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COMPARISON OF CROSS-REACTIVE ANTIGENS IN SINDBIS AND SEMLIKI
FOREST VIRIONS AND INFECTED CELLS

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ABSTRACT

Aliquots of rabbit hyperimmune antisera against either Sindbis or Semliki Forest virus were absorbed with purified Sindbis virus or chick embryo fibroblasts infected with Sindbis virus, or with purified SF virus or cells infected with SF virus. Residual antibody titers were determined by hemagglutination inhibition (HAI) and antibody-dependent, complement-mediated cytotoxicity (ADCC) assays. It appears that virus-infected cells remove ADCC detectable cross-reactive antibody more efficiently than does purified virus. This phenomenon was not due to the greater ability of virus-infected cells to absorb out antibody, nor was it due to the presence of antibody against normal, uninfected cells. HAI assays with the same absorbed antisera indicated that both virus and virus-infected cells removed cross-reactive antibody. Further characterization using radioimmune precipitation (RIP) indicated that cross-reactive antigenic determinants are present on viral E1 and on virus-infected cell E1. ADCC assays using the temperature sensitive mutant ts 20 indicate that ADCC-detectable cross-reactive determinants exist on E1 and possibly E2 in virus-infected whole cells.

On the basis of these data, there appear to be at least three antibody populations in anti-Sindbis or anti-SF: 1) a specific antibody population detectable by all assays employed. 2) a cross-reactive population detectable by HAI and RIP, which recognizes antigenic determinants on virus-associated and virus infected cell-associated E1, and 3) a second cross-reactive population, detectable by ADCC, which recognizes antigenic determinants on infected cell associated E1 and possibly E2.

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ABBREVIATIONS

ADCMC	Antibody-dependent, complement-mediated cytotoxicity
BHI	Brain-heart infusion broth
FCA	Freund's complete adjuvant
HAI	Hemagglutination inhibition
HBSS	Hanks' balanced salt solution
MEM	Eagle's modified minimal essential medium
RIP	Radioimmune precipitation

CHAPTER I

GENERAL BACKGROUND

Classification

Togaviridae is a family of animal viruses having an icosahedral nucleocapsid which contains infectious single-stranded RNA. The nucleocapsid, in turn, is surrounded by a lipoglycoprotein envelope. Until recently most members of this family were classified as arboviruses, based on their transmission vectors, hemophagous arthropods.

Togaviruses are in turn divided into two main groups, the A, or alphaviruses, and the B, or flaviviruses, based on serological classification. Alphaviruses, originally identified by Clark and Casals (12), are comprised of about 20 serologically related viruses. The two most intensively studied, and the ones used in this study, are Sindbis virus and Semliki Forest virus (SF).

Pathogenicity

Most alphaviruses cause relatively benign or inapparent infections in humans. However three, Venezuelan, Eastern, and Western Equine Encephalitis are capable of causing symptoms ranging from rash or flu-like symptoms, to encephalitis (17).

Mice have long been the animal of choice for pathogenicity studies. Both Semliki Forest and Sindbis virus are lethal for suckling mice. Adult mice are usually not susceptible to Sindbis virus, although lethal

brain-adapted strains have been isolated. Injection of Semliki Forest virus, especially by the intracerebral route still causes death within 10 days in adult mice.

Although the host range for Sindbis and Semliki Forest is extremely wide, injection with either virus usually results in subclinical infection in most other mammals. However, intracranial injection of Semliki Forest into rabbits or guinea pigs will cause fever and paralysis (3).

Virion Structure

Alphaviruses are among the least complex of the enveloped viruses, consisting of a single-stranded RNA genome surrounded by an icosahedral nucleocapsid. The nucleocapsid is in turn surrounded by a lipoglycoprotein envelope consisting of a host derived lipid bilayer containing two or three species of viral glycoproteins. The virion has a sedimentation coefficient of 280 S, a particle molecular weight of 70×10^6 , and a bouyant density of $1.21 \mu\text{g/ml}$ (31).

Viral RNA, Protein Synthesis and Maturation

Sindbis and Semliki Forest virus contain non-segmented, infectious RNA with a molecular weight of 4.5×10^6 as their genome. Two major species of viral mRNA are found within infected cells; the 42S species, which is thought to be equivalent to the virally packaged RNA, and the 26S species, which is derived from genomic RNA by internal replication and represents the $3' \frac{1}{3}$ of the 42S species. It is this 26S plus stranded RNA, with a molecular weight of 1.6×10^6 , from which structural proteins are translated (8, 25). Non-structural proteins are translated from the $5' \frac{2}{3}$ of the 42S species (9).

Formation of the structural proteins (Appendix) occurs through synthesis of a large polypeptide precursor derived from the whole 26S mRNA. This 130,000 dalton polypeptide is cleaved while still nascent to yield the capsid protein (M.W. = 30,000) and a precursor to the envelope proteins. This precursor is further cleaved to E1 (M.W. = 48,000) and PE2/NVP62 (M.W. = 54,000). In a final, rate-limiting step PE2/NVP62 is cleaved into E2 and E3. In the case of Semliki Forest, E3 is incorporated into the virion. However, in Sindbis, E3 is not incorporated but is released into the medium (47). Recently, a small, non-glycosylated non-structural protein has been discovered which is also translated from 26s mRNA. This protein, found to have a molecular weight of 4200 in Sindbis and 6000 in Semliki Forest, may be a signal peptide for facilitating transmembranal transport (47, 48).

Glycosylation of E1, E2, and E3 occurs during the final stages of maturation and is mediated by host glycosylases (40). The carbohydrate moiety of Sindbis and Semliki Forest glycoproteins consists of two non-identical oligosaccharide sidechains. The "simple" sidechain consists of six to eight residues of mannose and n-acetylglucosamine and is added to the protein relatively early in glycosylation. The longer, "complex" chain is variable in length and is composed of 16-20 residues of mannose, galactose, n-acetylglucosamine, fucose and sialic acid (39). E1 and E2 contain 8-12% carbohydrate, while E3 is approximately 45% carbohydrate (44).

Transfer of carbohydrates to the polypeptides is accomplished in bloc by a lipid carrier, dolichol (43, 39). The carbohydrate moiety is attached to the polypeptide through a β -n-glycosidic linkage between

asparagine and n-acetylglucosamine. After transfer, the precursor polysaccharide is processed by sequential trimming of terminal glucose and mannose moieties to yield either the high mannose "simple" sidechain or, after further modification by the addition of various sugar residues, the "complex" sidechain (43).

Maturation and budding occurs first with the insertion of E1 and PE2/NVP62 as a non-covalently bound complex into the host cell membrane about 2 h. post-infection. These glycoproteins diffuse laterally over the cell surface and increase in concentration to a maximum about 6-8 h. post-infection. Virus nucleocapsids are assembled in the cytoplasm and migrate to the underside of the cell membrane by diffusion. There they interact with a transmembranyl portion of PE2 and are fixed into position (21). Capsid protein, E1, E2, and E3 are found in a molar ratio of 1:1:1:1, indicating a specific recognition and attachment of capsid protein to the complex (25).

Final cleavage and glycosylation of the viral glycoproteins occurs after the insertion of the complex into the cell membrane. It appears that after insertion of the E1:PE2/NVP62 complex, but before the final budding, a number of maturational events occur both in protein biosynthesis and glycosylation (41, 23). These events appear to alter the conformation of the viral glycoproteins to a large extent and provide for a significant delineation between the cell associated E1:PE2/NVP62 complex and the final virion associated E1:E2 complex. Sefton et al. (41) have used lactoperoxidase iodination to determine the location of the viral glycoproteins within the cell membrane. Their studies have shown that PE2 is inaccessible to iodination, even though it is present

in the membrane in amounts approximately equal to E2. E2, on the other hand, is the most heavily iodinated of the viral proteins, both on the cell surface and on the virion. Since PE2, as a precursor to E2, is intrinsically a good substrate for iodination, these authors concluded that prior to cleavage PE2 is buried inside the cell membrane and is only exposed on the cell surface after cleavage. E1 was found to be iodinated to a similar extent both on the cell surface and on the virion. It was concluded that E1 does not undergo significant alterations during the budding process. However, other experiments involving analysis of the carbohydrate moiety of these glycoproteins suggests that E1 does in fact undergo maturational changes during budding. These same authors (40), have shown that there is more fucose and galactose associated with cellular E1 than with virion associated E1 and suggest that two populations of E1 could exist in the infected cell, one fully glycosylated and associated with E2, and one partially glycosylated and associated with PE2. That the degree of glycosylation may involve conformational changes has recently been demonstrated by Kaluza et al. (23). Using antisera against non-glycosylated, partially glycosylated, and fully glycosylated SF viral proteins, these authors were able to demonstrate that the degree of glycosylation influenced the antigenicity of E2 and E1. Since the carbohydrates themselves do not contribute to the antigenicity of the glycoproteins (23), these changes must be due to alterations in the conformation of the glycoproteins caused by the final glycosylation step, which affects the presentation of certain antigenic determinants. Furthermore, these authors found that antiserum against normal, cell-associated viral glycoproteins cross-reacted with partially

glycosylated viral NVP62 and E1, and that this antiserum contained antibodies specific for these immature proteins. On the basis of these data Kaluza et al. (23) suggested that two distinct forms of E1 exist on the cell and that the final conformational change responsible for the changes in antigenicity occur relatively late in glycoprotein maturation. In an accompanying paper (24), Kaluza and Pauli further corroborated their findings of conformationally induced exposure of antigenic sites by showing that reduction of E1 and E2 by 2-mercaptoethanol exposed previously hidden sites on the virion which were specific for the nonglycosylated NVP62 and E1 from infected cells in which glycosylation was inhibited.

Immunological Relationships

Togaviruses are divided into two subgroups (alpha- and flaviviruses) on the basis of neutralization, hemagglutination inhibition, and complement fixation (10, 12). A number of studies have been undertaken to categorize further each group into subsets based on cross reaction by these three serological criteria. Generally it has been found that while there is broad alphavirus or flavivirus intragroup cross reaction to capsid antigens, as measured by complement fixation, there is much more specific and less marked cross-reaction to envelope antigens (14). In the case of Semliki Forest and Sindbis virus, cross-reaction is only seen using HAI or antibody-dependent. complement-mediated cytolysis (27). There is no detectable cross-reaction by neutralization between these closely related viruses. Some of the viral proteins which carry the antigenic determinants recognized in these assays have been fairly well characterized. It has been shown that neutralization activity is

associated with E2, while hemagglutination inhibition may be attributed to E1. The evidence to support this comes mainly from work by Dalrymple et al. (16), in which viral proteins were isolated without loss of biological activity by disruption with Triton X-100 and subsequent isoelectric focusing. These isoelectrically focused proteins migrated as three separate peaks with isoelectric points of 3, 6 and 9 (referred to as pI3, pI6, and pI9). The peaks were identified with viral proteins by SDS-PAGE comparison with purified virus. pI3 was found to be capsid protein by this criterion. pI6 was found to migrate with E1 and to contain hemagglutinating activity. pI9 migrated with E2 and antisera against this glycoprotein neutralized infectious virus.

Antisera made from these three isoelectrically focused peaks were used to further characterize the immunological relationships among the viral proteins. Using radioimmune precipitation, Dalrymple et al. (16) were able to show that anti-pI3 reacted only with capsid proteins, anti-pI6 reacted against whole virus, pI6, and showed some cross reaction against Western equine encephalitis virus (WEE) virus. pI9 reacted against whole virus, to a lesser extent against pI9, and not at all against WEE. On the basis of this evidence the authors concluded that pI3 was the capsid antigen and contained antigenic determinants which were responsible for broad alpha-group cross reactions. Antigenic determinants responsible for more specific subgroup cross-reactions were thought to be contained on pI6, which was analogous to E1. Finally, pI9 was thought to be E2 and to contain determinants responsible for Sindbis virus specific reactions. In addition, the structural configuration of

pI9 on the surface of the virus was thought to play an important part in antigenicity since anti-pI9 reacted to a greater extent with whole virus than with isolated pI9.

The role of E1 in hemagglutination was also shown by Yin (51) using temperature sensitive mutants. Ts 23, a temperature sensitive mutant with a lesion in the E1 protein was found to be unable to hemagglutinate at the non-permissive temperature although hemagglutination proceeded normally at the permissive temperature.

Cross Protection

Numerous investigators (11, 13) have shown that immunization with a particular togavirus will confer immunity to subsequent challenge by another togavirus within the same group, i.e., alphaviruses.

