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# IMMUNOGENIC CHARACTERIZATION OF THE DENGUE VIRUS SPECIFIED NONSTRUCTURAL GLYCOPROTEIN gp48 (NV3, SOLUBLE COMPLEMENT FIXING ANTIGEN)

FINAL REPORT



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Efforts have been concerned with mechanisms of the protective immune response to the flavivirus nonstructural protein NS1 and to identification of protective NS1 domains for incorporation into possible future subunit flavivirus vaccines. This report summarizes work performed during the funding period and includes data from experiments initiated in our laboratory as well as from those performed in collaboration with our colleagues in other institutions.

*Evidence of protective immunity to NS1.* We had earlier reported that immunization with yellow fever (YF) NS1 protects mice and monkeys against lethal YF infection and that mice immunized with dengue (DEN)-2 NS1 are similarly protected against this flavivirus (Schlesinger et al, 1985,1986,1987). These observa-

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(*19. Abstract continued).* tions have since been confirmed and extended. Immunization of mice with a YF NS1 recombinant fusion protein expressed in E. coli (Cane & Gould, 1988) or in a vaccinia vector (Putnak & Schlesinger, 1990) conferred partial protection against YF encephalitis in mice. Similarly, immunization of mice with recombinant DEN 4 NS1 expressed in baculovirus (Zhang et al, 1988; C-J Lai, personal communication) or vaccinia vectors (Falgout et al, 1990) has resulted in complete protection of mice against lethal challenge with DEN 4. That anti-NS1 antibody alone is sufficient to account for protection induced by recombinant DEN 4 NS1 was demonstrated in passive transfer experiments with mouse sera raised against the recombinant protein (Falgout et al, 1990). Vaccination of monkeys with a baculovirus-expressed polyprotein construct (C-M-E-NS1) has provided limited but definite protection (as determined by abrogation of viremia) in some animals (Eckels et al, in preparation) thus extending, for the first time, recombinant results from the mouse flavivirus encephalitis model to primate infection.

*Mechanism of protection by anti-NS1 antibody.* Cumulative data from assays which measure the capacity of anti-NS1 monoclonal antibodies (Mab) to sensitize cells to complement-mediated cytolysis (CMC) suggests a correlation between such activity and anti-NS1 Mab protective capacity. We used Mab and monospecific polyclonal sera against YF NS1 and virion envelope E protein as probes to detect these antigens on the surface of YF-infected mouse neuroblastoma (Neuro 2a) and human adenocarcinoma (SW13) cells (Schlesinger et al, 1990). Surface NS1 and E were detected on these cells by radiobinding and immunofluorescent assays. Using plasma membrane-impermeable cross-linking agents we showed that NS1 exists on the cell as a heat-labile homopolymer, a form which would be expected to most efficiently result in activation of complement by bound antibody. Of great interest is the observation that monospecific rabbit anti-NS1 serum, but not monospecific rabbit anti-E serum or anti-E Mab, sensitized YF-infected Neuro 2a and SW13 cells to CMC. Productive 17D YF infection was reduced 10 to 100-fold in synchronously infected cells maintained in medium containing an IgG2a anti-YF NS1 Mab, 1A5 (we had earlier reported that Mab 1A5 conferred protection against YF in mice, Schlesinger et al, 1985) or monospecific rabbit anti-NS1 serum in the presence, but not absence, of complement.

In an attempt to extend these observations to in vivo conditions, we prepared the F(ab)2 moiety of the protective complement-fixing anti-NS1 Mab 1A5 and measured its capacity to affect replication of brain-associated virus after intracerebral YF challenge. Since complement binds to the Fc portion of IgG, its removal should abrogate complement fixation but not antigen binding. F(ab)2 of Mab 1A5 or an IgG2a myeloma control protein (PC-5) was prepared by pepsin digestion and affinity chromatography. F(ab)2 1A5 retained antigen-binding capacity as determined by its ability to abrogate CMC by intact 1A5 and to interfere, on an equimolar basis with unlabeled IgG 1A5, with the binding of radiolabeled intact 1A5 to YF-infected cells. To conserve materials in pilot experiments, we measured levels of titratable brain-associated virus in small numbers (5-6) of mice rather than mortality rates (which would require considerably larger groups). A 10-fold reduction in titers of brain-associated virus was observed among mice passively immunized with IgG 1A5 whereas there was no difference in

*(19. Abstract continued).* titratable brain virus among F(ab)2 1A5 and control immunized animals. Experiments to test the protective capacity of intact 1A5 in mice depleted of complement by cobra venom treatment are in progress.

*Structural requirements for NS1 protective immunogenicity.* Knowledge concerning the molecular basis of NS1 antigenicity or protective immunogenicity is scanty. We showed that cell surface NS1 exists as a homopolymer and that all 12 cysteine residues (which are highly conserved among all flaviviruses) are involved in intramolecular bonding. To initiate experiments to determine structural requirements for the retention of NS1 protective immunogenicity, we subjected purified authentic YF NS1 to a variety of conditions which alter the conformation of the protein and characterized the antibody response in Balb/c mice immunized with these NS1 products. Immunization with native NS1 induced a strong IgG2a cytolytic response. Although diminished, cytolytic activity was still present in sera from mice immunized with SDS-denatured NS1 suggesting that the capacity to elicit cytolytic (and therefore presumably protective) antibody is not dependent on strict preservation of protein configuration. However, information concerning the extent to which this is true will await assay results with reduced or fragmented forms of NS1 (these experiments are in progress). It is of interest that antibody elicited by each of these NS1 preparations recognized NS1 in its polymeric form as determined by Western blot analysis. Experiments to directly determine protective immunogenicity of such altered forms of NS1 using conventional challenge models are planned.

It is not known whether cytolytic activity of an anti-NS1 antibody depends entirely on its subclass specificity or whether, as with neutralizing epitopes of the E protein, only certain regions of NS1 subserve such activity. Results of NS1 epitope mapping experiments using biotinylated anti-YF NS1 Mab and rabbit antisera produced against authentic and recombinant 17D YF NS1 (the latter prepared by Charles Rice) suggest that complement-mediated lysis is subserved by selected NS1 domains and that the capacity of an antibody to sensitize YF infected-cells to CMC depends on both its subclass and epitope specificity. However, attempts to identify such NS1 cytolytic (protective) epitopes after chemical fragmentation or enzymatic digestion have met with little success. YF NS1 fragments produced by cyanogen bromide or N-chlorosuccinimide fragmentation failed to react with anti-NS1 Mab by immunoprecipitation or Western blot assays. Several partial tryptic peptides of DEN 2 NS1 separated by HPLC appeared to react with two monoclonal antibodies, one of which is protective. However, unavoidably high peptide loss during collection required substantially more NS1 protein than is available from current sources to pursue this approach. Using a commercially available kit (PEPSCAN), we synthesized all 404 possible overlapping hexapeptides of 17D YF NS1 as well as a 98 amino acid segment of DEN 2 NS1, shown by J.R. Putnak to be Mab-reactive. Screening of these hexapeptides with all available anti-YF or DEN 2 NS1 Mab failed to identify any reactive hexapeptides. These data suggest that protective epitopes of NS1, as with the flavivirus E protein, are largely discontinuous (or at least involve relatively long stretches of the protein) and are probably conformationally dependent.

*(19. Abstract continued). NS1/E protein subunit vaccines.* In anticipation of studies to test the protective capacity of a mixed NS1/E subunit preparation in mice, we purified 17D YF E by immunoaffinity chromatography, using a flavivirus group-reactive Mab as the ligand (Brandriss et al, 1990). Purified material was identified as a 33 Kd protein (E33). Rabbits immunized with E33 produced neutralizing antibody to 17D YF and DEN 2 in high titer and mice immunized with this protein were protected against lethal YF challenge. Using comparable methods DEN 2 E protein was prepared as well and was shown to elicit high titer neutralizing antibody reponses in rabbits. Unlike with YF E33 however, this response was type specific.

The affinity for phospholipid membranes of NS1 and E proteins suggests the possibility that their incorporation individually or in combination into liposomes of appropriate composition might mimic the in vivo state in which they are recognized by the immunologic apparatus. Such preparations might have the additional advantage of allowing for simultaneous carriage of novel adjuvants. In preliminary experiments we prepared phosphatidylcholine liposomes and demonstrated the incorporation of chromatographically purified DEN 2 NS1 and E proteins into this carrier. Studies designed to characterize the physical nature of entrapped viral proteins and to determine their protective immunogenicity in this form are planned.

*Future directions.* Taken together, these results suggest that antibody against the NS1 protein could offer a critical alternative to direct virus neutralization by contributing to the elimination of infected cells and avoidance of possible virus persistence. Protective anti-NS1 antibodies might also be advantageous against dengue infection since viral growth in macrophages is not enhanced by such antibody.

Despite in vitro evidence of a correlation between protective and lytic capacities of anti-NS1 antibody and the apparent requirement of an intact Fc portion for in vivo activity, the mechanism(s) of NS1-induced immunity remains poorly understood. If immune recognition of cell surface NS1 is critical to the protective effect, is protection simply the result of diminished virus replication attending cell death, or might other events such as antigenic modulation or premature release of interfering immature virion play a role? Protection by some anti-DEN 2 NS1 Mab which do not fix complement (Henchal et al, 1988) suggests consideration of other mechanisms e.g. ADCC. Target cells used in these assays may not be representative of cells involved in natural infection: expression and immune recognition of NS1 in/on macrophages, the primary site of virus replication in dengue, should be investigated.

Advances in the construction of protective recombinant NS1 preparations should allow new approaches for characterization of antigenic and protective immunogenic properties of NS1. Optimal formulation of NS1 in flavivirus subunit vaccines will depend on this information.

## FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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## INTRODUCTION

We and others have shown that passive transfer of monoclonal antibodies against the YF and DEN nonstructural protein NS1 protects mice against lethal challenge with the respective viruses (Schlesinger et al, 1985; Gould et al, 1986; Henchal et al, 1988). Similarly, active immunization with purified authentic NS1 conferred protection against lethal infection in rhesus monkeys (yellow fever, Schlesinger et al, 1986) and mice (dengue encephalitis, Schlesinger et al, 1987). Most recently, protective immunization of mice with recombinant yellow fever or dengue virus NS1 has been demonstrated (Cane & Gould, 1988; Putnak & Schlesinger, 1990; Falgout et al, 1990). Although the mechanism(s) of such protection is presently unclear, cumulative data from our laboratory suggests a correlation between protection and the capacity of anti-NS1 antibody to sensitize flavivirus-infected cells to complement-mediated cytolysis. Taken together, the NS1 data provide evidence of an important alternative to direct viral neutralization in the protective immune response against flavivirus infection and argue for inclusion of this protein in possible future subunit flavivirus vaccines. This report summarizes work performed during the funding period (1987-1990) and includes data from experiments initiated in our laboratory as well as from those performed in the laboratories of our colleagues in other institutions as collaborative efforts .

## EXPERIMENTAL RESULTS

### 1.0 Recombinant flavivirus NS1: protection experiments.

1.1 17D YF NS1/vaccinia. Recombinant NS1-NS2A-NS2B was expressed in

vaccinia virus (Vr) and physical and immunogenic properties of the NS1 moiety were studied (Putnak & Schlesinger, 1990). NS1 was correctly processed as evidenced by its tendency to polymerize, to be expressed on the cell surface, and to be secreted. Immunization of mice with this recombinant preparation elicited non-neutralizing, complement-fixing cytolytic antibody and conferred partial protection against lethal intracerebral challenge of mice with 17D YF. Figure 1A shows results of two experiments in which mice were immunized with Vr YF NS1-NS2A-NS2B and challenged. Prechallenge sera from individual mice were assayed for cytolytic antibody against NS1 and vaccinia proteins expressed on the surface of 17D YF or wild-type vaccinia-infected cells, respectively. Cytolytic antibody directed against both vaccinia and YF NS1 proteins was elicited by Vr YF NS1-NS2A-NS2B immunization Figure 1B. Anti-NS1 cytolytic activity was higher among protected animals (21% vs 10%) but, perhaps given the small number of fatalities, this difference was not statistically significant ($p=0.1$). Vaccinia-specific cytolytic antibody activity was unrelated to survival or to anti-NS1 antibody activity.

1.2 DEN 4 NS1/vaccinia. Recombinant vaccinia viruses expressing truncated versions of NS1 or the complete NS1 gene and various N-terminal flanking sequences consisting of truncated NS2A moieties were prepared (Figure 2) (Falgout et al, 1990). These were tested for cell surface expression and protective capacity in mice. We found that NS2A is not required for cell surface expression of rNS1 since monospecific anti-(authentic) NS1 rabbit serum sensitized cells infected with Vr NS1 lacking a flanking NS2A sequence to complement-mediated cytolysis (CMC)(Table 1). However, consistent with earlier findings (Falgout, et al, 1989) that NS2A does appear to influence nativity of NS1, we found that truncation of the NS2A C-terminus was associated with marked reduction in the protective capacity of immunization with Vr NS1. Mice immunized with Vr NS1-NS2A, which expresses correctly processed NS1, were

completely protected against lethal DEN 4 challenge whereas immunization of mice with Vr NS1-NS2A in which 85% of the NS2A C-terminus was deleted (NS1-15%NS2a) resulted in only partial protection (Table 2). In these experiments the degree of protection correlated with the titer of complement-fixing cytolytic antibody against NS1. That such antibody is sufficient to explain the observed protection is seen in the capacity of a pool of serum from mice immunized with Vr NS1-NS2A to confer protection in passive immunization experiments (Table 3).

1.3 DEN 4/baculovirus. A Baculovirus vector was used to express recombinant (Br) virion and nonstructural DEN 4 pr teins which had earlier been shown to confer protection against lethal DEN 4 encephalitis in mice (Zhang et al, 1988). Constructs consisted of Br C-prM/M-E-NS1 or E, and the effect of immunization of monkeys with lysates of such Br-infected Sf9 cells was determined (Eckels et al, in preparation). An adjuvant was not used. All animals inoculated with the NS1-containing construct developed immunoprecipitating anti-NS1 antibody and we found that most of these sera had cytolytic activity as well (Table 4A). Although low levels of anti-virion antibody, as measured by ELISA, was present in serum from all monkeys immunized with these Br constructs, only 1/9 monkeys developed measurable neutralizing antibody (at low titer; 1/10) and none had detectable hemagglutinating antibody activity. Lysates of Sf9 cells infected with wild-type baculovirus served as a control. All animals were challenged with a subcutaneous dose (10.3-10.5 pfu) of DEN 4 and blood was sampled on 10 consecutive days for viremia. Table 4B summarizes these data. Among control animals (3 monkeys) the mean duration of viremia was 4.7 days (range: 4-6 days) which was little different from that observed among unprotected Br recipients (4.1 days, range: 1-6 days). Complete absence of detectable viremia was considered evidence of protection and was observed in two monkeys (4IJ, 4GV). A strong anamnestic hemagglutination-inhibiting (HAI) antibody response was elicited among "unprotected"

animals but not in the two animals without viremia. It is thought that such reduced HAI response in monkeys #4IJ and #4GV reflects the absence of substantial replication of challenge virus and thus presents additional indirect evidence of vaccine efficacy in this study. A definite explanation for the essentially absent neutralizing antibody response to the E protein is not at hand: it is worth remarking, however, that failure to elicit a measurable neutralizing antibody response with these and other E protein-containing sequences in vaccinia vectors has been a common finding despite the protective capacity of such constructs (Bray et al, 1989). Presumably, immunization with such Vr E constructs primes for a prompt neutralizing response upon challenge. Because the recombinant material was delivered in the form of a crude cell lysate it is not known what the actual dose of the respective proteins was. Using HPLC methods we are presently attempting to prepare purified native DEN 4 NS1 and E as standards against which to measure the concentration of the respective proteins in the Br-infected Sf9 lysates.

1.4 DEN 2/baculovirus (MicroGeneSys). Characterization of the antibody response in rabbits immunized with Br DEN 2 NS1 has been presented in detail in the Midterm Report and will be briefly summarized. MicroGeneSys provided a preparation of partially purified recombinant DEN 2 NS1 extracted from lysates of Br-infected Sf9 cells. The company would give no details concerning the method(s) used to accomplish the separation except to indicate that it involved removal of all genetic material to protect proprietary information. Our analysis of their material revealed a triplet band (ca. 52-55 kd) by SDS-PAGE which probably reflects differences in glycosylation between Br and authentic NS1 (ca. 42-46 kd). More importantly none of our anti-NS1 monoclonal antibodies reacted with this protein by Western blot analysis. Rabbits were immunized with the rNS1 emulsified in Freund's adjuvant in a multi-dose schedule. Rabbits immunized in parallel with authentic DEN 2 (purified by immunoaffinity

chromatography from lysates of DEN 2-infected Vero cells, Schlesinger et al, 1987) served as a control. Control rabbits generated a brisk and sustained high titer antibody response to authentic NS1 as determined by ELISA and Western blot (see Figures 8-11, Midterm Report). Additionally, sera from these control rabbits exhibited broad DEN serotype-reactive, complement-fixing antibody titers (up to 1/16,000 to DEN 2) as well as cytolytic activity in high titer. In contrast, rabbits immunized with the recombinant NS1 developed a sluggish, relatively weak antibody response to NS1 which had little CF activity (1/64) and no cytolytic activity. Failure to elicit such activity together with relatively weak binding to native authentic NS1 raised doubts about this material as a vaccine candidate and work with it was terminated. In light of present information concerning the possible role of NS2A in NS1 processing it is possible that absence of this moiety from the construct might have contributed to the poor immune response observed. However, it is more likely that the extraction method(s) used was the most important factor in this regard.

2.0 Mechanism of protection by anti-NS1 antibody.

The mechanism of such protection in the absence of anti-virion antibody is unclear, but evidence that NS1 is associated with the plasma membrane (Stohlman et al, 1975) and an apparent correlation between protective and cytolytic capacities of anti-NS1 antibodies (Schlesinger et al, 1985), suggest that protection might result from immune recognition and destruction of infected cells bearing surface NS1.

