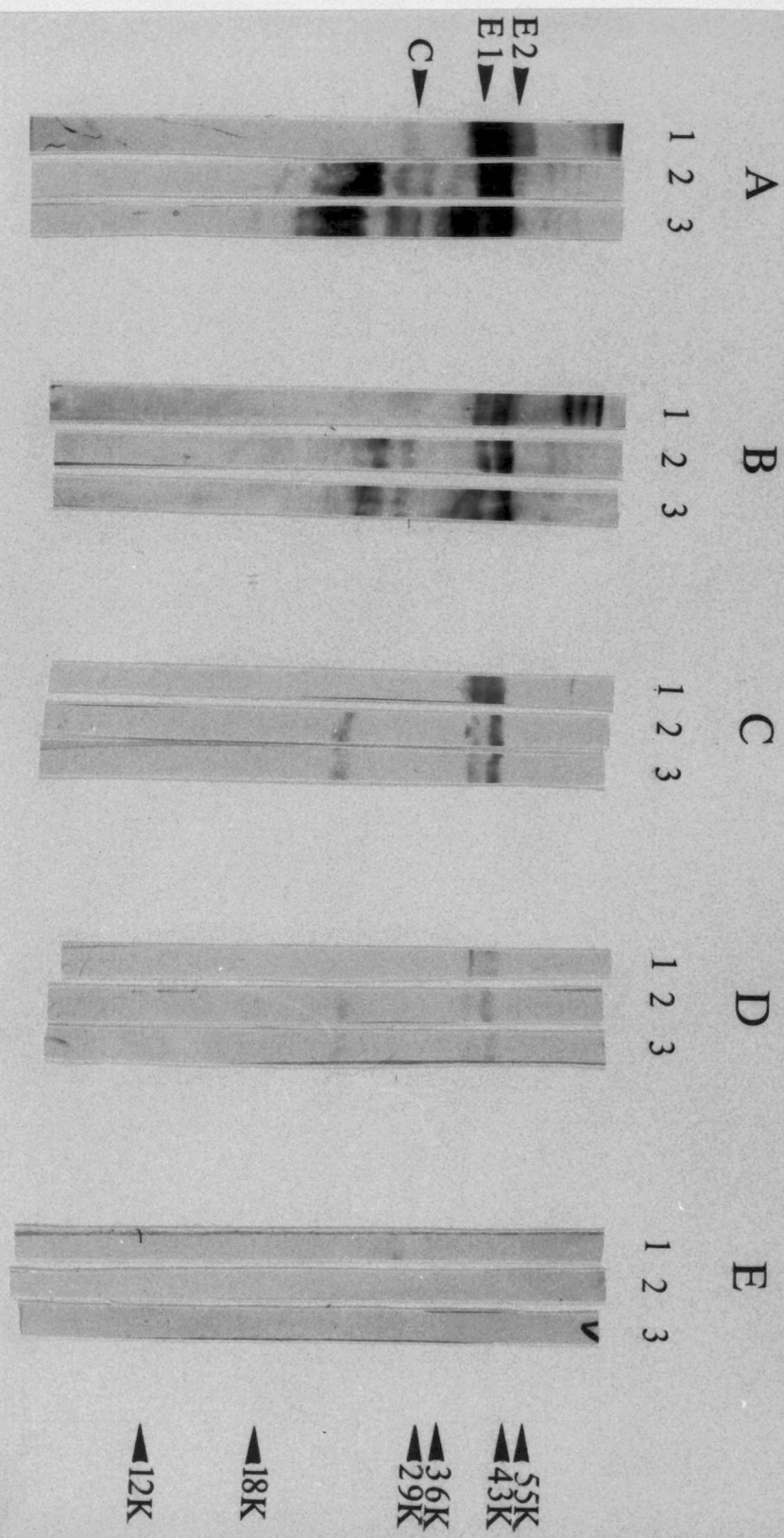
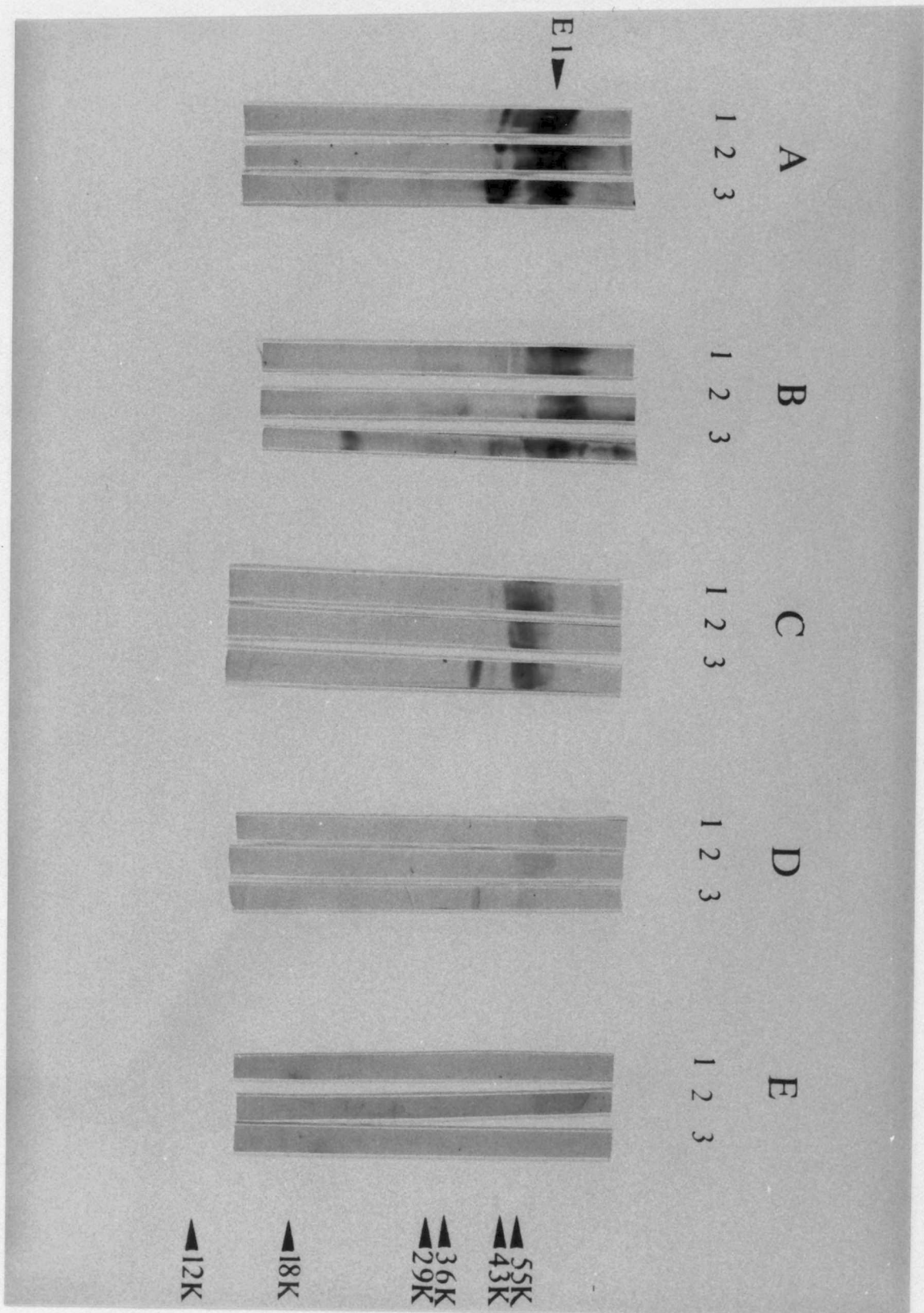