This phenomenon, cross-protection, is observed in most instances, when the more closely related members of a group (by complement fixation, neutralization, and hemagglutination inhibition) are tested against each other. Even so, the majority of these viruses do not exhibit marked relationships by these serological criteria, even though good cross-protection is conferred (11). Most importantly, cross-protection may be elicited without the detection of cross-neutralizing antibody (37).

The mechanism for cross-protection appears to be a T-cell mediated event (34), since adoptive transfer of Sindbis virus immune spleen cells, in the absence of detectable antibody, will confer protection against a lethal challenge by SFV. Whether or not the same antigen is recognized by immune T cells and cross-reactive antibody has not been resolved.

Temperature Sensitive Mutants

A number of different series of Sindbis virus temperature sensitive mutants have been isolated. In addition to the original series isolated by Burge and Pfefferkorn (7), two other series, one derived from the HR strain (45), and one from the prototype AR 339 strain (1) have been isolated. Temperature sensitive mutants of Semliki Forest virus have also been derived (26, 46).

These ts mutants have been sorted into groups on the basis of complementation tests and on the basis of physiological defects. Mutants have been designated RNA- or RNA+ on the basis of incorporation of radioactive uridine into virus-specific RNA at the non-permissive temperature. Mutants incorporating 0-10% uridine are classified as RNA- while those incorporating 60-100% are classified as RNA+. Mutants incorporating intermediate amounts of uridine (10-60%) are designated RNA+.

RNA+ mutants have been found to fall into one of three complementation groups: C, D, or E. These three groups have been characterized biochemically, and have been found to correspond to defects in the processing of one of the three viral proteins. Mutants of group C have been found to be defective in cleavage of the precursor "ts 2" protein into capsid protein and viral glycoprotein precursors. Group D mutants have been shown to have a defective E1 protein since cells infected with Group D mutants fail to hemadsorb at the non-permissive temperature (51) and fail to bind anti-E1 antibody (2). In addition, Group D mutants also fail to cleave PE2 to E2 and E3 (20). This latter phenomenon is thought to be due to improper orientation of the E1-PE2

complex. Group E mutants fail to cleave PE2 to E2 and E3. The sole representative of this group, ts 20, has been shown to contain normal amounts of functional E1 on the surface of infected cells (41).

The Major Histocompatibility Complex

The involvement of the major histocompatibility complex (MHC) in alphavirus infections appears to be two-fold. In addition to the well-defined role of MHC in immune recognition, the MHC may also play a role unique to togaviruses, that of a virus receptor.

Immune recognition of virus-infected cells by cytotoxic T-cells involves recognition of syngeneic MHC antigens as well as virus defined antigens on the surface of infected cells (52). Presumably MHC restriction is also operative in alphavirus infections. The relative paucity of data regarding cytotoxic T-cell recognition of alphavirus-infected cells does not yet permit any firm conclusions to be drawn. At present, it is known that cytotoxic T-cells recognize alphavirus-infected cells and that cross-reactive cytotoxic T-cells appear to play a role in in vivo cross-protection (34). The location and identity of the viral antigens involved, as well as the exact role of MHC antigens in cytotoxic T-cell recognition, is unknown.

Recently Mullbacher et al. (30) have shown that cytotoxic T-cells against Sindbis virus-infected cells are completely cross-reactive by in vitro cytotoxic T-cell assays. However, antisera against these viruses can only block homologous cytotoxic T-cell activity. According to these authors, cytotoxic T-cells appear to recognize the same antigens as does antibody, however, cytotoxic T-cell recognition is much less specific. These findings are in contrast to those found in the influenza system.

Various authors (4, 5) have demonstrated that influenza immune cytotoxic T-cells contain two populations of reactive cells: one population recognizes type-specific antigens, probably HA determinants, and a second cross-reactive population is directed against the M protein. It is interesting in this regard that the findings of Peck et al. (35) also provide circumstantial supporting evidence for the presence of a distinct subpopulation of cross-reactive cytotoxic T-cells against Sindbis and SF virus infected target cells.

Recently, a second role has been ascribed to the MHC in togavirus infections. Helenius et al. (19) in 1978 reported that both human and mouse MHC determinants acted as specific cell surface viral receptors for SFV. This conclusion was based on the observation that detergent isolated spike protein from SF virus inhibited complement-mediated lysis of mouse cells by anti-H-2K and anti-H-2D antibodies, that isolated human HLA-A and HLA-B antigens inhibited binding of SFV glycoproteins to cells, and that soluble SF virus glycoproteins complexes selectively bound to H-2K and H-2D proteins. This conclusion has recently been seriously questioned as a result of data from Oldstone et al. (32). These authors have examined the kinetics of togavirus penetration and replication in murine F9 and PCC4 teratoma cells, which do not express MHC determinants and have found no difference in viral replication or yield in these cells compared to cells which do express MHC antigens. In light of these findings, it appears that while SF virus may in fact bind to MHC determinants, it is not a specific binding and is not directly related to penetration and replication of the virus.

Rationale

The following project was undertaken as the result of previous work by King et al. (27) which suggested that HAI and ADCMC assays might be detecting two separate antibody-antigen recognition systems. We decided to determine if this was the case by absorbing anti-SF and anti-Sindbis antisera with various combinations of purified virus and virus-infected cells in hopes of being able to delineate the cross-reactive antigens involved. This characterization also employed radioimmune precipitation and use of temperature sensitive mutants to attempt to determine the viral glycoprotein(s) containing cross-reactive determinants. The results of this work, and other work involving recognition of cross-reacting determinants by the cellular immune system (34, 35) are expected to culminate in the elucidation of immune recognition of alpha virus cross-reactive determinants.

CHAPTER II

CHARACTERIZATION OF VIRION AND CELL-ASSOCIATED ANTIGENS BY ANTIBODY-DEPENDENT, COMPLEMENT-MEDIATED CYTOLYSIS AND HEMAGGLUTINATION INHIBITION ASSAYS

Abstract

Rabbit hyperimmune antisera against Sindbis or Semliki Forest (SF) virus were absorbed with purified Sindbis virus or Sindbis virus-infected cells, or with SF virus or SF virus-infected cells. Residual antibody titers were determined by hemagglutination inhibition (HAI) and antibody-dependent, complement-mediated cytolysis (ADCMC) assays. It appears that absorption with virus-infected cells removes ADCMC detectable cross-reactive antibody more efficiently than does absorption with either virus. HAI assays with the same absorbed antisera indicated that both virus and virus-infected cells removed HAI-detectable cross-reactive antibody. On the basis of these data, there appears to be a cross-reactive antigen present on virus-infected cells which is detected by ADCMC. This antigen is distinct from the cross-reactive antigen detected by HAI.

Introduction

Sindbis virus and Semliki Forest (SF) virus are two closely related alphaviruses. Sindbis and SF virions contain two or three glycoproteins, E1, E2, and, in the case of SF virus, a third small glycoprotein, E3 (21, 38). The corresponding E3 in Sindbis infected cells is not incorporated into the virion, but is released into the medium (47). Maturation of either virus involves the insertion of partially glycosylated

E1 and the immediate precursor of E2 and E3, PE2/NVP62, into the host cell membrane as a non-covalently bound dimer (6, 21, 42, 18). This complex undergoes extensive conformational changes prior to incorporation into the free virion, involving proteolytic cleavage from PE2 to E2 and E3 (20, 41) and glycosylation (23, 40). Since cleavage of PE2 is rate limiting, taking up to 20 min (22, 38), it seems likely that there is a significant delineation between the immature PE2-E1 complex exposed on the cell surface and the mature E1-E2-(E3) complex found on the free virion. The conformational differences between PE2-E1 and E1-E2-(E3) manifest themselves as differences in antigenic presentation (23, 24).

We are interested in elucidating the mechanism(s) of cross-protection in vivo and immunological cross-reactivities in vitro between these two viruses (27, 34, 35). Using antibody-dependent, complement-mediated cytolysis (ADCMC), hemagglutination inhibition (HAI), neutralization and radioimmune precipitation assays, we attempt to determine and compare the viral induced antigens recognized in those cross reactions. Furthermore, by using antisera absorbed with infected cells or purified virions we are exploring the differences in antigen presentation between the viral specific glycoproteins on infected cells and those on free virions. On the basis of our data, there appear to be two cross-reactive subpopulations of antibody in antisera to Sindbis or SF virus sera. One subpopulation, detected by ADCMC, recognizes antigenic determinants predominantly, if not exclusively on infected cells, while the second population, detected by HAI, recognize determinants on both infected cells and mature virus.

Materials and Methods

Cells and Cell Culture. Primary chick embryo cells (CEF) were prepared from 9-10 day old embryos as previously described (27).

Virus Purification. Four hundred and fifty cm² roller bottles (Corning) were seeded at 1×10^6 cells/ml in 100 ml Medium 199 containing 5% calf serum (Flow Labs), and incubated 3-4 days at 37° until confluent. After washing the monolayers with Hanks balanced salt solution (HBSS), they were infected at a multiplicity of infection of 0.01 in 0.1 ml Brain-heart infusion (BHI) (Difco) with either Sindbis, strain AR339, or SF, Casals prototype strain. Virus was allowed to adsorb for 45 min, after which 20 ml Medium 199 plus 1% calf serum was added. After 18 h, for Sindbis, or 16.5 h, for SF, the virus-containing medium was removed, centrifuged at 3000 x g for 10 min and the clarified supernate frozen at -70°. After approximately 1500 ml had been collected, the virus-containing supernate was thawed, and virus precipitated by addition of 10% (w/v) polyethylene glycol and solid NaCl to give a final concentration of 0.5 M NaCl. After stirring at 4° for 1 h, the virus suspension was centrifuged at 10,000 x g for 45 min and the pellet suspended in TNE (Tris-HCl 0.05 M, pH 7.6; NaCl 0.1 M; EDTA 0.001 M) plus 0.1% calf serum. After centrifuging at 500 x g for 10 min, 20 ml virus suspension was layered on top of 10 ml 15% (w/v) sucrose in TNE + 0.1% calf serum and centrifuged at 90,000 x g for 2.5 h onto a cushion of 60% sucrose in TNE + 0.1% calf serum. The partially purified virus was collected from the 15%-60% interface by monitoring absorbance at 240 nm through an Isco gradient collector. The collected peak was diluted 1:1 in TNE, layered on a 32 ml 20-60% continuous sucrose gradient in TNE and centrifuged for 16 h at 90,000 g. The virus

band was collected as before, diluted 1:1 with TNE, pelleted at 100,000 x g for 2.5 h, resuspended in 1 ml TNE and sonicated for 30 sec. Virus titer was then assayed on CEF monolayers and viral protein concentration determined by a modified Lowry procedure (35). Recovered infectious virus was usually 25-30% of the original titer. Approximately 1×10^{12} pfu corresponded to 2 mg protein. Virus preparations were then frozen at -70° until further use.

Antisera. Two 250 μ g/inj. of purified virus emulsified in Freund's complete adjuvant (FCA) were injected into 6-8 week old New Zealand white rabbits. The first injection was made intramuscularly into the gastrocnemius, followed 3 weeks later by an intraperitoneal injection. Three days later, an intravenous injection of 250 μ g purified virus in TNE without FCA was given. Sera were collected 9 days later, heat-inactivated at 56° for 1 h, and absorbed twice with 1×10^8 fresh cells/ml.

Antisera Absorption. Two ml of antisera were absorbed two to three times with either 1×10^{11} pfu/ml of the appropriate purified virus or with 1×10^8 virus infected cells/ml. Infected cells were obtained by infecting monolayers in 450 cm^2 roller bottles with a multiplicity of infection of 30-50. Seven and a half hours later infected cells were washed once with HBSS, once with Saline A (8% NaCl, 0.04% KCl, 0.1% glucose, 0.04% phenol red and 0.5% NaHCO_3 , pH 7.6) containing 1 mM EDTA, and then incubated with 10 ml Saline A and EDTA for 15 min at 37° on a roller bottle apparatus. After removing the cells by flushing the roller bottle with a syringe and 14 gauge cannula, they were washed in suspension

three times with HBSS. Antisera were absorbed for 1 h at 37° with rocking, and then overnight at 4°. Residual virus was pelleted out of the antisera by centrifugation at 100,000 x g for 2 h, while cells were removed by centrifugation at 800 x g for 20 min. Absorbed antisera were frozen at -70° until used. No particular difference in absorption profiles was seen between cells obtained by EDTA removal and cells obtained by scraping monolayers directly into HBSS.

Labeling Conditions. Fresh cells were diluted to 5×10^7 cells/ml in Medium 199 and 150 $\mu\text{Ci Na}_2^{51}\text{CrO}_4$ (New England Nuclear) in 0.85% saline added per ml. Cells were incubated in suspension for 1 h at 37° with occasional shaking. Labeled cells were then counted, diluted to 3×10^6 cells/ml and 1 ml plated per well in 24 well plates. Cells were allowed to form monolayers overnight at 37° and then used in ADCMC assays as described below.