2.1 Cell surface expression of NS1. Results of experiments designed to characterize the physical and antigenic nature of plasma membrane-associated NS1 and E proteins have been published (Schlesinger et al, 1990) and will be briefly described. To initiate study of the possible role of complement-mediated cytolysis in protection,

we measured the capacity of anti-yellow fever virus NS1 or E protein monospecific serum or monoclonal antibodies to bind to YF-infected cells and of anti-YF NS1 or E serum to sensitize them to cytolysis. Although both anti-NS1 and anti-E antibody bound to infected cells (Figure 3), complement-mediated cytolysis was observed only with anti-NS1 antibody (Table 5) suggesting that NS1, but not E, may exist as an integral rather than peripheral membrane protein. Greater binding by anti-NS1 antibody suggests that larger amounts of NS1 than E protein are associated with the cell membrane. Earlier studies by Winkler et al (1988,1989) indicated that NS1 exists as a heat-labile dimer in flavivirus-infected cells. To test whether this form is present on the plasma membrane we compared YF-infected cells treated with the cell membrane-impermeable cross-linking reagent BS3 to BS3-treated purified NS1. We reasoned that NS1, a lysine-rich protein, should be susceptible to BS3 cross-linking in its dimeric but not monomeric form. Figure 4A shows results of BS3 treatment of purified NS1. Unheated untreated NS1 (lane 1) is represented by monomeric and polymeric forms, which upon boiling, were seen in monomeric form only (lane 3). BS3-treated NS1 (lanes 2 and 4) largely retained the polymeric form even after heating (lane 4). Similarly, polymeric NS1 predominated in unheated lysates of infected cells (Figure 4B, lanes 1-4), but only monomeric NS1 remained after heating lysed untreated infected cells (lane 5). Treatment of the cell surface with increasing concentrations of BS3 resulted in the formation of heat-stable polymerized NS1 (lanes 6-8). Rabbit serum against host cell proteins or E protein did not react with the high molecular weight forms of NS1 suggesting that they are homopolymers of this protein. Antibody bound to polymerized NS1 might be expected to activate complement more efficiently than antibody bound to E protein since the affinity of complement binding increases with immune complex size.

To test whether recognition of cell surface NS1 by complement fixing protective

Mab would affect virus replication, we measured virus output from YF-infected cells grown in the presence of anti-NS1 Mab or monospecific rabbit anti-NS1 serum. A possible in vitro correlate of anti-NS1 antibody activity in-vivo (see section 2.2) was suggested by results of these experiments (Table 6). Peak titers of progeny YF were reduced 10 to 100-fold when infected cells were treated with complement-fixing, anti-NS1 monoclonal antibody or monospecific anti-NS1 rabbit serum and complement (Table 6). The capacity of anti-NS1 antibody to sensitize flavivirus-infected cells to CMC suggests the possibility of similar recognition of plasma membrane-associated flavivirus antigen by effector arms of the cellular immune response, e.g., cytotoxic T cells (Tc). Reports of H-2 or HLA-restricted T cell killing of flavivirus-infected targets are supportive in this regard (Kesson et al, 1987; Bukowski et al, 1989). However, antigen specificity of the Tc cells does not appear to involve NS1 (Bukowski et al, 1989).

2.2 In vivo evidence of a role for complement in protective anti-NS1 antibody activity. We have postulated a role for complement-mediated cytolysis in protection conferred by anti-NS1 antibody. To initiate experiments to test this hypothesis, we prepared the F(ab)2 moiety of a previously described (Schlesinger et al, 1985) protective complement-fixing IgG2a monoclonal anti-YF NS1 antibody (1A5) and measured its capacity to affect replication of brain-associated virus after intracerebral YF challenge. An IgG2a mouse myeloma protein, PC-5, served as a control. We reasoned that since complement binds to the Fc portion of IgG, its removal should abrogate complement fixation but not antibody binding. Monoclonal antibody or mouse myeloma protein was subjected to pepsin digestion using a previously described protocol (Parham, 1983). Fc fragments and undigested IgG were separated from F(ab)2 by Staph protein A column chromatography. Figure 5 shows a representative SDS-PAGE analysis of such a separation. To prove that the purified 1A5 F(ab)2 retained

antigen recognition, we measured its capacity to interfere with NS1-binding and cytolytic activity of intact 1A5 antibody. 1A5 IgG was prepared by Staph protein A chromatography and radioiodinated. Figure 6 shows results of a competition binding assay in which we measured the capacity of equimolar concentrations of unlabeled IgG or F(ab)2 1A5 to compete with binding of radiolabeled 1A5 to live YF-infected cells in situ. Binding was interfered with by pretreatment of YF-infected cells with equimolar concentrations of unlabeled IgG or F(ab)2 1A5. On a molar basis, the competition by each preparation was equal. Similarly, binding of F(ab)2 1A5 to YF-infected cells was confirmed by its capacity to abrogate IgG 1A5-sensitization of infected cells to CMC (Figure 7). To test whether the protective capacity of IgG 1A5 depends on the integrity of the Fc moiety, we compared IgG and F(ab)2 1A5 for their capacity to affect YF replication in mouse brain. In preliminary experiments we showed that 8 days after intracerebral challenge, maximal differences in titratable brain virus were observed between IgG 1A5 and control-immunized mice (log10: control-5.90; 1A5-3.48; 4E9(anti-E)-$<$2.00). In these experiments an IgG2a monoclonal antibody (L9) against the respiratory syncytial virus (RSV) G protein served as a control and the antibodies were delivered by intraperitoneal injection. However, we subsequently determined that peripheral injection of F(ab)2 1A5 resulted in extremely rapid clearance of the antibody from the blood (t 1/2 less than 24 hrs) as measured by NS1-specific ELISA. Moreover, the efficiency with which F(ab)2 crosses the blood-brain barrier is unknown. For these reasons we decided to deliver the antibodies by intracerebral injection: mice were challenged at day 0 followed by intracerebral injection of antibody into the contralateral hemisphere on day 1. Brains were harvested on day 8 and individually titered for infectious virus. Figure 8 shows cumulative results of three individual experiments. The quantity of infectious virus was significantly reduced among mice which received intact IgG 1A5 (log10: 4.41 vs 5.55, $p<0.001$, Mann-Whitney U test). The considerable overlap in titers of brain-associated virus among IgG 1A5-immunized

the control and F(ab)2-immunized groups. We do not know whether measurement of titratable brain virus 8 days after challenge is a suitable proxy for subsequent morbidity or mortality: this approach was adopted to conserve hard-won materials (especially purified F(ab)2 fragments) and in the hope that this model can be further exploited to search for histologic evidence of complement and antibody deposition at sites of virus replication. The apparent requirement of an intact Fc portion for interference with viral replication by the 1A5 antibody supports but does not prove our hypothesis that complement fixation is necessary for this antibody to protect. The Fc portion is also required for ADCC activity, although no data concerning flaviviruses has been published in this regard. To more directly test the putative role of complement activation in the mechanism of protection conferred by anti-NS1 antibody we are presently determining the capacity of IgG 1A5 to protect complement deficient mice. Purified cobra venom (naja naja) is used to decomplement Swiss white mice prior to passive transfer of 1A5 or control anti-RSV monoclonal antibody and virus challenge. Mice treated with heat-inactivated venom serve as controls. Using commercially available complement deficient and sufficient mouse serum, we have developed a sensitive RIA to detect complement in the serum of our test animals. We find that through the fifth day of cobra venom treatment no complement is detectable but by the sixth day it is. This can best be explained by the appearance of anti-venom antibody by this day. It is doubtful that raising the dose of venom, already at the threshold of toxicity, can overcome such an antibody effect. However, it is hoped that if complement is required for the protective effect of 1A5 antibody, its early depletion will be reflected in reversal of interference with virus replication by this antibody. Pilot experiments with this protocol are in progress.

3.0 NS1 structural requirements for retention of protective immunogenicity.

Considerable advances have been made in our knowledge of the secondary structure of the flavivirus E protein and recognition of molecular correlates of virulence and neutralization subserved by this protein are emerging. In contrast, other than knowledge of the deduced primary amino acid sequence of NS1 (10 flaviviruses have been sequenced to date) little is known about the molecular basis of NS1 antigenicity or immunogenicity. All flavivirus NS1 proteins tend to form homopolymers, a state which may confer requisite hydrophobicity for their observed membrane association (Winkler et al, 1989). NS1 sequence determinations reveal considerable homology and even where divergences appear, hydrophobicity profiles are largely preserved. The following sections summarize attempts to define some elements of NS1 molecular structure and immunogenic and antigenic correlates. Because of the availability of a large panel of monoclonal antibodies to 17D YF, much of the work was initiated with this virus (we are planning to prepare a large panel of monoclonal antibodies to DEN 4 NS1 to further exploit the recombinant DEN 4 NS1 constructs available to us through our collaborative arrangement with C-J Lai and colleagues (NIH).

3.1 NS1 disulfide bonds: evidence of intramolecular bridging. As with the E protein, NS1 bears 12 cysteine residues which are highly conserved among all flaviviruses. We adopted the method of Nowak and Wengler (1987) to determine to what extent these cysteine residues are involved in intramolecular bonding. Figure 9 presents evidence that, as in the case of the E protein, 6 intramolecular disulfide bridges are present: alkylation of either YF or DEN 2 NS1 could be accomplished only if the protein was first reduced.