Figure 22. Immunoblot reactions of CNBr-treated SFV E1 with polyclonal antisera and MAbs. Gel pieces containing SFV E1 were incubated in covered jars with CNBr vapors for two days. Panels A-E are as in figures 20 and 21. Lane 1 in all panels represents incubation in water only; lane 2, incubation in 70% formic acid; and lane 3, incubation in 0.5 mg/ml CNBr in 70% formic acid.



The results from these digestions suggest that the epitope defined by these MAbs may be conformation-dependent, either continuous or discontinuous.

CHAPTER V

DISCUSSION

The E1 glycoprotein of alphaviruses has already been implicated in the in vivo cross-protection across subgroups (Wolcott, 1982; Wolcott, 1985), and is, therefore, of interest in the development of a cross-reactive vaccine.

In the procedures outlined in this thesis, eight MAb to alphavirus proteins were identified and four of these were found to be cross-reactive with the E1 glycoprotein of SIN or SFV as presented on immunoblots and by ADCMC and ELISA assays. These MAbs did not neutralize either virus in vitro, but three of them (IgG isotypes) were passively protective against SFV challenge in vivo.

The ADCMC assay appears to be one assay that may be useful in selecting MAbs that are protective and cross-protective across alphavirus subgroups in vivo, since several reports have indicated a positive correlation between the ability to lyse infected cells in the presence of added complement and passive in vivo protection against homologous alphavirus challenge (Schmaljohn, 1982; Mathews, 1985; Boere, 1986; Stanley, 1986). The four cross-reactive MAbs to the E1 glycoprotein were able to lyse SIN- or SFV-infected cells in the presence of complement to high titer (Table 3), but only three of these (IgG isotypes) were passively protective, i.e., the IgM MAb appeared to be non-protective (Table 6). Thus, the ADCMC assay appears to be an indicator of cross-protection by MAbs among alphaviruses as well as

homologous protection by MABs. It appears, however, that the isotype of the MAB must be considered in in vivo protection experiments.

Boere, 1986 reported similar findings regarding MAB isotype with SFV and homologous protection, while Mathews, 1982 observed that there was no apparent correlation between MAB isotype and protection against VEE, although all his protective MABs were of the IgG isotype.