Serological Assays. Hemagglutination inhibition: HAI assays were done in microtiter plates essentially as described by Clarke and Casals (12). The HAI titer was taken to be the highest dilution which caused complete inhibition of hemagglutination by four units of infected mouse brain antigen in triplicate samples. A unit of antigen was taken to be the last dilution of antigen which caused complete hemagglutination on prior hemagglutination assays. Controls included uninfected mouse brain antigen, normal serum, antiserum without addition of antigen, and buffer alone.

Neutralization: Titration of virus neutralizing activity was done by the plaque reduction assay. Two-fold dilutions of antisera were made in Medium 199 without calf serum and an equal volume of virus at approximately 1000 pfu/ml was added. The virus:antibody mixture was allowed to incubate at room temperature for 1 h, after which 0.2 ml aliquots were added to monolayers in 60 mm diameter plates. Virus was allowed to adsorb for one-half hour at 37° and then overlaid with Lac-10 agar (0.5% lactoalbumin hydrolysate, 0.1% yeast extract, 1.1% bactoagar, 125 units penicillin, 125 µg streptomycin, 10% HBSS, 15 mg cysteine, 12 mg histidine and 0.14% NaHCO₃; pH 7.4). Plates were incubated for 48 h at 37° and stained with neutral red. The number of viral plaques at each dilution were counted in triplicate samples and the neutralizing titer taken as the reciprocal of that dilution which caused a 50% reduction in plaque number compared to plates containing virus alone, or virus plus preimmune or normal serum.

Antibody-dependent, complement-mediated cytotoxicity: ADCMC assays were performed as follows: ⁵¹(Cr)-labeled monolayers in 24 well plates were infected at a multiplicity of infection of 10 with the appropriate virus. Seven and a half hours post-infection, the infected cells were washed with HBSS, and 0.5 ml antiserum at the appropriate dilution and 0.5 ml guinea pig complement (Flow Labs) (1:15 final dilution) were added. After one hour incubation at 37°, the supernate was removed, the monolayer washed with HBSS, and the cells suspended in 1 ml Saline A plus EDTA containing 0.25% trypsin. The supernate and cells were counted in a

Beckman 4000 gamma spectrometer and the specific ^{51}Cr release determined according to the following formula:

% specific ^{51}Cr release =

$$\left(\frac{\text{CPM in supernate}}{\text{CPM in supernate} + \text{cells}} \right)_{\text{exp}} - \left(\frac{\text{CPM in supernate}}{\text{CPM in supernate} + \text{cells}} \right)_{\text{control}} \times 100$$

Controls included non-infected cells plus a 1:5 dilution of antiserum and complement, infected cells plus complement alone, and infected cells plus Medium 199 alone.

Results

Specific-antibody against Sindbis or SF virus was removed to a greater extent by absorption with homologous virus, or with cells infected with the homologous virus than with heterologous virus, or with cells infected with heterologous virus. Specific^a antibody was assayed in Sindbis antisera by performing ADCMC assays against ^{51}Cr -labeled monolayers which had been infected with Sindbis virus. As can be seen in Figure 1A, when Sindbis antiserum was absorbed with Sindbis virions a four-fold reduction in titer and a 34% decrease in the plateau ^{51}Cr -release was seen. Similarly, when Sindbis antiserum was absorbed with Sindbis-infected cells

^aDefinitions used: Homologous absorption: absorption of antiserum with the same virus, or with CEF infected with the same virus, as that used to produce the antiserum. Heterologous absorption: absorption of antiserum with an alphavirus, or CEF infected with an alphavirus, different than the one used to produce the antiserum. Specific antibody: antibody which was assayed using antigens from the immunizing virus, e.g., antibody detectable in anti-Sindbis antiserum against Sindbis-infected CEF, or Sindbis HAI antigens. Cross-reactive antibody: antibody which was assayed for using antigens from a second alphavirus, e.g., antibody detectable in anti-Sindbis antiserum against SFV-infected CEF, or SFV HAI antigens. Non-specific antibody: antibody detectable in assays which were not directed against togavirus antigens, e.g., against uninfected cell antigens.

Figure 1. Titration of specific antibody by ADCMC.

Anti-Sindbis (A), and anti-SFV (B) were assayed for specific antibody by ADCMC against ^{51}Cr -labeled CEF monolayers. Antisera were absorbed twice with 1×10^8 infected CEF/ml, or twice with 1×10^{11} pfu purified virus/ml prior to assay.

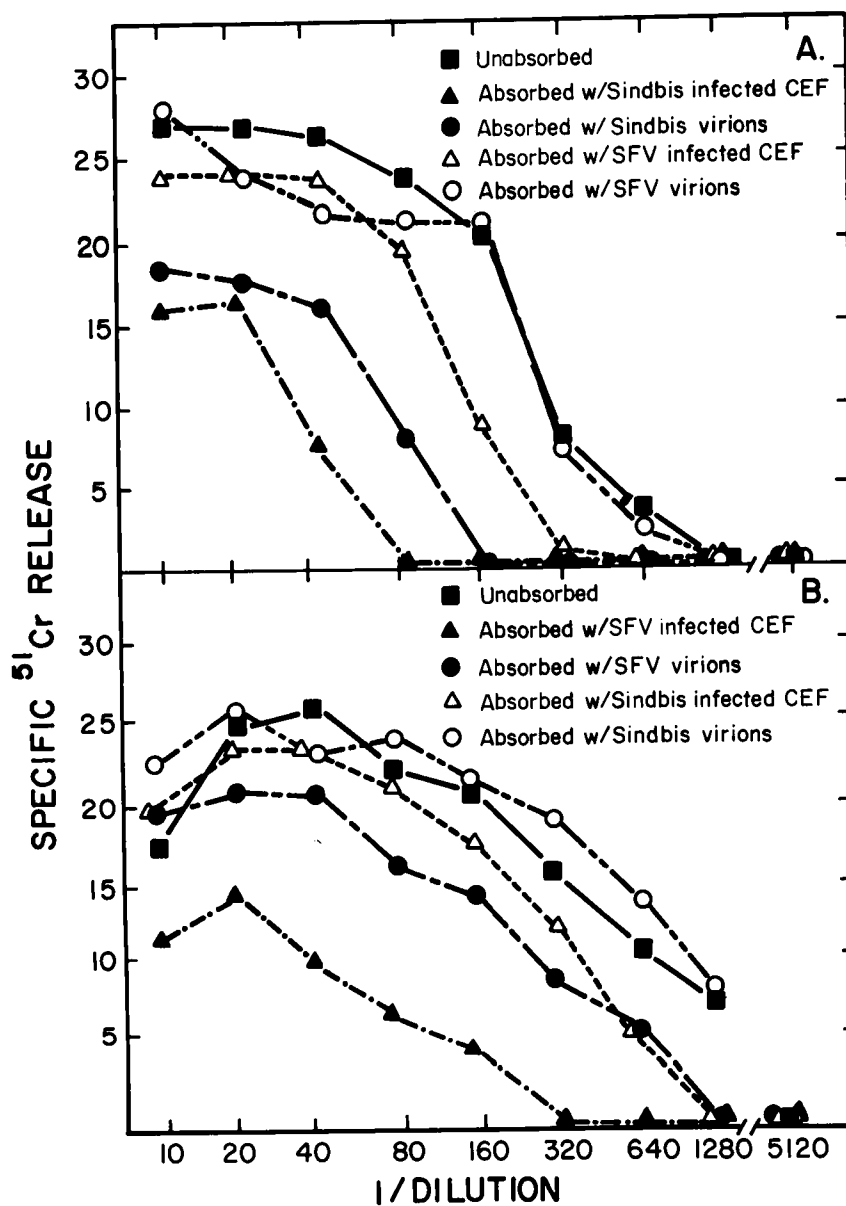


Figure 1

and eight-fold reduction in titer and a 40% decrease in the plateau $^{51}\text{(Cr)}$ -release was seen. Absorption of Sindbis antiserum with SF virion or SF-infected cells did not reduce the titer nor the level of $^{51}\text{(Cr)}$ -release to the same extent (no reduction in titer and 11% reduction in $^{51}\text{(Cr)}$ -release for antiserum absorbed with SF virions; a two-fold reduction in titer and 15% reduction in $^{51}\text{(Cr)}$ -release for antiserum absorbed with SF-infected cells). The same general pattern of titer reduction was seen in reciprocal experiments using anti-SF (Figure 1B). Both the titers and the amount of $^{51}\text{(Cr)}$ -release were relatively unaffected after heterologous absorption (anti-SF absorbed with Sindbis virus or Sindbis-infected cells). Absorption of anti-SF with SF-infected cells resulted in a seven-fold reduction in titer and a 40% decrease in $^{51}\text{(Cr)}$ -release. Absorption with SF virions was much less efficient in removing specific antibody (two-fold reduction in titer and 16% decrease in $^{51}\text{(Cr)}$ -release).

These data suggested that homologous absorption was more efficient in removing specific antibody than was heterologous absorption. However, the inability to exhaustively absorb out specific antibody, coupled with the two- to four-fold difference in titer reduction between antiserum absorbed with homologous virus and antisera absorbed with cells infected with the homologous virus, suggested additional experiments. To further substantiate the ability of homologous virus or cells infected with homologous virus to remove specific antibody, antisera were diluted 1:4 in TNE buffer and absorbed three times with either Sindbis virions, Sindbis-infected cells, SF virions, or SF-infected cells. After each absorption specific HAI antibody titers were assayed. As can be seen in Table 1, only homologous absorption removed antibody. Heterologous

TABLE 1. Specific Hemagglutination-Inhibition Antibody Titers of Diluted Antisera Before and After Absorption With Virus or Infected Cells^a

Anti-Sindbis absorbed with				
Number of Absorptions	Sindbis Virus	Sindbis Infected Cells	SFV Virus	SFV Infected Cells
Unabsorbed	80 ^b	80	80	80
1	<40	<40	80	80
2	<40	<40	80	80
3	<40	<40	80	80

Anti-SFV Absorbed with				
Number of Absorptions	Sindbis Virus	Sindbis Infected Cells	SFV Virus	SFV Infected Cells
Unabsorbed	640	640	640	640
1	320	640	<40	40
2	640	640	40	<40
3	320	640	<40	<40

^aAntisera were identical to those used in ADCMC assays shown in Figure 2, except that antisera were absorbed with 50 μ l gander RBC and an equal volume of Kaolin prior to assay. HAI assays were done in microtiter by the method of Clarke and Casals (2). The pH of Sindbis HAI antigen was 6.0, while that of SFV HAI antigen was 6.2.

^bAverage of triplicate samples.

absorption had no effect on specific antibody titers, even after three absorptions. The inability of heterologous absorption to remove specific antibody was further corroborated by ADCMC (Figure 2). Antisera absorbed with heterologous virus or with cells infected with heterologous virus still contained detectable specific antibody after three rounds of absorption. In contrast, specific antibody was below the limits of detection in antisera absorbed with the homologous virus, or with cells infected with the homologous virus. Background lysis was approximately 5.8%.

Cross-reactive antibody detectable by ADCMC was removed more efficiently by cells infected with either Sindbis or SF than by either free virus. In contrast to the titer reduction pattern seen when specific antibody was assayed, these same absorbed antisera showed a different pattern when assayed for cross-reactive antibody, e.g., anti-Sindbis assayed against ^{51}Cr -labeled cells infected with SF. As can be seen in Figure 3A, anti-Sindbis sera absorbed with either SF-infected cells, or with Sindbis-infected cells do not contain ADCMC-detectable cross-reactive antibody. However, anti-Sindbis sera absorbed with either SF or Sindbis virions showed much greater amounts of residual cross-reactive antibody (titers of 80% of the unabsorbed titers, and plateau ^{51}Cr -release values within 20% of the unabsorbed values). Reciprocal experiments using anti-SF sera demonstrate the marked ability of virus-infected cells to remove cross-reacting antibody (Figure 3B). Again, while there was no detectable antibody in infected-cell absorbed antisera, antisera absorbed

Figure 2. Titration of specific antibody by ADCMC after exhaustive absorption.

Anti-Sindbis (A), and anti-SFV (B) were diluted 1:4 in TNE buffer and absorbed three times as in Figure 1. It should be noted that dilution and variability in complement activity from lot to lot cause some decrease in maximum ^{51}Cr release.