With the E protein cumulative evidence indicates that the induction of a protective neutralizing antibody response to E depends largely on retention of conformation maintained by the integrity of its disulfide bonds (Wengler & Wengler, 1989). Moreover, tryptic fragments smaller than 42 kd failed to elicit neutralizing antibody. To initiate a similar analysis of NS1 we subjected YF NS1 to various denaturing treatments including SDS solubilization, dithiothreitol reduction (with or without alkylation), and tryptic digestion. Trace amounts of residual undigested protein were removed by HPLC gel chromatography. Similar amounts of these preparations as well as undenatured YF NS1 were emulsified in complete Freund's adjuvant before intraperitoneal inoculation of Balb/c mice. Ovalbumin, (native or SDS-denatured) was given in the same schedule as controls. An additional control group consisted of mice given a single intraperitoneal dose of live 17D YF at 3 weeks of age. Mice were primed at 3 weeks of age, boosted with the respective protein preparation in solution (PBS) at 5 weeks and bled at 6 weeks. This immunization schedule using similar NS1 preparations had previously been shown to protect Swiss white mice against lethal YF or DEN 2 challenge (Schlesinger et al, 1985, 1987). Sera from groups of mice were pooled for analysis. Each protein preparation elicited NS1-specific antibody as measured by ELISA and Western blot analysis (Figure 10). Sera from animals immunized with native and SDS-denatured or reduced NS1 reacted with both monomeric and polymerized forms of NS1 but sera from animals immunized with fragmented NS1 reacted only with NS1 in its polymerized form. Because cytolytic activity appears to be associated with the protective capacity of anti-NS1 antibody, we sought to determine cytolytic antibody titers and relative contributions of each IgG subclass to the total IgG anti-NS1 response among sera from the various groups. For the latter assay subclass-specific rabbit anti-mouse IgG antisera were used according to previously described methods (Coutelier et al, 1986, 1987). A panel of monoclonal anti-YF NS1 antibodies (Schlesinger et al, 1983), with representation in each subclass,

served as standards. Immunoaffinity-purified NS1 was used in the solid phase. Figure 11A,B shows results of subclass-specific and cytolytic assays, respectively, performed with sera from mice immunized with native or SDS-denatured NS1 or with control sera from mice immunized with SDS-denatured ovalbumin or live 17D YF (assay of sera from mice immunized with reduced or fragmented NS1 are in progress). NS1-immunized mice and mice immunized with a single dose of live YF virus produced anti-NS1 IgG antibody: among NS1-immunized animals each of the subclasses was represented. The NS1-specific IgG antibody response was lowest among 17D YF-immunized mice. Immunization with SDS-denatured NS1 elicited a somewhat lower response than with the native protein. A prominent IgG2a response was seen in each group and in one experiment IgG2b and IgG2a responses were equivalent and predominated. Cytolytic activity was greatest in sera from mice immunized with native NS1. Although diminished, cytolytic activity was still present in sera from mice immunized with SDS-denatured NS1 suggesting that the capacity to elicit cytolytic (and presumably protective) antibody is not strictly dependent on protein conformation. However, the extent to which this is true will await assay results with reduced or fragmented NS1. Further studies to measure the protective capacity of these and other altered forms of NS1 are planned. The anticipated panel of anti-DEN 4 monoclonal antibodies (in preparation) together with novel recombinant DEN 4 protein constructs may offer alternatives to our previously unsuccessful (see below) chemical and enzymatic methods to map protective NS1 determinants.

3.2 Identification of protective NS1 epitopes.

3.2.1 Correlation of complement-mediated lysis and protective capacity of anti-NS1 monoclonal antibodies. Table 7 summarizes relevant characteristics of available ant i-YF and DEN 2 NS1 Mab produced in our laboratory or in those of our

collaborators. Figure 12 compares lytic activities and protective capacities among available anti-YF NS1 Mab. There was an apparent correlation between protective and lytic capacities among the antibodies although it must be emphasized that the protection results are from different laboratories (E. Gould and our own, using different strains of 17D YF). In general, at least three categories of anti-NS1 Mab activity emerge from these assay results; lytic-protective, non-lytic protective, and non-lytic non-protective. However, that some non-lytic anti-NS1 Mab are protective (Gould's Mab 492, 863, 925, 428 and Henchal's IgG1 Mab) suggests that other mechanisms of antibody-dependent immunity may be operating. Among these, antibody-dependent cell-mediated cytotoxicity (ADCC) would be expected but, to date, we have been unable to demonstrate ADCC using these antibodies in assays employing primary mouse macrophages or a continuous mouse macrophage cell line (P388D1) as effectors and YF or DEN-infected neuro 2a or SW-13 cells as targets.

3.2.2 NS1 epitope mapping: competition binding assays. It is presently unclear whether CMC is subserved by specific NS1 domains or whether such activity is simply determined by an anti-NS1 antibody's complement-fixing capacity without regard to the NS1 domain recognized. Preliminary data from our laboratory is inconclusive. Results of competitive binding assays employing biotinylated anti-YF NS1 Mab are shown in Table 8. This assay measures the capacity of protective and non-protective anti-YF NS1 Mab to block binding of biotin-labeled complement-fixing, protective anti-NS1 Mab. Binding avidities among this panel of Mab were quite similar (data not shown). Although the assay was limited by denaturation of the majority of the Mab after biotinylation, a number of conclusions are suggested by these data: 1) At least 2 separate epitopes are recognized by protective cytolytic Mab; 2) a number of non-protective Mab (4E2, 2G2) recognize a potentially "protective" site(s); 3) two protective antibodies (428, 925) which are not active in our CMC assay appear to

define an epitope distant from at least one which does subserve complement-mediated lysis; 4) two antibodies (428, 492) of an isotype (IgG2a) expected to fix complement do not compete with the cytolytic Mab and are not cytolytic. Of interest in regard to the last is failure of rabbit serum prepared against recombinant YF NS1 (provided by Dr. Charles Rice) to sensitize cells to complement-mediated lysis or to interfere with binding to NS1 of biotinylated cytolytic protective Mab above (1A5, 8G4, 423, 871). Rice's recombinant YF NS1 was constructed as a TrypE fusion protein and differs from authentic NS1 by deletion of the first 91 amino acids from the NS1 amino terminus. In contrast, rabbit serum that we prepared to authentic YF NS1 was cytolytic and did compete with protective lytic Mab (see figures 3 & 4, midterm report). Both rabbit anti-YF NS1 sera bind to authentic NS1, immunoprecipitate NS1, and stain YF-infected SW13 and Vero cells in an indirect immunofluorescent assay. Taken together, these results are consistent with the view that complement-mediated lysis is subserved by selected NS1 domains and that the capacity of an antibody to sensitize YF-infected cells to complement-mediated lysis depends both on its subclass and specificity. It is tempting to speculate that the cytolytic domain(s) are located at the NS1 amino terminus, but masking of other NS1 regions by the TrypE fusion protein could also account for the results.

3.2.3 Peptide mapping. Our approach to identification of NS1 immunoreactive domains had largely assumed that at least some are represented by continuous epitopes or that, if discontinuous, some regions might be close enough to each other to allow preservation of antibody recognition after fragmentation of the complete protein. Methods used for fragmentation were entirely empirical and have included chemical cleavage with cyanogen bromide (CnBr, methionyl peptide) and N-chlorosuccinimide (NSC) (Lischwe & Ochs, 1982), tryptophanyl peptide, or enzymatic digestion with trypsin (lysinyl, argininyl peptide). NS1 for this purpose was purified by

immunoaffinity chromatography by methods reported earlier from our laboratory (see midterm report for fragmentation methodology). Initially, immunoaffinity-purified radioiodinated YF and DEN 2 NS1 were subjected to CnBr fragmentation followed by immunoprecipitation with representative anti-NS1 Mab or monospecific antisera. None of the fragments generated were precipitated. The method was modified by exposing NS1-containing acrylamide gel slices to CnBr vapors (Zingde et al, 1986) in an effort to minimize formic acid-induced denaturation. The resulting fragments were electroeluted into SDS-acrylamide gels followed by transfer to nitrocellulose paper and immunoblotting. Again, no reactivity was detected with any of the available monoclonal or monospecific anti-NS1 probes. We had found markedly heightened sensitivity of detection of intact NS1 using polyvinylidene difluoride membranes (Immobilon, Millipore) in Western blots, but use of this support also failed to detect NS1 fragments (CnBr, NSC, tryptic) reactive with any of the available anti-NS1 antibodies.

In parallel experiments, we have purified DEN 2 NS1 by reverse-phase HPLC (Figure 13A) and generated reproducible tryptic peptide maps. Several peptides appeared to react with two anti-NS1 Mab, one of which is protective (Figure 13B). However, these results were inconsistent probably due in large measure to the small amounts of available NS1 and the marginal sensitivity of the immunoassays at the NS1 concentrations used. Additionally, protein retrieval from collection tubes after acetonitrile evaporation was complicated by substantial and apparently irreversible binding to the plastic or glass surface leading to substantial loss. This problem has been commented upon by others and represents an example of the highly empirical nature of this methodology (L'Italien, 1986). The approach of Geysen et al (1984) was used to systematically synthesize all possible short (Hexa-) peptides of YF as well as part of DEN 2 using sequences provided by Charles Rice, Vincent Deubel, and Robert

Putnak. This computer-assisted method allowed rapid concurrent synthesis and immunoassay (ELISA) of all 404 possible overlapping hexapeptides covering the total 409 amino acid sequence of 17D YF NS1. Results were disappointing: screening of YF NS1 hexapeptides with each available anti-YF Mab or monospecific rabbit and monkey anti-YF NS1 sera failed to demonstrate a single convincingly positive peptide on multiple tests. Similarly, no reactivity with any DEN 2 NS1 Mab was detected against amino acid residues 261-359 of DEN 2 NS1 shown by R. Putnak to be reactive with several Mab as well as monospecific rabbit anti-NS1 sera in a Western blot analysis of recombinant NS1 produced in E. coli. Correct synthesis of control peptides included in the "PEPSCAN" kits suggests that our failure to detect reactive NS1 peptides was not the result of error in synthesis. We conclude that possible continuous NS1 epitopes recognized by these antibodies are likely to be larger than 6-mers. The expense and uncertainty of this approach led us to abandon it. Taken together, our results strongly suggest that the available protective YF and DEN 2 NS1 Mab are directed at discontinuous and probably conformationally determined epitopes.

4.0 Subunit NS1/E vaccines: preliminary immunogenic characterization of 17D YF and DEN 2 E proteins and E/NS1 incorporation into liposomes.