In a second serological procedure, namely ELISA, the MABs recognized SIN and SFV virion epitopes, and reactivity correlated with the ADCMC data. However, caution must be exercised in comparing these data, since different batches of crude ascites fluid were used in the ADCMC assays and ELISA, i.e., the MABs were not purified or concentrated from the ascites. Therefore, the relative amounts of MAB present in one batch of ascites fluid compared to another may differ considerably. One would assume that the specificity would not change between batches (Hunt, 1985).

MAB 32-15-1-45 appears to be specific for an E1 epitope present on both SFV virions and infected cells (Figures 13 and 3, respectively) and SIN-infected cells (Figure 3), but not SIN virions (Figure 13). The other three cross-reactive MABs recognize SIN and SFV epitopes in either purified virions (Figures 14-16) or in infected cells (Figures 4-6), although SIN-infected cell epitopes appeared to be recognized better than SFV-infected cell epitopes.

Contrary to early reports that neutralization epitopes were confined to the E2 glycoprotein (Dalrymple, 1976), later reports suggested that MABs to E1 could neutralize virus infectivity in vitro

(Chanas, 1982; Mathews, 1982; Clegg, 1983; Schmaljohn, 1983; Boere, 1984; Hunt, 1985; Yamamoto, 1985; Stanley, 1986). In the present study, the anti-E1 MAbs did not neutralize virus infectivity but could be used in vivo to passively protect mice from SFV challenge. Again, caution must be exercised in comparing protection and other data, since different batches of ascites fluid were used in these experiments. In addition, a comparison of the relative efficiency of protection by different MAb preparations is difficult because the amounts of specific Ab were not quantitated (Hunt, 1985).

Based on the results obtained with the digestion and immunoblot procedure, it can be suggested that the epitopes defined by these MAbs may be dependent on their three-dimensional conformation. Such conformation could be shaped from the folding of a linear (continuous) sequence of amino acid residues or consist of foldings of different segments of the protein (discontinuous). Recently it was shown that the treatment of the virus glycoproteins with beta-mercaptoethanol apparently destroys the disulfide bonds with the epitope(s) recognized by these MAbs ([Wolcott] Nicholas, manuscript in preparation), further suggesting that three-dimensional conformation is important for reactivity with Ab and may involve intrachain disulfide bonds or discontinuous segments of the protein which are placed in juxtaposition by disulfide bonds close nearby.

Three different patterns of recognition by these four cross-reactive MAbs are shown by immunoblotting with virions or infected cells, depending on the presence or absence of

beta-mercaptoethanol ([Wolcott] Nicholas, manuscript in preparation).

(1) MAb 32-15-1-45 reacts with the cellular form of SFV E1 and both, SIN and SFV virion forms of E1, regardless of reduction. These data suggest that MAb 32-15-1-45 could be specific for a linear epitope, i.e., not dependent on disulfide bond formation. (2) MAbs 37-113-2-3 and 37-139-2 recognized the cellular and virion forms of SFV E1 and the virion form of SIN E1, but only when unreduced, suggesting that the epitopes recognized by these two MAbs is dependent on the conformation induced by disulfide bond formation and it different from the cross-reactive determinant defined by MAb 32-15-1-45. (3) MAb 37-114-2-20 recognized all cellular and virion forms of SIN and SFV E1 but preferred the unreduced forms.

Because the samples were heat-treated twice (once before digestion and once after digestion), most secondary structure was destroyed except for the disulfide bonds which require beta-mercaptoethanol treatment to be broken. All MAbs tested still recognize the twice-boiled form of E1 on immunoblots (Figures 20, 21, 22, Panels C and D, lane 1), further suggesting that the epitopes are dependent upon the disulfide bond formation. The length between the epitope and the disulfide bond must be small, however, because the heat treatment would have destroyed any other secondary structure present.

One can assume that if the epitopes are linear, enzymatic digestion followed by immunoblotting might yield fragments that are reactive with at least one of the MAbs. Staining the blots with amido black or with homologous polyclonal antiserum did indicate reactive,

small fragments ($M_r = 17K - 30K$), but it is unclear (except in the CNBr gels) which of these small fragments may have been generated from the digestion of the E1 glycoprotein. It is possible that a linear epitope would have been cleaved and is not reactive with the MAb on an immunoblot, perhaps due to conformational constraints imposed by the nitrocellulose. However, it is also possible that cleavage resulted in the generation of large fragments (M_r approximately 20K) of the E1. Time course digestions, which will be stained by the MAb may provide an answer to these possibilities. It is unlikely, however, that if the epitope is linear and dependent on folding in a particular conformation, enzymatic digestion might yield a fragment which would fold the correct way because it would no longer be influenced by constraints imposed by the intact, native protein.

If the epitopes are discontinuous, i.e., formed by peptide segments brought together by intrachain disulfide linkages, then one would strongly suspect that the epitope would be disrupted during digestion. The time course experiments described above may help to discriminate between linear or non-linear epitopes.

Another alternative to resolve the conformation problem would be to study enzymatic fragments or synthetic peptides in solution as opposed to fixation in ELISA or immunoblots. In solution, fragments should have the freedom of motion to arrange in all possible conformations including that of the native epitope. Fragments in solution would appear to be a better choice to block ADCMC recognition

of infected-cell epitopes or to inactivate the MAbs in protection studies.

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