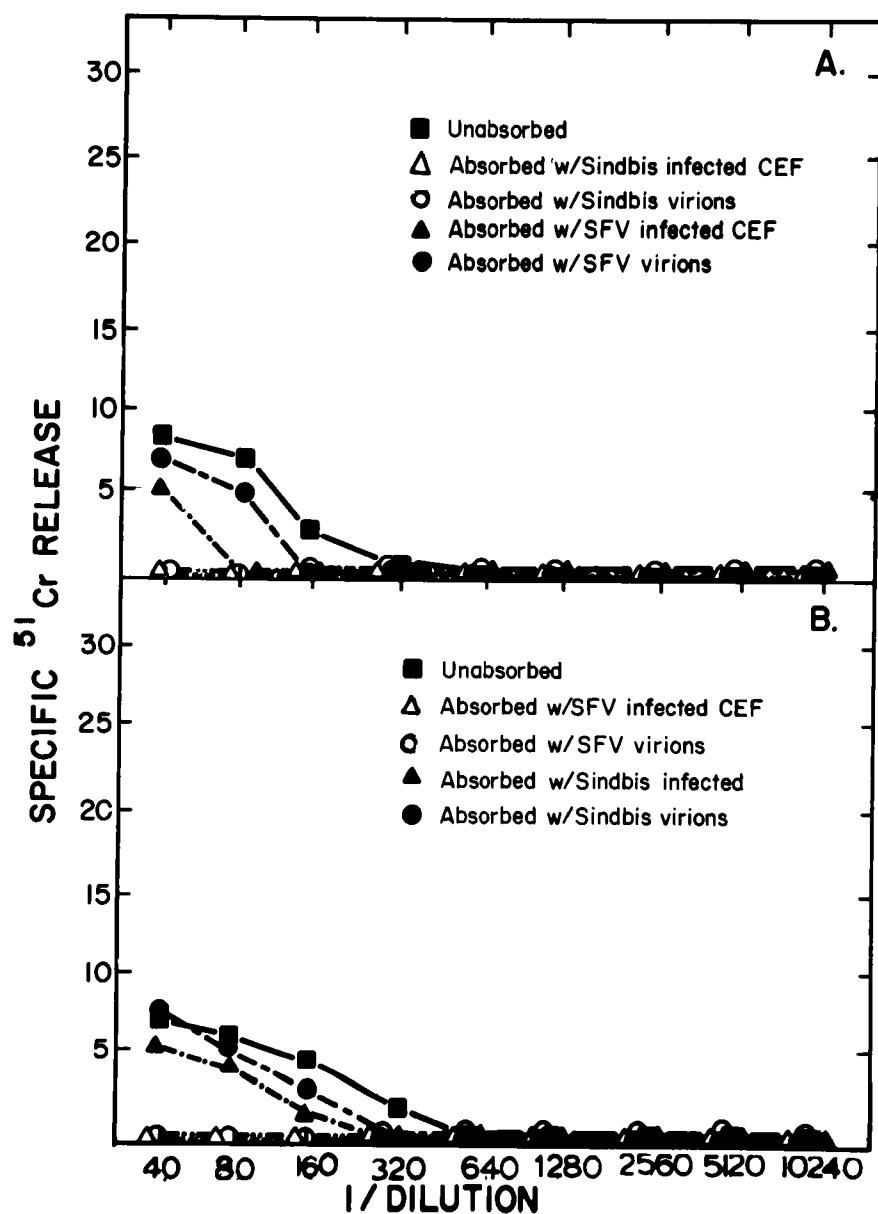


Figure 2

Figure 3. Titration of cross-reactive antibody by ADCMC.

Anti-Sindbis (A), and anti-SFV (B) were assayed for cross-reactive antibody by ADCMC against ^{51}Cr -labeled CEF monolayers. Antisera was identical to that used for Figure 1.

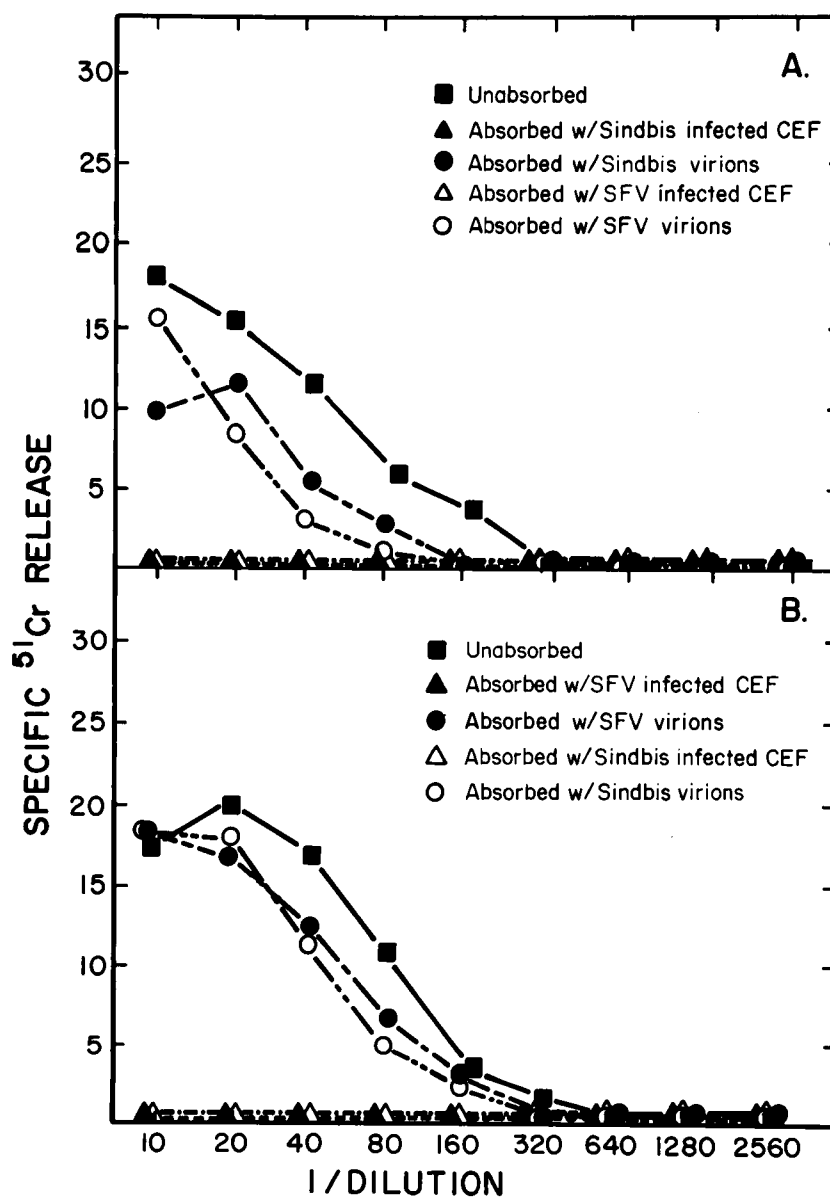


Figure 3

with either virus still had titers of 80% of the unabsorbed antisera, and ^{51}Cr -release values within 15% of the ^{51}Cr -release values of unabsorbed antisera.

There may be two cross-reactive antibody populations present in anti-Sindbis and anti-SF sera. Other investigators (16) have shown that mature virus contains cross-reacting determinants on E1 which are demonstrable by HAI. We therefore ran HAI assays on the same absorbed antisera used in ADCMC assays (Table 2). Specific antibody was removed more efficiently by homologous absorption, in agreement with ADCMC assays. When cross-reactive antibody titers were assayed, a different pattern of titer reduction was observed. Cross-reactive antibody titers were reduced four-fold or greater after absorption with cells infected with either virus. However, in contrast to ADCMC results, absorption with the heterologous virus (anti-Sindbis absorbed with SF virus, or anti-SF absorbed with Sindbis virus), also resulted in a reduction of cross-reactive HAI titers equal to those of antisera absorbed with infected cells. Taken together, these findings are consistent with the existence of two cross-reactive antibody populations. One population, assayable by ADCMC, is removed most efficiently by virus-infected cells. The other population, assayable by HAI, is removed to a similar extent by both infected cells and free virus.

The enhanced ability of virus-infected cells to remove cross-reactive antibody was not due to an intrinsic deficiency in the ability of free virus to remove antibody. It was noticed throughout these assays that, in general, absorption with infected cells decreased titers to a greater extent than absorption with free virus. The possibility existed that the

TABLE 2. Hemagglutination-Inhibition Titers of Virion and Infected Cell-Absorbed Antiserum^a

	Anti-SFV		Anti-Sindbis	
	Specific	Cross-Reactive	Specific	Cross-Reactive
Unabsorbed	1280	40	640	80
Absorbed w/SFV infected cells	160	<10	320	<10
Absorbed w/SFV virions	320	20	640	20
Absorbed w/Sindbis infected cells	1280	<10	80	10
Absorbed w/Sindbis virions	640	<10	160	40

^aConditions were the same as those described in Table 1.

failure of free virus to remove ADCMC detectable cross-reactive antibody was due to some difference in the ability of free virus to remove antibody during absorption. While it was impossible to assay the total amount of ADCMC-detectable cross-reactive antigen on free virus compared to that on infected cells, it was possible to arrive at an estimate of the comparative ability of virus and infected cell absorption to remove specific antibody. This was accomplished by assaying for residual neutralizing antibody, which recognizes non-cross-reacting determinants on E2 (16), after virion or infected cell absorption. Any gross differences in the ability of free virus and infected cell absorption to remove antibody should be reflected in differences in the residual amount of neutralizing antibody after absorption. As can be seen in Table 3, absorption of antisera with virus reduces neutralizing antibody titer to a similar, if not a greater extent than does infected cell absorption. In addition, comparison of HAI titers between virus absorbed and infected cell absorbed antisera (Table 2) (anti-SF absorbed with either SF virus or SF-infected cells, or anti-Sindbis absorbed with either Sindbis virus or Sindbis-infected cells) show similar degrees of specific antibody reduction.

If we make the assumption that any cross-reactive antigenic determinants would be present on the same molecules as antigenic determinants recognized by specific antibody, it seems likely that differences in cross-reactive antibody reduction observed in ADCMC assays between infected cells and free virus is not due to some deficiency in the ability of free virus to remove antibody.

The ability of virus infected cells to remove cross-reactive antibody was not due to normal cell-associated antigens. Although antisera were absorbed twice with uninfected chick embryo fibroblasts prior to absorption

TABLE 3. Neutralization Titers on Virion and Infected Cell-Absorbed Antisera^a

	Anti-SFV		Anti-Sindbis	
	Specific	Cross-Reactive	Specific	Cross-Reactive
Unabsorbed	15,000 ^b	<10	15,000	<10
Absorbed w/SFV infected CE cells	3,200	N.T. ^c		
Absorbed w/SFV virions	2,000	N.T.		
Absorbed w/Sindbis infected cells			800	N.T.
Absorbed w/Sindbis virions			139	N.T.

^aNeutralization titers were obtained by the plaque reduction techniques explained in Materials and Methods.

^b50% endpoint titer by plaque reduction.

^cNot tested.

with virus or virus-infected cells, it was still a possibility that antigens on normal chick embryo fibroblasts were in some way responsible for removing cross-reactive antibody. To determine whether or not this was the case, antisera were absorbed twice with fresh, uninfected cells and then an additional three times with uninfected cells harvested in the same manner as virus-infected cells. Additional absorption with uninfected cells had little effect on either specific or cross-reactive ADCMC titers (Figure 4).

Discussion

Previous work by King, et al. (27) suggested the possibility that HAI and ADCMC assays were detecting two separate antigen-antibody recognition systems. We decided to determine if this was the case by absorbing both anti-Sindbis and anti-SF sera with various combinations of infected cells or purified virus and assaying for remaining antibody by either HAI or ADCMC. Our results indicate that while absorption with homologous virus-infected cells, or homologous virus removes antibody responsible for the specific ADCMC reaction more efficiently than does absorption with heterologous virus-infected cells or heterologous virus, cross-reactive antibody can be removed most efficiently by cells infected with either virus. Absorption with either free virus had a much less pronounced effect on cross-reactive ADCMC titers. When these same absorbed antisera were assayed by HAI, the pattern of titer reduction was somewhat different. As with ADCMC assays, HAI assays showed that homologous virus, or cells infected with the homologous virus were best able to remove specific

Figure 4. Titration of antisera absorbed with normal CEF by ADCMC.

Anti-Sindbis (A), and anti-SFV (B) which had been absorbed three extra times with uninfected CEF (o, Δ) were compared to antisera which had been absorbed in the standard manner ($\bullet$, $\blacktriangle$). Circles, assayed for specific antibody; triangles, assayed for cross-reactive antibody.