4.1 YF and DEN 2 E proteins. In anticipation of studies to test the protective effect of immunization with NS1/E subunit mixtures in mice, we purified 17D YF E from lysates of infected Vero cells by immunoaffinity chromatography using a flavivirus group-reactive Mab as the ligand (Brandriss et al, 1990). Purified material could not be identified as intact E but bore antigenic determinants of E as defined by selective reactivity with anti-E monoclonal antibodies. The reactivity correlated with a 33 kd band (E33), the predominant component on polyacrylamide gel electrophoresis. Rabbits hyperimmunized with E33 produced neutralizing antibody to YF in titers

similar to those obtained with a single dose of 17D YF vaccine (Table 9). The rabbit anti-sera had a high degree of cross-neutralizing activity against DEN 2, whereas the human antisera, as expected, failed to neutralize dengue. Rabbits immunized with high dose live 17D YF produced neutralizing antibody to 17D YF in high titer, but cross-neutralizing activity against dengue was lower than that seen in rabbits immunized with E33. This E protein fragment, then, appears to express a group-reactive determinant, suggesting the possibility of heterotypic protection after vaccination. Mice actively immunized with E33 were protected against lethal intracerebral challenge with YF: 16/20 E33-immune mice survived vs. 6/33 control ovalbumin-immune mice ($p < 0.001$).

Using similar methods we purified the DEN 2 E protein (Schlesinger, unpublished) and measured the level and specificity of neutralizing antibody generated in rabbits immunized with this protein in Freund's adjuvant. Comparable levels of neutralizing antibody (1/1000 PRNT50) developed in rabbits immunized with either live DEN 2 virus or the purified E protein. In contrast to results with YF E33 however, neutralizing antibody was largely DEN-specific with little or no neutralizing activity against YF.

4.2 liposomal carriers. The affinity for phospholipid membranes of NS1 and E proteins suggests the possibility that their incorporation individually or in combination into liposomes of appropriate composition might best mimic the in vivo state in which they are recognized by the immunologic apparatus. Such carrier systems might have the added advantage of enabling the simultaneous incorporation of novel adjuvants currently in development in a number of laboratories. In preliminary experiments, DEN 2 NS1 and E proteins isolated by immunoaffinity chromatography were radioiodinated and further purified and partially renatured by elution from gel slices

after SDS PAGE in non-reducing, unheated Laemmli buffer. Phosphatidylcholine liposomes were prepared by a conventional evaporation method with entrapment of labeled protein upon solubilization in octylglucoside followed by dialysis to remove the detergent. Association of liposome and viral protein was determined by demonstrating co-flotation of 125I-labeled protein and 14C-labeled liposome as shown in Figure 14,A-G. Studies to characterize the physical nature and protective immunogenicity of such viral protein-carrier systems are planned.

## CONCLUSION

Cumulative evidence indicates that the immune response to the YF, DEN 2 and DEN 4 nonstructural protein NS1 is protective. Protection conferred by passive transfer of monoclonal anti-NS1 antibody or of monospecific mouse serum raised against recombinant NS1, indicates that antibody elicited by active immunization with NS1 is sufficient for this effect. Anti-NS1 antibody provides an alternative to direct viral neutralization in the defense against these viruses and may be especially important to immune recognition and destruction of infected cells bearing surface NS1. That the immune enhancement phenomenon should not be an issue with anti-NS1 antibody further emphasizes the potential value of incorporating this protein into candidate subunit dengue vaccines. Cell surface expression of NS1 raises the possibility of ADCC or cytotoxic T-cell mechanisms in the protective immune response to NS1 immunization but experimental evidence to support these possibilities has not been presented. Indeed the mechanism of protection provided by anti-NS1 antibody remains poorly understood. The Fc portion of anti-NS1 antibody appears to be needed for protection and cumulative data suggest a correlation between in vitro complement-mediated cytolytic activity of anti-NS1 antibody and protective capacity. However, some monoclonal antibodies without this quality have been shown to be protective as

well. If immune recognition of cell surface NS1 is central to the protective effect, is protection simply the result of diminished virus replication attending cell death, or might other events such as premature release of interfering immature virion play a role? Little is known about the structural requirements for retention of antigenicity and protective immunogenicity of NS1. Cumulative data from monoclonal antibody screening of peptide fragments of yellow fever and dengue NS1, as well as overlapping hexapeptides of this protein, have, in our hands, failed to provide evidence that protective NS1 epitopes are expressed as continuous determinants. To the contrary, it seems highly probably that NS1 recognition by protective antibody is conformationally dependent. The recent availability of protective recombinant NS1 preparations offers the possibility of new approaches to the characterization of important antigenic and immunogenic domains of NS1 and as well should allow more efficient production of the protein for incorporation into experimental subunit vaccine preparations. Among these, liposomes might be especially attractive vehicles for carriage of "protective" flaviviral proteins (E, NS1, prM/M) since each are membrane-associated and might therefore be expected to assume native configuration in this form.

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Table 1. Cell surface expression of VrNS1 determined by sensitivity to complement-mediated cytolytic assay

| SW13 cells infected with Vaccinia | | % specific $^{51}$Cr release[a] using: Anti-Den 4 MIAF | | | Rab anti-Den 2 NS1 | | | Rab anti-vaccinia | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Exp 1 | Exp 2 | Exp 3 | Exp 1 | Exp 2 | Exp 3 | Exp 1 | Exp 2 | Exp 3 |
| rec[b] | A | 19 | 16 | 5 | ND | 14 | 13 | 31 | 70 | 29 |
| | B | 31 | 28 | 14 | ND | 33 | 25 | 38 | 67 | 32 |
| | C | 18 | 0 | 3 | ND | 0 | 0 | 45 | 49 | 39 |
| | D | 3 | 0 | 1 | ND | 0 | 0 | 35 | 62 | 35 |
| | E | 1 | 0 | 0 | ND | 0 | 0 | 45 | 80 | 59 |
| Vaccinia | | 2 | 0 | 0 | ND | 0 | 0 | 41 | 45 | 59 |
| DEN 4 | | 14 | 18 | 21 | ND | 17 | 30 | 0 | 0 | 0 |

[a] Calculated using spec release = Exp-spon/max-spon x 100 where PC-5 ascites is spontaneous control for MIAF and normal rabbit serum the spontaneous control cfor rabbit antisera. Ascites and rab anti-DEN2 NS1 used at 1/100; rab anti-vaccinia at 1/200; complement at 1/20

[b] Vaccinia recombinant: A=NS1-NS2A (nt 2346-4128); B=NS1,100% (nt 2346-3476); C=NS1,99% (nt 2346-3461); D=NS1,97% (nt 2346-3446); E=NS1,92% (nt 2346-3396).

Table 2. Protection of mice against dengue type 4 encephalitis by immunization with recombinant vaccinia viruses

| Virus | Expression of authentic NS1 | Cytolytic antibody titer | Survivors/ total (%) |
|---|---|---|---|
| vC-M-E-NS1-NS2a | + | 1/160 | 18/18 (94) |
| vC-M-E-NS1-15%NS2a | - | ND | 15/15(100) |
| vNS1-NS2a | + | 1/320 | 28/28(100) |
| vNS1-15%NS2a | - | 1/40 | 20/30 (67) |
| vRSVG/NS1-15%NS2a | - | 1/40 | 36/57 (63) |
| negative control | - | <1/10 | 8/52 (15) |

Table 3. Protection against DEN4 encephalitis in mice by passive transfer of anti-VrNS1-NS2A mouse serum

| Mouse serum | Protective transfer: survivors/total (%) |
|---|---|
| anti-DEN 4 immune ascites | 4/4 (100) |
| Vr NS1-NS2A | 12/18 (67) |
| VrSC8 (neg. control) | 2/17 (12) |
| PBS (sham immune) | 1/8 (13) |

Table 4A. Cytolytic antibody titers in sera from monkeys immunized with Br E or C-M-E-NS1

| Immunogen | Cytolytic antibody titer (geometric mean) |
|---|---|
| BrE | < 1/20 |
| Br C-M-E-NS1 | 1/130 |
| Wt Baculovirus control | < 1/20 |

Table 4B. Challenge of monkeys inoculated with DEN-4 E and C-M-E-Ns1 recombinants 4 weeks following the third dose

| Monkey number | Immunogen | Viremia (days) | HAI ab at week: 0 | 2 | 4 | 8 |
|---|---|---|---|---|---|---|
| 4IJ | E | 0 | <10 | 20 | 40 | 10 |
| 4GV | C-M-E-NS1 | 0 | <10 | 10 | 160 | 80 |
| | | (0)[a] | (<10) | (10) | (80) | (30) |
| 070D | E | 6 | <10 | 160 | 160 | 320 |
| 927C | E | 4 | <10 | 1280 | 1280 | 640 |
| 3J8 | C-M-E-NS1 | 3 | <10 | 2560 | 2560 | 320 |
| 003D | C-M-E-NS1 | 1 | <10 | 2560 | 2560 | 1280 |
| 200D | C-M-E-NS1 | 6 | <10 | 80 | 640 | 160 |
| 819C | C-M-E-NS1 | 4 | <10 | 2560 | 2560 | 2560 |
| 190D | C-M-E-NS1 | 5 | <10 | 1280 | 2560 | 2560 |
| | | (4.1)[a] | (<10) | (700) | (1000) | (500) |
| 058D | baculo wt | 4 | <10 | <10 | 1280 | 2560 |
| 373D | | 6 | <10 | 20 | 5120 | 2560 |
| 2Y3 | | 4 | <10 | 80 | 320 | 320 |
| | | (4.7)[a] | (<10) | (10) | (1000) | (1000) |

[a] Mean

Table 5

Complement-mediated lysis of YF-infected SW-13 or Neuro 2a cells

| Rabbit serum[a] against: | % specific $^{51}Cr$ release from YF-infected cells: SW-13 | Neuro 2a |
|---|---|---|
| YF NS1 | 54 | 16 |
| YF E | 2 | 2 |
| SW-13 | 34 | - |
| Neuro 2a | - | 72 |

[a] Sera were used at 1/100 final dilution

Table 6

Growth of YF in tissue culture in the presence of anti-NS1 antibody and complement

| Antibody[a] | Complement[b] | Cell | |
|---|---|---|---|
| | | Neuro 2a | SW-13 |
| PC5 | - | 6.3[c] | 7.0 |
| | + | 6.2 | 7.1 |
| 1A5 | - | 6.4 | 7.0 |
| | + | 4.1 | 5.3 |
| Preimmune rabbit serum | - | 6.2 | 7.1 |
| | + | 6.1 | 7.1 |
| Rabbit anti-YF NS1 serum | - | 6.3 | 7.2 |
| | + | 5.2 | 6.3 |

[a] Cells were infected for 1 hour after which residual virus was removed and antibody (1/100) and complement (1/20) were added. PC-5 is a mouse myeloma protein and 1A5 an IgG2a anti-NS1 monoclonal antibody.