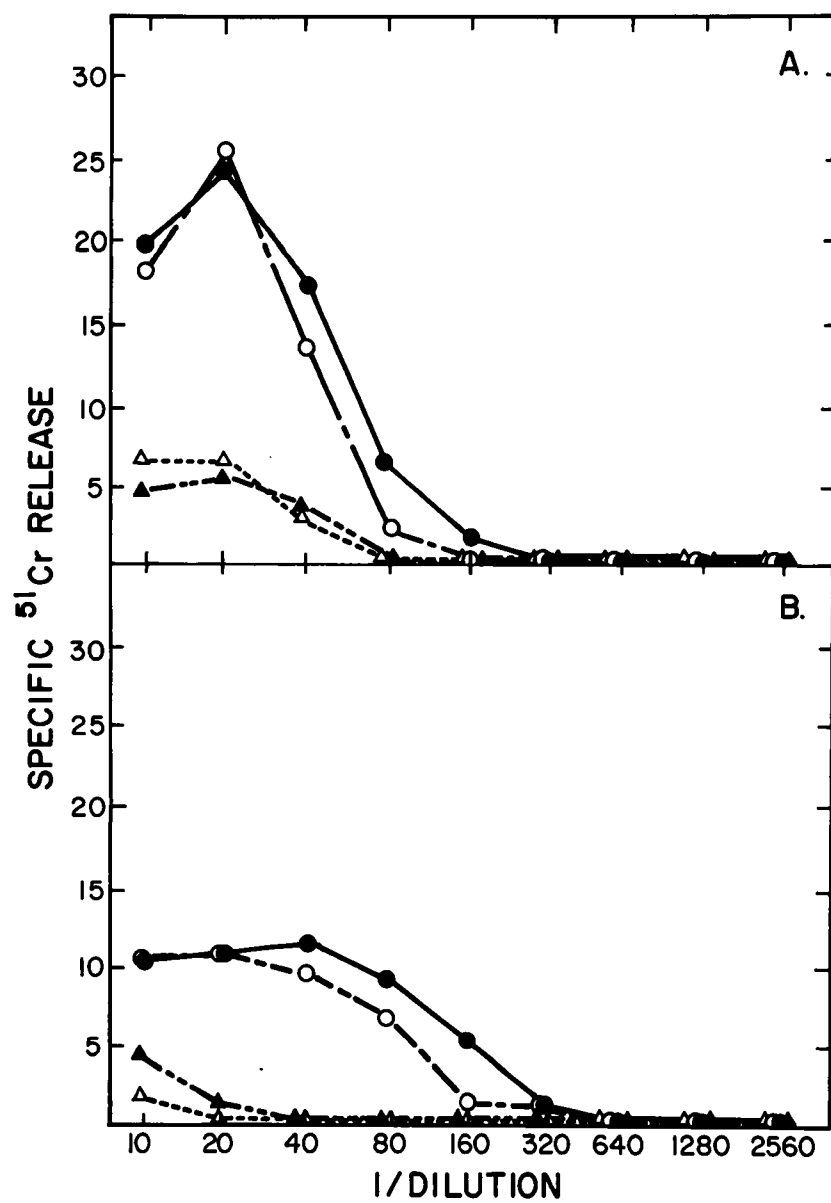


Figure 4

antibody. In contrast to ADCMC assays, heterologous virus absorption, as well as absorption with cells infected with either virus significantly reduced cross-reactive titers.

This pattern of titer reduction was seen with both anti-Sindbis and anti-SF sera. It does not seem to be due to some deficiency in the ability of virus to absorb out antibody since neutralization titers of virion absorbed antisera were reduced by similar, if not greater, amounts as virus-infected cell absorbed antisera. In addition, antisera in which specific antibody titers were not completely removed by homologous virus or infected cells showed similar levels of titer reduction in HAI assays. These data suggest that both free virus and virus-infected cells are able to absorb out antibody and that the greater degree of cross-reactive antibody reduction seen after infected cell absorption is not due to some intrinsic deficiency in the ability of virus to remove antibody.

While absolute, qualitative distinctions between antigenic determinants on virus or virus-infected cells could not be made, there does appear to be quantitative differences between the ability of infected cells and free virus to remove ADCMC-detectable cross-reactive antibody. The antisera used were against whole virus and virus-infected cells and would be expected to contain a broad range of antibody against various antigenic determinants. In this context, the small, but detectable decrease in ADCMC-detectable cross-reactive antibody after virus absorption may be due to removal of the same antibody population seen in HAI assays. Infected cells, on the other hand, may contain additional antigenic determinants and are thus able to remove both cross-reactive populations.

The apparent inefficiency of SF virus to remove specific antibody from undiluted anti-SF sera is of some concern even though subsequent ADCMC

assays on diluted antisera, and HAI assays, indicate that homologous virus is capable of removing specific antibody. One difference between Sindbis virus and SF virus is the presence of E3 on the surface of SF virions. It is possible that E3 on SF virus in some way lowers the efficiency of antibody binding during absorption of antisera.

Rabbit hyperimmune antisera did show cytotoxicity to uninfected CEF by ADCMC. Therefore all antisera were absorbed twice with fresh uninfected CEF prior to assays. It was still possible that the additional absorptions with infected cells might be further reducing some anti-CEF antibody. This possibility was ruled out by absorbing antisera an additional three times with uninfected cells harvested in the same manner as infected cells and demonstrating the lack of any change in titer.

Finally, the cross-reactivity described could not be attributed to contaminating virus during immunization, or to inadvertent mixing of antisera, since cross-neutralization assays failed to detect cross-neutralizing antibody in high titered serum.

The observation that HAI cross-reactive antibody is removed by heterologous virus, but not homologous virus is puzzling. It is known that virions contain HAI cross-reactive determinants on E1 (16). In addition, preliminary radioimmune precipitation (RIP) assays (Chapter III) show that both viral and cellular E1 are precipitated by cross-reactive antibody. It seems that HAI cross-reactive antibody is present in the antisera but is not being absorbed out by homologous virus. Since absorption with homologous virus involves the binding of specific antibody as well as any cross-reactive antibody to the virus, it is possible that the additional binding of specific antibody in some way, either by steric

hinderance or by increased avidity, blocks the binding of cross-reacting antibody. Heterologous absorption involves only the binding of cross-reactive antibody and so is not affected by such factors.

In contrast to HAI cross-reactive antibody, ADCMC cross-reactive antibody can only be removed efficiently by infected cells. Since virion associated cross-reactive antigen can be demonstrated by HAI but not by ADCMC, it seems probable that cross-reactive antibody may be comprised of two subpopulations, one population recognizing cross-reactive antigens on both infected cells and virus, and a second population recognizing cross-reacting antigens predominantly on infected cells. In this context, it may be significant that ADCMC assays involved titration of cross-reactive antibody on whole infected cells, while HAI and RIP assays involve titration against extracted antigens.

There are a number of possible candidates for this cell-associated cross-reactive antigen. PE2/NVP62 is the immediate precursor to E2 and E3 and is known to be in the membrane of infected cells (6, 42). However, since this glycoprotein does not seem to be detectable by either lactoperoxidase iodination (41) or ferritin-conjugated antibody (42), it is unlikely that it would be accessible to recognition by lytic antibody.

Evidence by Dalrymple (14) has suggested the presence of three or four antigenic determinants on the surface of alphaviruses, based on radioimmune precipitation studies of Sindbis, Western equine encephalitis virus (WEE), and Eastern equine encephalitis virus (EEE). In addition to an antigenic determinant recognized by specific antibody two, or possibly three, determinants appear to be recognized by cross-reaction between Sindbis and WEE. It may be the case in the Sindbis-SF system, that one class of cross-reactive antibody recognizes antigens on both the intact

virus and the infected cell, and is assayable by HAI. A second class of cross-reactive antibody would recognize antigenic determinants on the surface of infected cells and would be assayable by ADCMC. However, for this hypothesis to be tenable, one would have to argue that this second class of cross-reacting antigen is much more efficiently recognized on the surface of infected cells, and that it is not due in some way to the presence of capsid antigens, which are known to be broadly cross-reactive (15).

Another more likely possibility is that conformational changes in one or both of the two main glycoproteins prior to budding sequester antigenic determinants on the mature virion which were initially exposed on the infected cell. The explanation is consistent with recent observations by Kaluza et al. (23, 24) in which antibody against infected cells were found to contain antibodies against antigenic determinants which were inaccessible on the mature virus. These antibodies were found to be directed against antigenic sites on E1 and E2 which were exposed on the cell surface prior to final glycosylation events, but were masked in mature virus due to carbohydrate induced changes in conformation. It may be that this subpopulation of antibody is also responsible for cross-reactive ADCMC activity against infected cells.

There appears to be at least three populations of antibody present in anti-Sindbis or anti-SFV antisera. A major population comprised of antibody specific for the homologous virus, a cross-reactive population which reacts with antigenic determinants on mature, fully glycosylated E1 and is assayed by HAI, and a second cross-reactive population, assayed by ADCMC, that recognizes antigenic determinants only on infected cells, possibly partially glycosylated E1 and/or E2.

CHAPTER III

PRELIMINARY CHARACTERIZATION OF VIRION AND CELL-ASSOCIATED ANTIGENS BY RADIOIMMUNE PRECIPITATION

Abstract

Radioimmune precipitation of antigens from Sindbis virus, SF virus, or virus-infected cells was performed using both homologous and heterologous antisera. The results indicate that there are cross-reactive antigenic sites on E1 and that antibody directed against these cross-reactive sites may be removed by either virus or virus-infected cells.

Introduction, Materials and Methods, Results and Discussion

Sindbis and Semliki Forest (SF) are two alphaviruses which have been shown to contain cross-reacting antigens by several methods (10, 16, 27). Both viruses contain two glycoproteins on the surface of free virions, E1 and E2. SF virus contains, in addition, a third small glycoprotein, E3. Its counterpart in Sindbis virus is not incorporated into the virion and is believed to be released into the medium upon viral budding (47). Although E1 has been implicated as the glycoprotein containing cross-reactive antigenic determinants in other alphavirus systems (16), the location of the cross-reacting determinants in the Sindbis-SF virus system, as well as its presence on infected cells has not been determined. By using radioimmune precipitation (RIP) of viral and infected cell antigens with homologous and heterologous antisera, we

attempt to show that E1 contains at least one cross-reactive antigenic determinant and that this determinant is present on both the virus and virus-infected cells.

Virus was grown, harvested and purified as previously described (Chapter II). Antigens for RIP consisted of virus which had been labeled with 34 μCi (^3H)-labeled amino acids/ml (96 Ci/mmol, average specific activity) (Amersham) under steady state labeling conditions in Eagle's modified minimal essential medium (MEM) deficient in those amino acids used for labeling, and containing 1% calf serum (Gibco). After purification, virus was treated with 2% Triton X-100 (Rohm-Haas) for one hour at room temperature, sonicated for 30 seconds, and centrifuged at 100,000 x g for one hour to remove capsids. Infected cell antigen was prepared by infecting confluent monolayers in 60 mm diameter plates with the appropriate virus at a multiplicity of infection of 200. After seven and a half hours incubation at 37° in MEM containing 2.5 $\mu\text{g/ml}$ actinomycin D and 1% calf serum, infected monolayers were washed twice with Hanks balanced salt solution (HBSS) and 1 ml of the same medium used for virus labeling containing with 50 μCi (^3H)-amino acids and 2.5 $\mu\text{g/ml}$ actinomycin D was added. After two hours incubation, the labeling medium was removed, the monolayer washed twice with HBSS, and 1 ml 2% Triton X-100 added. The infected cells were solubilized during repeated passage through a 20 gauge needle and subsequent incubation for one hour at room temperature. After brief sonication, the suspension was centrifuged at 200 x g for 20 minutes to remove cell debris, followed by centrifugation at 100,000 x g for one hour to remove viral capsids. The supernate was removed and frozen at -70°. Radiolabeled antigen was centrifuged at 100,000 x g immediately prior to the assay to remove any aggregated

material. Rabbit hyperimmune antisera (Chapter II) were absorbed twice with 1×10^8 uninfected cells/ml prior to use. In some cases, antisera were absorbed with either 1×10^{11} pfu/ml of purified virus, or 1×10^8 virus-infected cells/ml as described in Chapter II. RIP assays were done using 25 μ l ^3H amino acid-labeled antigen, 25 μ l hyperimmune rabbit antisera, both diluted in 100 μ l TNE (Tris-HCl, 0.05 M, pH 7.6; NaCl 0.1M, EDTA 0.001 M) containing 0.1% Triton X-100. After incubation for one hour at 37° and subsequent incubation at 4° for four to six hours 25 μ l Staph A (Pansorbin, Calbiochem) were added. The mixture was allowed to incubate overnight at 4°. The cells, with the attached immune complexes were washed three times in ice cold TNE, with the sample being transferred to a new tube after the first wash. After centrifugation, the final pellet was solubilized in 50 μ l stacking gel buffer containing 2% SDS, 2% 2-mercaptoethanol, 2 M urea and 10% sucrose and bromphenol blue. Following incubation for 30 minutes at 37°, the mixture was heated in a water bath at 95° for three minutes, centrifuged to remove cells, and loaded onto a 20% polyacrylamide slab gel with a 5% stacking gel. Electrophoresis was for six hours at 10 mA/gel in a discontinuous buffer system (33). The gel was then impregnated with diphenyloxazole (Enhance, NEN), dried, and autoradiographed on prefogged Kodak X-Omat film (28).

As can be seen in Figure 5, anti-Sindbis virus serum appears to precipitate the E1 glycoprotein from both purified SF virus and from SF-virus-infected cells. The E1 protein of SF virus apparently has antigenic sites that are shared by a Sindbis virusprotein. When the reciprocal experiment was done, i.e., when anti-SF virus serum

Figure 5. Radioimmune precipitation of SFV antigens.

Twenty-five microliters of ^3H -amino acid-labeled glycoproteins from purified virions or virus-infected cells were reacted with 25 μl of either homologous or heterologous antisera. (1) untreated virions, (2) virion antigens precipitated with homologous antiserum, (3) virion antigens precipitated with heterologous antiserum, (4) SFV-infected cells, (5) SFV-infected cells precipitated with homologous antiserum, (6) SFV-infected cells precipitated with heterologous antiserum, (7) virion antigens precipitated with preimmune serum, (8) SFV-infected cell antigen precipitated with preimmune serum.

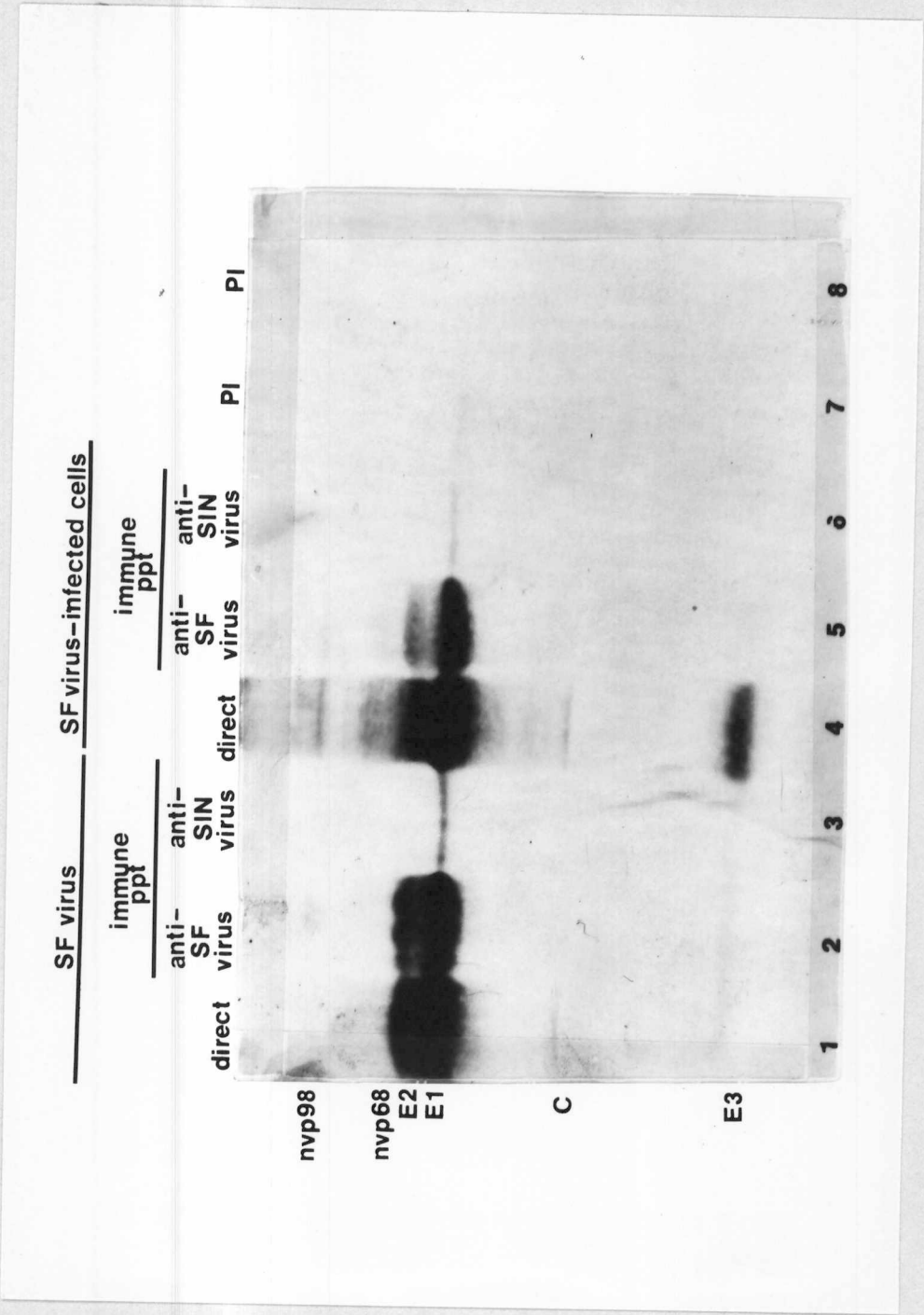


Figure 5

is used to immune precipitate proteins from Sindbis virus or Sindbis virus-infected cells, the E1 protein is also immunoprecipitated (Figure 6). The E1 glycoprotein of Sindbis virus therefore appears to have antigenic sites that are shared by a similar glycoprotein on Semliki Forest virus.

Even though E1 and E2 are both precipitable by homologous antiserum, E1 appears to be precipitated in proportionately far greater quantities by homologous antiserum than does E2. Previous experiments with Sindbis virus (16) also suggest that Triton X-100 extracted E2 is less precipitable than E1 with rabbit antisera. Although heterologous antiserum precipitation of E1 is clearly visible in our experimental system, the recognition of cross-reactive antigenic determinants on E2 by the same techniques cannot be ruled out. E2 immune precipitates by heterologous antiserum may be below the limits of detection.

Although antigen extracted from either free virus or virus-infected cells were immune precipitated by cross-reacting antisera, we were not certain whether the virion associated E1 and cell associated E1 glycoproteins were precipitated by the recognition of the same antigenic site on the molecule. Theoretically, subpopulations of antibody recognizing a separate antigenic site could yield the same results. We had previously found (Chapter II) that virus infected cells appear to contain a cross-reactive antigen which was not detected (or poorly detected) on free virions. This was based on ADCMC assays using antisera which had been absorbed with either free virus or virus-infected cells. It was therefore necessary to determine whether the cross-reactive antigenic determinants on E1 as seen by RIP were common to virions and

Figure 6. Radioimmune precipitation of SFV antigens.

Twenty-five microliters of ^3H -amino acid labeled glycoproteins from purified virions or virus-infected cells were reacted with 25 μl of either homologous or heterologous antisera. (1) untreated virions, (2) virion antigens precipitated with homologous antiserum, (3) virion antigens precipitated with heterologous antiserum, (4) SFV-infected cells, (5) SFV-infected cells precipitated with homologous antiserum, (6) SFV-infected cells precipitated with heterologous antiserum, (7) virion antigens precipitated with preimmune serum, (8) SFV-infected cell antigen precipitated with preimmune serum.

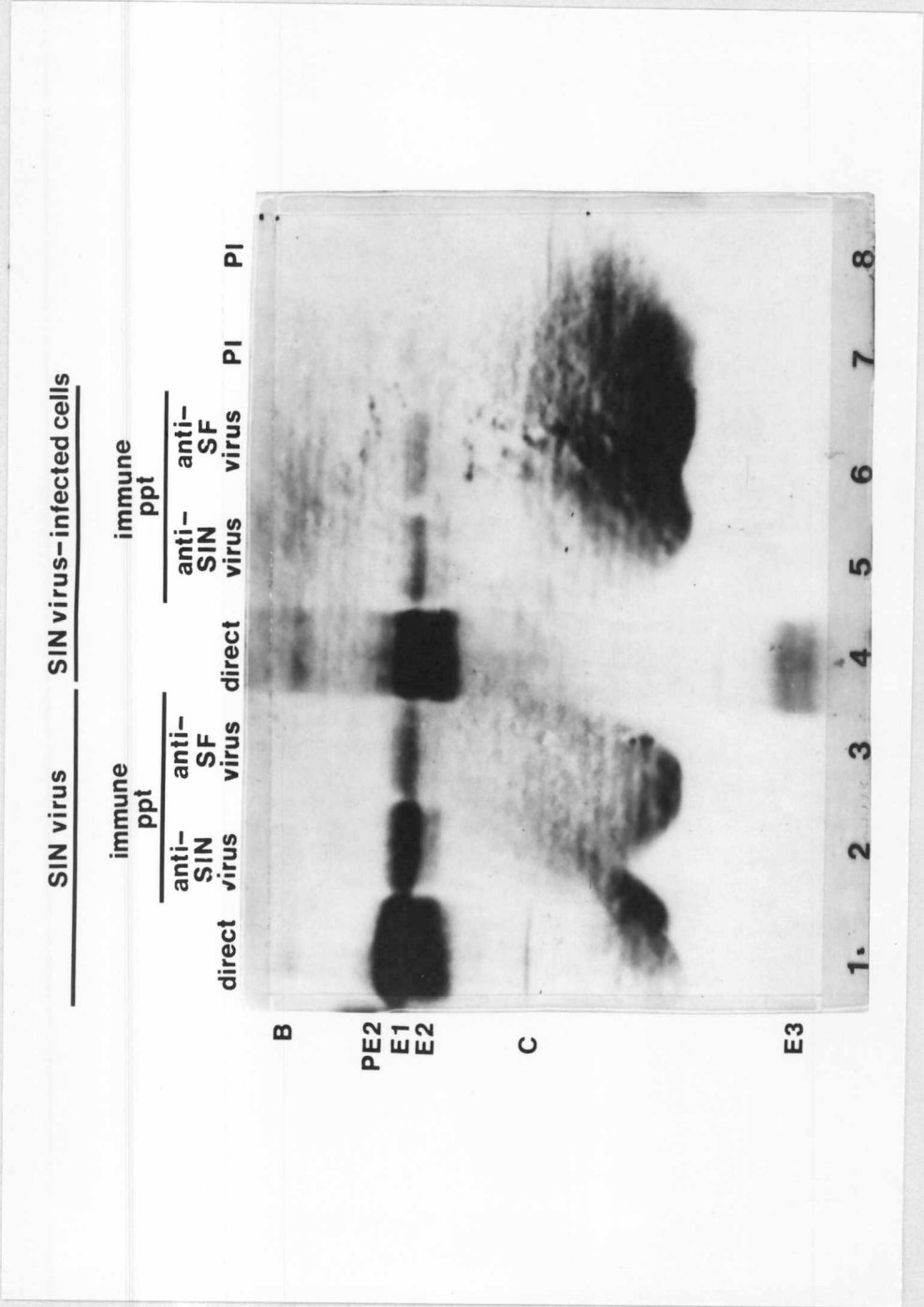


Figure 6

infected cells, or consisted of two antigenic determinants, one predominantly on virions and the other predominantly on infected cells. This was tested by using antisera which had been absorbed with either SF or Sindbis virions, or with SF virus-infected or Sindbis virus-infected cells in RIP experiments. As can be seen in Figure 7A, anti-SFV which had been absorbed with SF virions contained very little antibody against SF virus (lane 1), but did contain some reactivity towards SF virus-infected cell antigen (lane 2). Anti-SFV absorbed with SF virus-infected cells failed to precipitate either SF virus antigen or SF virus-infected cell antigen (lanes 5 and 6). Both absorbed antisera failed to precipitate Sindbis virus antigen or Sindbis virus-infected cell antigen (lanes 3, 4, 7, and 8). Anti-SFV absorbed with Sindbis virus (Figure 7B) was still able to precipitate both SF virus and SF virus-infected cell antigen (lanes 1 and 2). Anti-SFV absorbed with Sindbis virus-infected cells also precipitated SF virus antigen (lane 5). However, it appears that absorption of anti-SFV with Sindbis virus-infected cells reduces the capability of the antisera to precipitate SF virus-infected cell antigen (lane 6). As in 7A, anti-SFV absorbed with either Sindbis virus or Sindbis virus-infected cells fail to precipitate the cross-reactive antigen (lanes 3, 4, 7, and 8). The reciprocal experiments using anti-Sindbis sera are shown in Figures 8A and 8B. While the distinctions between the ability of homologous virus and homologous virus-infected cells to absorb out antibody against virus and infected cell antigen are not clear (lanes 1, 2, 5, and 6), the inability of antisera to precipitate cross-reactive antigen after absorption with either virus or either kind of virus-infected cells is

Figure 7. Radioimmune precipitation of viral antigens using absorbed anti-Semliki Forest serum.