[b] (-) = heat inactivated complement
(+) = active complement

[c] $\text{Log}_{10}$ PFU/ml tissue culture supernatant at 48 hrs

Table 7. Characteristics of anti-NS1 monoclonal antibodies

**DEN 2**

| Clone | Immunizing DEN Strain | Ig | ELISA titer ($-\log_{10}$) vs DEN 2 (NGC) NS1 | Protection |
|---|---|---|---|---|
| *16-25/3 | D80-100 | G1 | < 2 | - |
| *20-1/1 | " | G1 | < 2 | - |
| *27-12/4 | " | G1 | 5.8 | + |
| *34-23 | " | G1 | 4.8 | - |
| *40-21/9 | " | G2b | 5.6 | - |
| *47-10/10 | " | G2a | 2.7 | - |
| *63-15 | " | G1 | 5.6 | + |
| *68-5/16 | " | G1 | 5.3 | + |
| *101-47 | " | G1 | 2.3 | - |
| **6A8 | NGC | M | ND | + |
| **9A9 | " | G1 | > 5.0 | - |
| **4D11 | " | G1 | 3.7 | - |
| +D7-4E9 | " | G1 | > 5.0 | - |

**17DYF**

| Clone | Immunizing YF Strain | Ig | ELISA titer ($-\log_{10}$) vs 17D YF | Protection |
|---|---|---|---|---|
| #863 | Porterfield | G1 | > 7.0 | + |
| #925 | " | G1 | 5.0 | + |
| #871 | " | G2a | 6.0 | + |
| #979 | " | G2a | 6.0 | + |
| #999 | " | G2a | 6.0 | + |
| #993 | " | G2a | 6.0 | + |
| #917 | " | G2a | 6.0 | + |
| #992 | " | G2a | 5.0 | + |
| #423 | " | G2a | 6.0 | + |
| #492 | " | G2a | < 2.0 | + |
| #428 | " | G2a | 5.0 | + |
| #924 | " | M | < 2.0 | + |
| *1A5 | Connaught | G2a | > 7.0 | + |
| *8G4 | " | G2b | 6.0 | + |
| *4E3 | " | G1 | > 7.0 | - |
| *2G2 | " | G3 | ND | - |
| *2D10 | " | G1 | 6.0 | - |

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Table 8. Competitive Binding Assay

| | Unlabeled competitor anti-YF NS1 monoclonal antibody | Isotype IgG | Biotinylated anti-YF NS1 monoclonal antibody | | | |
|---|---|---|---|---|---|---|
| | | | 1A5 | 8G4 | 423 | 871 |
| Protective, cytolytic | 1A5 | 2a | +[a] | + | + | + |
| | 8G4 | 2b | + | + | + | + |
| | 423 | 2a | - | +/- | + | + |
| | 871 | 2a | + | + | + | + |
| | 992 | 2a | - | - | - | - |
| | 999 | 2a | - | - | - | - |
| | 979 | 2a | - | - | - | - |
| | 917 | 2a | - | - | + | - |
| Protective, not cytolytic | 428 | 2a | - | - | - | - |
| | 925 | 1 | - | - | - | - |
| Not protective not cytolytic | 2D10 | 1 | - | - | - | - |
| | 4E3 | 1 | + | + | + | + |
| | 2G2 | 3 | + | + | + | + |
| | 863 | 1 | - | - | - | - |
| | 492 | 2a | - | - | - | - |

[a] competition + = >75%; +/- = 25-50%; - = <1%

Table 9. Neutralizing antibody titers (reciprocal) in rabbits immunized with 17D YF virion or E p33 and in humans immunized with 17D YF vaccine

| Rabbit immunized with: | PRNT | |
|---|---|---|
| | 17D YF | DEN 2 |
| E p33[a] | | |
| #1 | 256 | 64 |
| 2 | 64 | 64 |
| 3 | 128 | 32 |
| 4 | 512 | 256 |
| 17D virion[b] | | |
| #1 | 1,024 | 32 |
| 2 | 2,048 | 32 |
| Humans immunized with 17D YF vaccine[c]: | | |
| #1 | >256 | <8 |
| 2 | 40 | <10 |
| 3 | >160 | <10 |

a 25ug x 3 b $10^6$ PFU I.V. c ca $10^{5.5}$ PFU S.C.

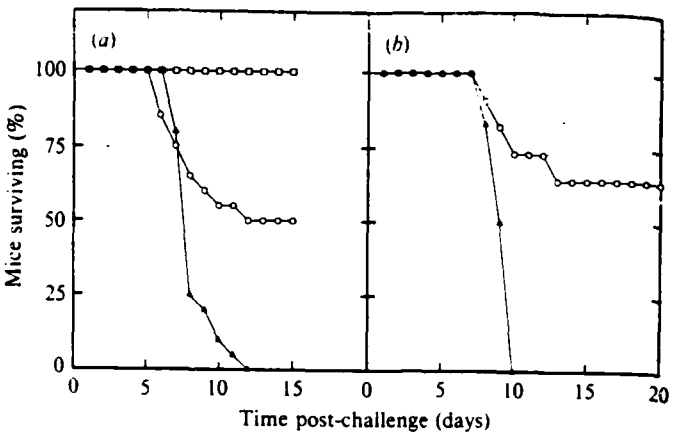


Figure 1A. Mouse protection assay. Mice immunized i.p. with 17D YF (□), a vaccinia virus recombinant expressing YF NS1 (○) or wild-type vaccinia virus (△) were challenged with 4 x $10^5$ p.f.u. of 17D YF i.c. (a) Experiment 1. (b) Experiment 2.

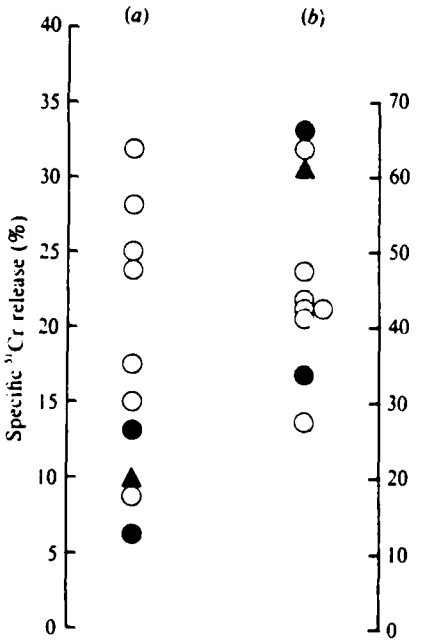


Figure 1B. Cytolytic antibody in recombinant-immunized mice. Complement-mediated cytolytic activity in the sera of mice bled immediately prior to challenge with 17D YF was measured by specific $^{51}Cr$ release from 17D YF-infected cells (a) or wild-type vaccinia virus-infected cells (b). Mice which survived i.c. challenge with 17D YF (○). Mice which died as a result of challenge (●). Mouse which died as a result of pre-challenge bleeding (▲).