Rabbit hyperimmune antiserum was absorbed four times with either 1×10^{11} pfu/ml. of the designated virus, or 1×10^8 cells/ml. of the designated infected cells. (A) anti-SFV absorbed with SFV virions (lanes 1-4) or SFV-infected CEF (lanes 5-8), (B) anti-SFV absorbed with Sindbis virions (lanes 1-4) or Sindbis-infected cells (lanes 5-8). Lanes 1 and 5, SFV virion antigen; lanes 2 and 6, SFV infected CEF antigen; lanes 3 and 7, Sindbis virion antigen; lanes 4 and 8, Sindbis-infected CEF antigen.

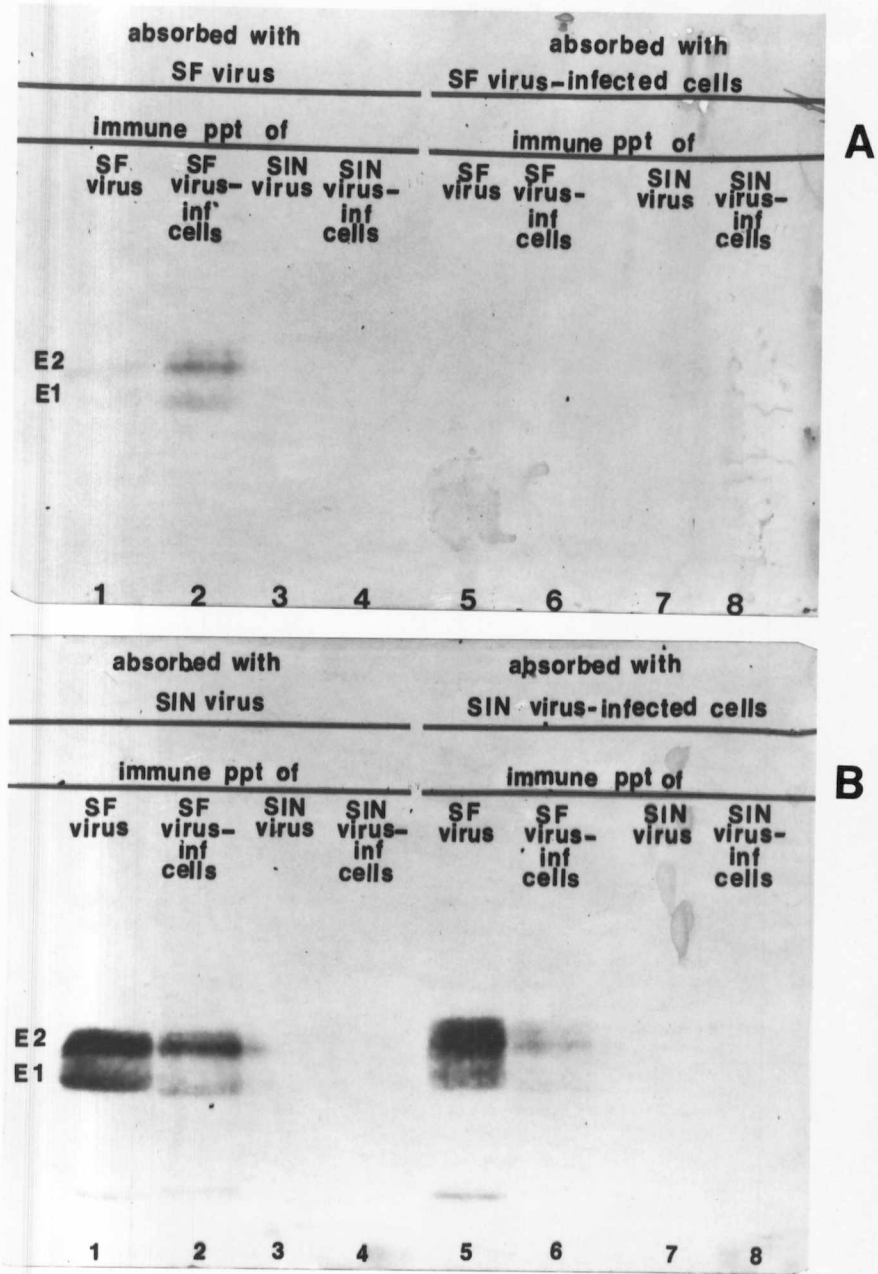


Figure 7

Figure 8. Radioimmune precipitation of viral antigens using absorbed anti-Sindbis virus serum.

Antisera was absorbed as described in Figure 9. (A) anti-Sindbis absorbed with Sindbis virions (lanes 1-4) or Sindbis-infected CEF (lanes 5-8), (B) anti-Sindbis absorbed with SFV virions (lanes 1-4) or SFV-infected CEF (lanes 5-8). Lanes 1 and 5, Sindbis virion antigen; lanes 2 and 6, Sindbis-infected cell antigen; lanes 3 and 7, SFV virion antigen; lanes 4-8, SFV-infected cell antigen.

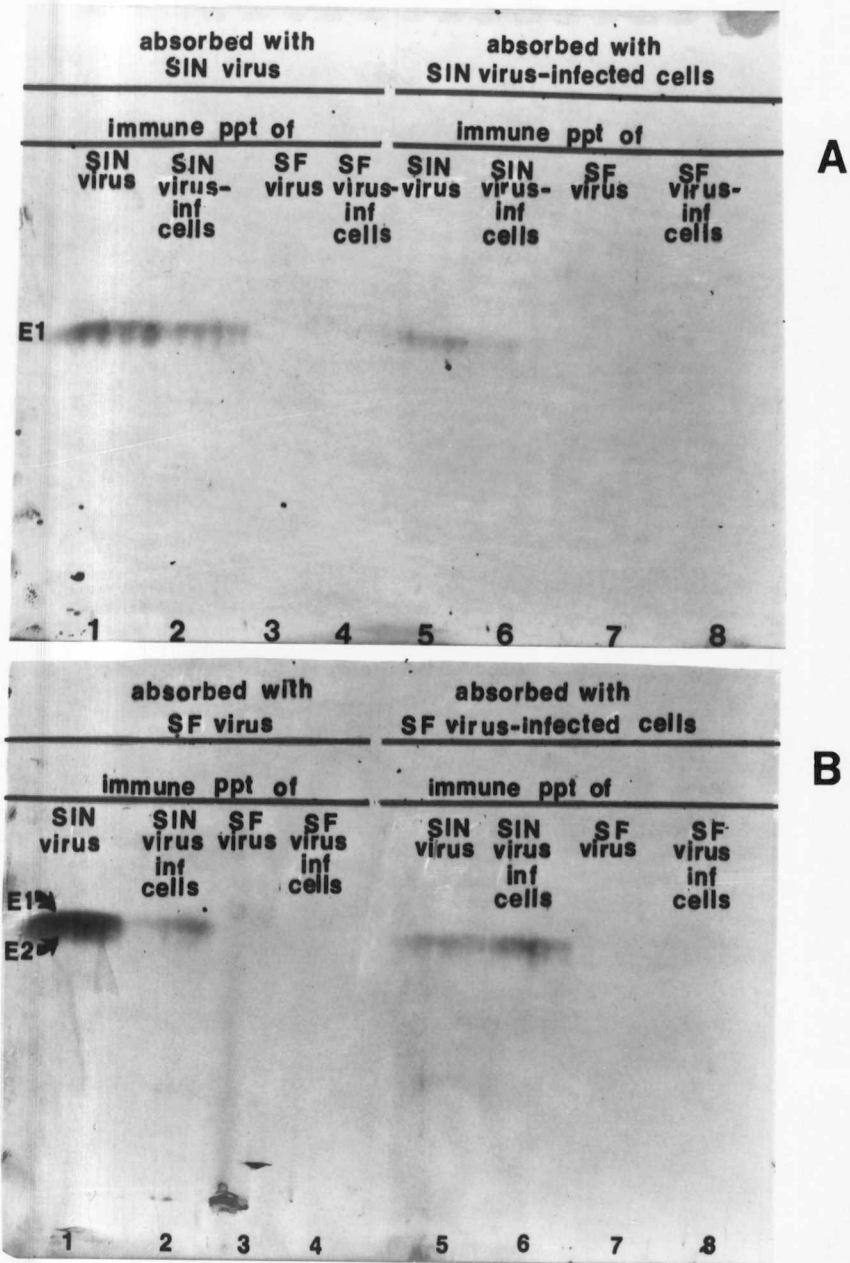


Figure 8

seen (lanes 3, 4, 7, and 8). Subsequent RIP assays, which included controls of unabsorbed antisera and antigen alone, also show the inability of either virus or infected cell absorbed antisera to precipitate cross-reactive antigen (data not shown). The results presented here suggest that both virion E1 and infected cell E1 are recognized by the same population of antibody, since antisera absorbed with either free virus or virus-infected cells failed to precipitate cross-reactive antigen. These results are similar to HAI results (Chapter II). However, a number of factors prevent firm conclusions from being drawn. The differential precipitation of E1 and E2 by homologous antiserum does not permit the possibility of cross-reactive determinants of E2 to be ruled out. In addition, the co-migration of Sindbis E1 and E2 in some gels does not allow unambiguous determination of the glycoprotein containing cross-reactive antigens. In general though, the data are consistent with other RIP studies (16) which suggest that E1 does contain cross-reactive determinants on the free virion.

It is interesting to note that while RIP experiments reported here and previous HAI assays (Chapter II) have suggested cross-reactive determinants to be present on both virus and virus-infected CEF, ADCMC assays detect the presence of cross-reactive determinants principally on infected CEF. The ADCMC assay is distinctive in that the antigens in question are assayed on the whole cell rather than as extracted antigens. This is important in light of other reports (16) which indicate that the structural configuration of alphavirus glycoproteins may play a major role in antigenicity (23, 24). In addition, it is possible that the detection of cell-associated cross-reactive antigens are below the level

of sensitivity for the RIP. The ADCMC assay relies on complement mediated cytolysis of infected cells, which is thought to involve one hit kinetics, and which may be sensitive enough to detect low levels of antibody. Finally Kaluza, et al. have recently shown that partially glycosylated, immature E1 and E2 contain antigenic sites which are not present on the mature virion associated E1 and E2 (23, 24). It is quite possible that these antigens are responsible for the cell-associated cross-reactivity seen by ADCMC and that their detection is enhanced by using living cells as the targets for antibody recognition rather than extracted antigens.

CHAPTER IV

PRELIMINARY ANTIGENIC CHARACTERIZATION USING TEMPERATURE SENSITIVE MUTANTS

Abstract

The RNA+ mutant of Sindbis virus, ts 20, which expresses E1 but not E2 on the surface of infected cells, was used to infect ⁵¹(Cr)-labeled cell monolayers for ADCMC assays. Results presented here indicate that cells infected with ts 20 and incubated at the permissive temperature were lysed to similar extents as cells infected with wild type Sindbis virus. There was also significant specific ⁵¹(Cr)-release from cells infected with ts 20 at the non-permissive temperature than with wild type infected cells.

Introduction, Materials and Methods, Results and Discussion

We have previously shown that rabbit hyperimmune antisera against both Sindbis and Semliki Forest virus appear to contain two subpopulations of cross-reactive antibody. One subpopulation recognizes cross-reactive determinants present on both free virus and infected cells. Radioimmune precipitation and hemagglutination inhibition experiments indicate that these cross-reactive sites include an antigenic determinant that is present on E1 and is similar, if not identical to that antigen characterized by Dalrymple et al. (16). A second cross-reactive site detected by antibody-dependent, complement mediated cytolysis (ADCC) appears to be predominantly on infected cells, since absorption of antisera with cells infected with either virus removes

much more lytic activity in the ADCMC assay, than does absorption with free virus. In an attempt to further define and characterize the nature of this second, cell-associated antigen, we made use of temperature-sensitive mutants of Sindbis virus which express only one of the two viral glycoproteins on the surface of infected cells.