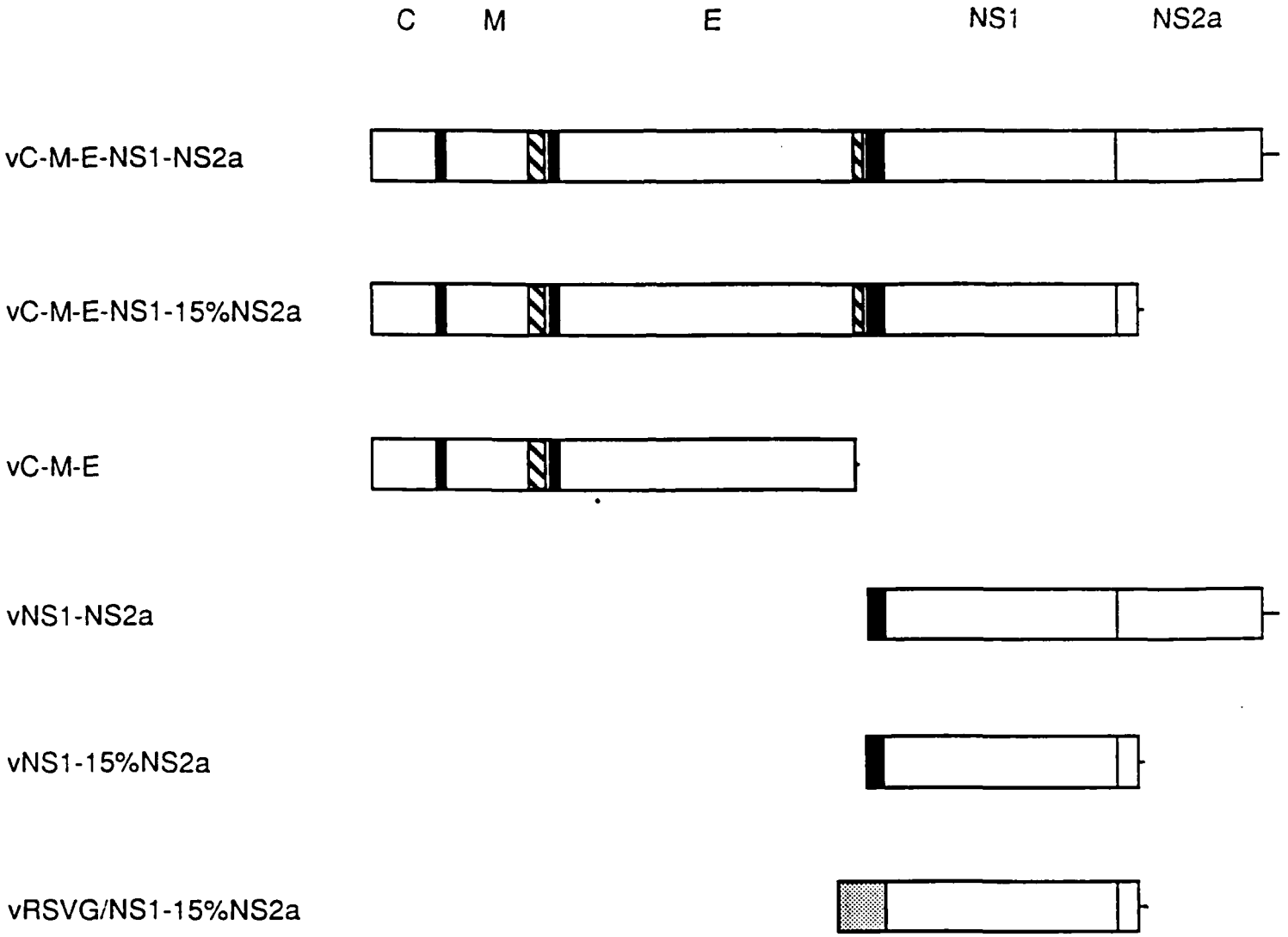


Figure 2. Polyproteins encoded by dengue 4 cDNA contained in vaccinia virus recombinants. The region of the dengue polyprotein expressed by each recombinant is shown, with the N-terminus at the left. Viral protein sequences are represented by boxes, while amino acid sequences at the C-termini encoded by the vector are shown by the thin horizontal lines. This vector-encoded "tail" is 3 residues for vC-M-E, 5 residues for vC-M-E-NS1-NS2a and vNS1-NS2a. The striped regions are stretches of hydrophobic amino acids in the DEN4 polyprotein that are thought to act as stop-transfer signals. The black regions are hydrophobic amino acid sequences in the DEN4 polyprotein that are thought to act as signal sequences. The stippled region is the N-terminal 71 amino acid sequence of the RSV G glycoprotein.

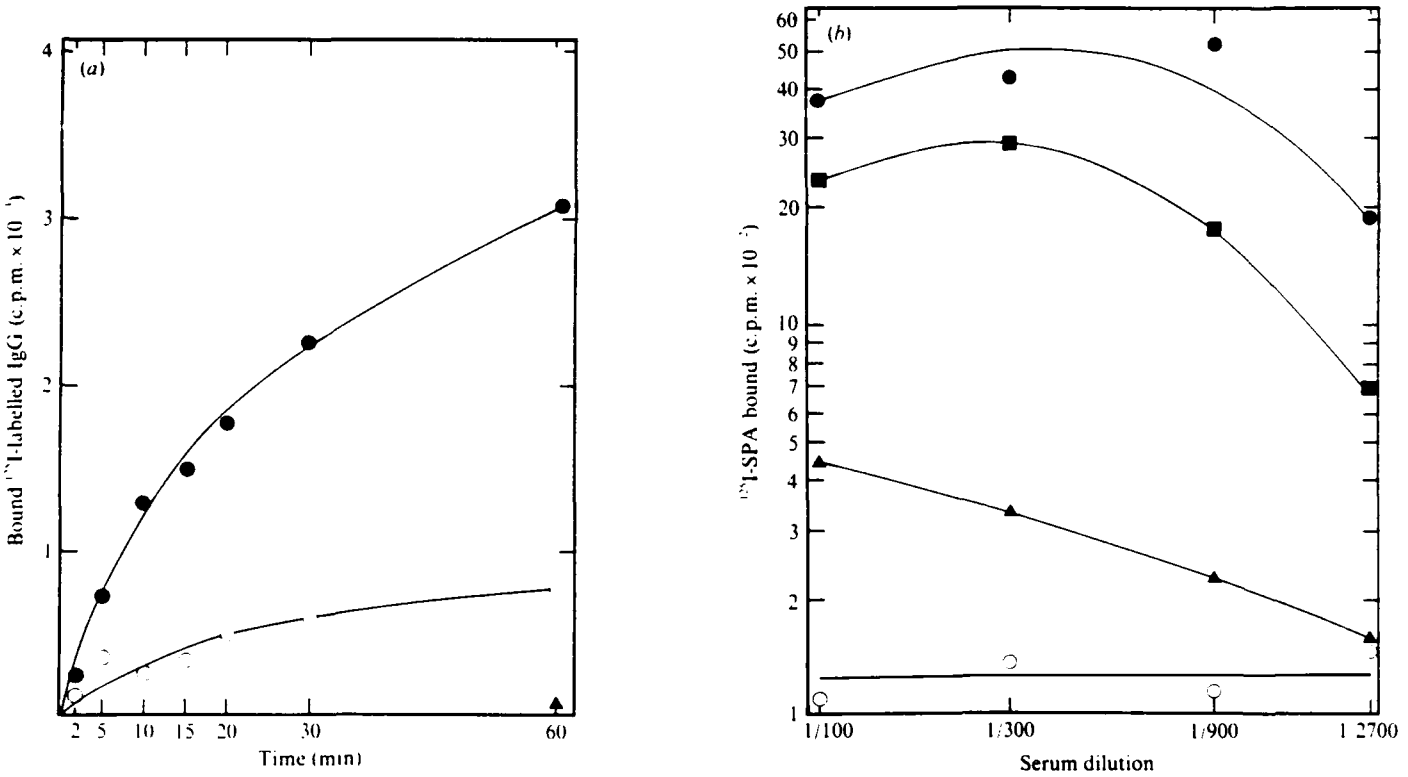


Figure 3. (a) Binding of equal amounts of (0.03 ug) radioiodinated anti-NS1 (●) anti-E (○) monoclonal antibody or myeloma control protein (▲) to YF-infected SW13. Cells were infected 24 h before the assay. (b) Binding of rabbit antiserum against YF NS1 (■), E (▲), or SW13 cell (●) to YF-infected SW13 cells. Cells were infected 24 h before the assay. Normal rabbit serum (○) served as a control. Radioiodinated *S. aureus* Protein A (SPA) was used to probe bound IgG.

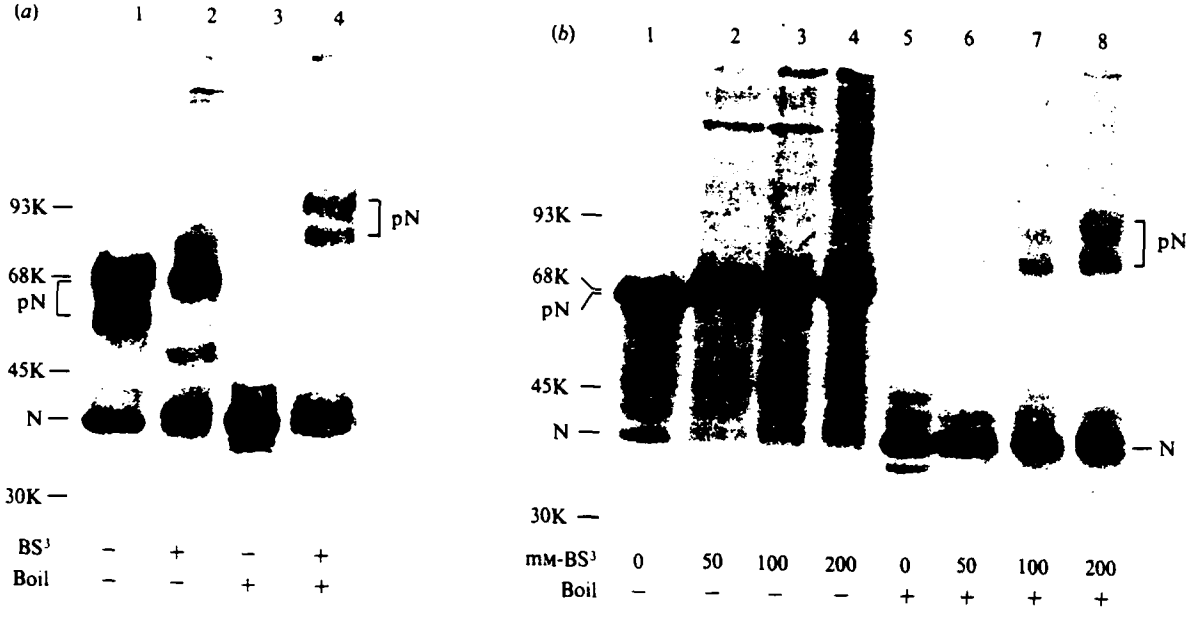


Figure 4. (a) Cross-linking of purified YF NS1. $BS^3$-treated or untreated NS1 (N) was prepared in Laemmli buffer and boiled (or not) before SDS-PAGE and Western blot analysis, as described using rabbit anti-YF NS1 serum to detect monomeric (N) and polymeric (pN) NS1. (b) Cross-linking of YF-infected SW13 cells *in situ*. Monolayers of YF-infected cells were cross-linked with $BS^3$ as described. Solubilized monolayers of treated (lanes 2 to 4 and 6 to 8) or untreated (lanes 1 and 5) YF-infected cells were boiled (lanes 5 to 8) or not (lanes 1 to 4) before SDS-PAGE and Western blotting with rabbit anti-YF NS1 serum.