Ts 20 is an RNA+ mutant of Sindbis (7), which does not allow cleavage of PE2 to E2 and E3 at the non-permissive temperature (42). However, E1 appears to be expressed normally on the cell membrane (50, 2). Using this mutant, we attempted to determine whether it was susceptible to lysis in ADCMC assays.

The ts 20 mutant (a gift from James Strauss, California Institute of Technology) was plaque purified and grown on primary chick embryo fibroblasts as previously described (27). The reversion frequency for this mutant was determined by taking the ratio of pfu titered at the non-permissive temperature (39°) to that determined at the permissive (29°) temperature and was found to be 8.34×10^{-5} . ADCMC assays were carried out as previously described (Chapter II), except that infected, ^{51}Cr -labeled monolayers were incubated at 39° for 7.5 hours, or 29° for 22 hours prior to the addition of complement and antiserum. Monolayers infected with AR339, the wild type strain, were assayed in parallel with the ts mutant-infected cultures.

As can be seen in Figure 9, cells infected with ts 20 and assayed at the permissive temperature for specific antigenic determinants (anti-Sindbis) or cross-reactive determinants (anti-SF) show approximately 22% greater plateau ^{51}Cr -release than their wild type

Figure 9. ADCMC of ts 20 and wild type Sindbis after incubation at permissive temperature.

⁵¹(Cr)-labeled cells monolayers were infected with either wild type virus or ts 20 and incubated at 29°. Twenty-two hours postinfection the appropriate antisera plus complement were added and specific ⁵¹(Cr)-release determined. ●, anti-Sindbis against ts 20 infected cells; ■, anti-SFV against ts 20 infected cells; ○, anti-Sindbis against Sindbis infected cells; □, anti-SFV against Sindbis-infected cells.

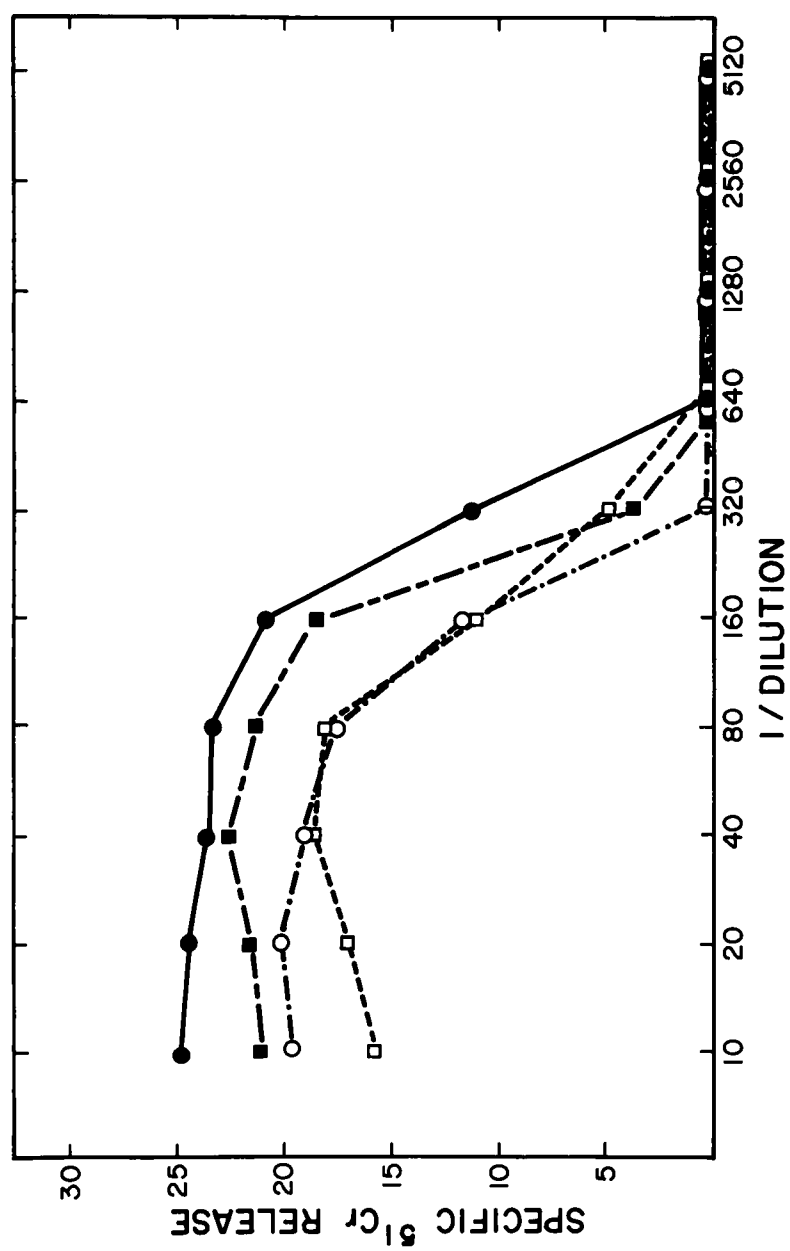


Figure 9

counterparts. In contrast, cells infected with ts 20 and incubated at the non-permissive temperature (Figure 10) show a reduced capability for lysis by either anti-Sindbis (50% less lysis than wild type) or anti-SF (33% less lysis than wild type). This approach has been extended by others in our laboratory who have repeated these results with infected CEF cells, and have also found the same pattern of reduced lysis in infected L-929 cells. Additional assays using a number of RNA⁺ and RNA⁻ from all five complementation groups have shown ts 20 to be the only mutant to express ADCMC detectable antigenic sites at the non-permissive temperature. This is in agreement with previous results (42, 50) which have shown that ts 20 is the only mutant that expresses detectable antigen on the surface of infected cells at the non-permissive temperature.

Cells infected with Sindbis mutant ts 20 and incubated at the non-permissive temperature express normal E1 on the cell surface based on the ability of cells infected with this mutant to hemadsorb (50) and to bind anti-E1 antibody (2) at the non-permissive temperature. E2 is not detectable on the surface of ts 20 infected cells at the non-permissive temperature (42).

If cross-reactive antibody were recognizing only E1, one would expect that complement-mediated lysis of ts 20 infected cells incubated at the non-permissive temperature would be similar to that of the wild-type. Alternatively, if only E2 were being recognized, one would expect very low levels of lysis at the non-permissive temperature. The observation that intermediate levels of lysis are seen, coupled with previous observations that only infected cells are capable of removing cross-reactive lytic activity, suggest the possibility that

Figure 10. ADCMC of ts 20 and wild type Sindbis after incubation at the non-permissive temperature.

Methods and Materials as in Figure 9 except that infected cells were incubated at 39° for seven and a half hours. Symbols as in Figure 9.

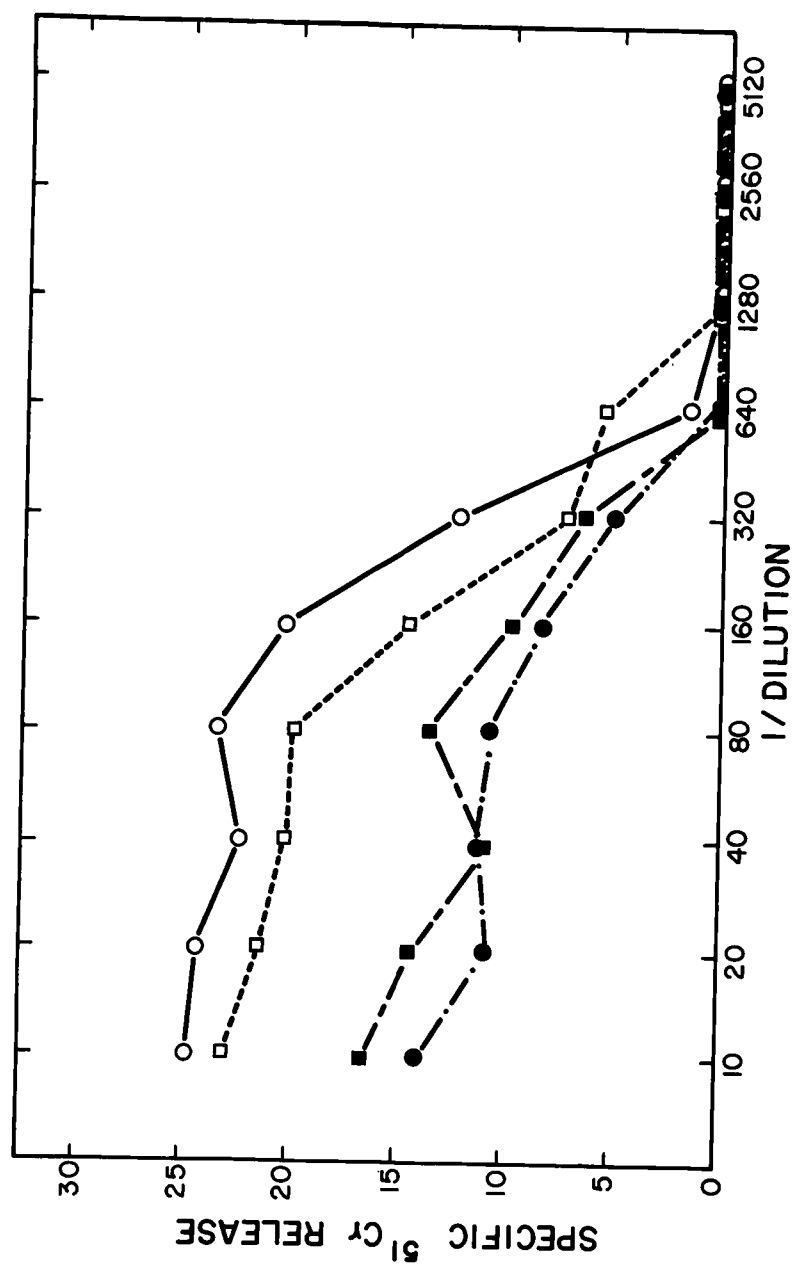


Figure 10

cross-reactive antibody assayed by ADCMC is recognizing antigenic determinants exclusive to the infected cell and located on both E1 and E2. It may be that these antigens are similar to those demonstrated by Kaluza et al. (23, 24), and represent partially glycosylated, immature forms of virion E1 and E2. If this was the case, cross-reactivity would be due to recognition of these immature cell-associated glycoproteins. Kaluza et al. have further shown that both E1 and E2 contain these "masked" determinants prior to complete glycosylation. Therefore, it is not unlikely that whole antisera against the virus contain subpopulations of antibody against immature E1 and E2, both of which are responsible for cross-reactivity in an additive manner. This hypothesis may be tested by using tunicamycin-treated virus-infected cells as targets for an ADCMC assay or, alternatively, raising antisera against tunicamycin-treated, virus-infected cells.

In conclusion, it appears that there are at least three distinct antibody subpopulations in anti-Sindbis or anti-SFV antisera; a population of specific antibody which recognizes determinants on both mature E1 and E2 and which is detectable by all the methods employed, a cross-reactive subpopulation which appears to recognize antigenic determinants on mature E1 and is assayable by HAI and RIP, and finally a second cross-reactive subpopulation which appears to recognize determinants that are predominantly expressed on infected cells, and may be present on both E1 and E2 prior to complete glycosylation and which may be recognized in an additive manner.

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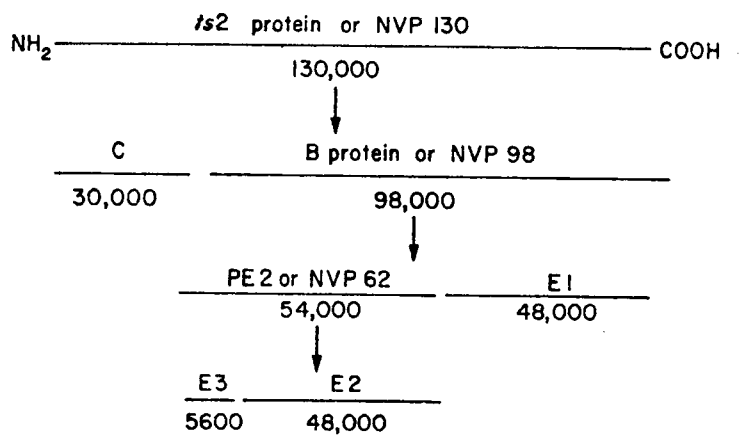
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APPENDIX

APPENDIX 1

Maturation of togavirus glycoproteins.



VITA

David W. Gates was born in Washington, DC. He was raised in California. He attended the University of California, received his Master of Science degree at the University of Kentucky and his Ph.D. at The University of Tennessee, Knoxville. He has been married for 10 years and has a five year old son, Moses, and a one month old daughter, Rivka.