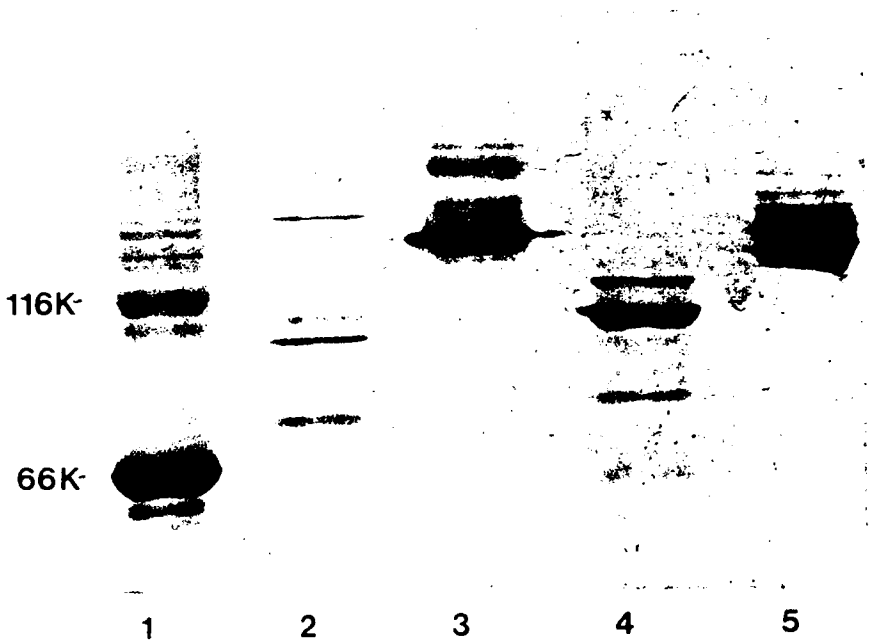


Figure 5. SDS-PAGE analysis of affinity-purified $F(ab)_2$ and IgG proteins lane 1: MW markers; lane 2: $F(ab)_2$ 1A5 (ca 110 kd), Fab 1A5 (ca 70 kd); lane 3: IgG 1A5; lane 4: $F(ab)_2$ PC-5; lane 5: IgG PC-5.

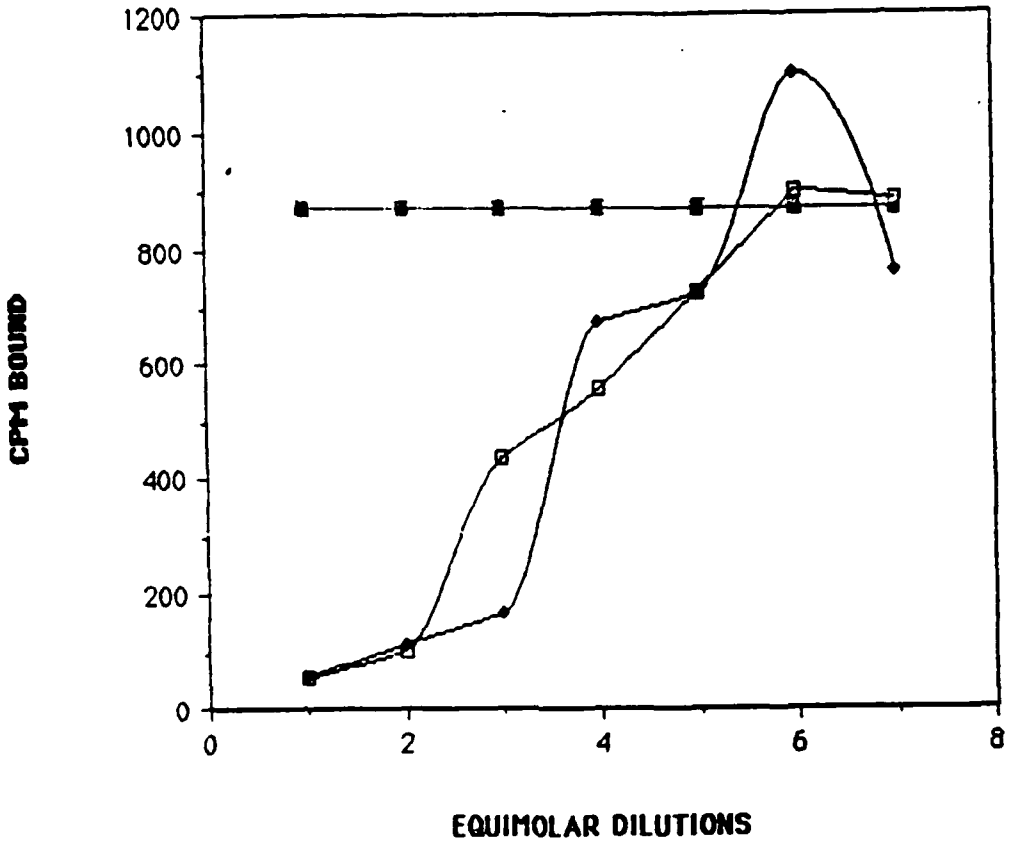


Figure 6. Inhibition of binding of radioiodinated anti-NS1 Mab 1A5 to cell surface NS1. YF-infected SW13 cells were grown in 96 well trays. $^{125}$I-labeled anti-NS1 Mab 1A5 (100,000 cpm) was added to the monolayers in the presence of serial dilutions of chromatographically purified IgG2a (♦) or F(ab)$_2$ (□) 1A5 or IgG2a control myeloma protein PC-5 (■). Undiluted, each protein was used in a final concentration of 840 ug/nl. Cells and added proteins were incubated for 1 hour before washing with PBS (4° C) and detergent lysis. All assays were performed in quadruplicate.

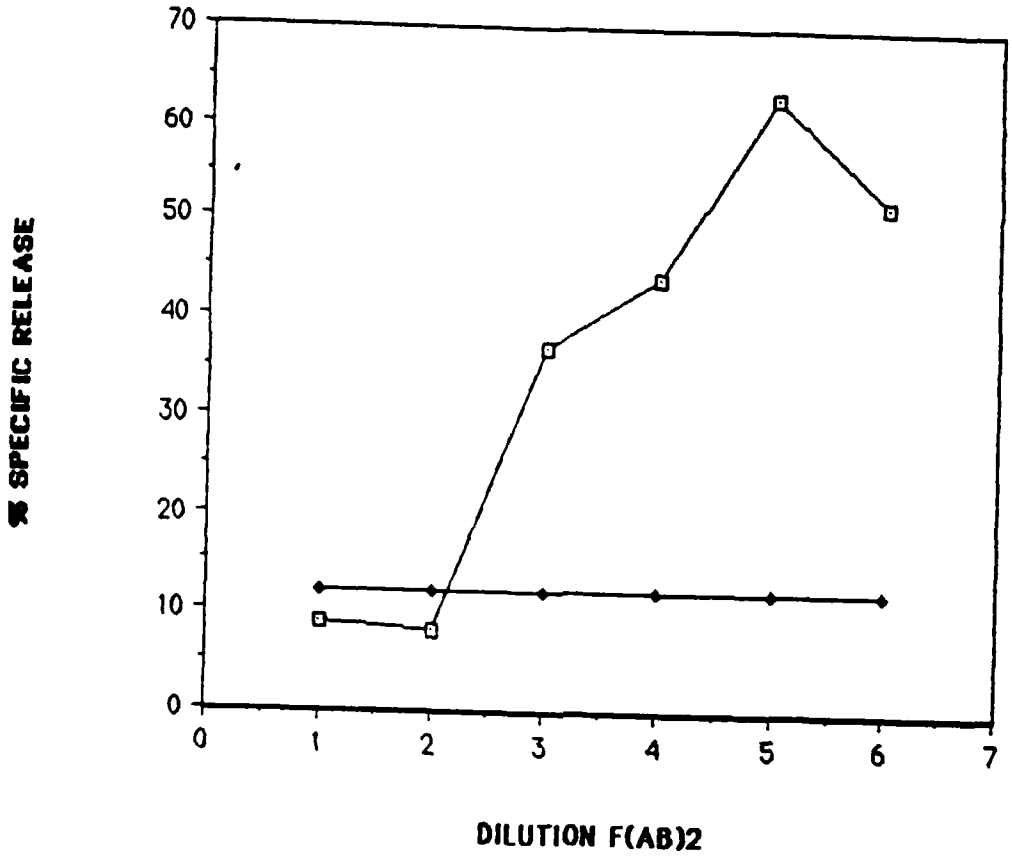


Figure 7. Inhibition of cytolytic activity of IgG2a anti-NS1 Mab 1A5 by $F(ab)_2$ 1A5. IgG 21 1A5 (ca 1nm) was added to YF-infected SW13 cells in the presence of serial 10-fold diltuions of $F(ab)_2$ 1A5 (□) and % specific $^{51}Cr$ release measured after addition of rabbit complement . Determination of lytic activity of serially diluted $F(ab)_2$ alone (◆) was performed in parallel. Ca. 1 nM $F(ab)_2$ was used at the lowest dilution. All assays were performed in quadruplicate.

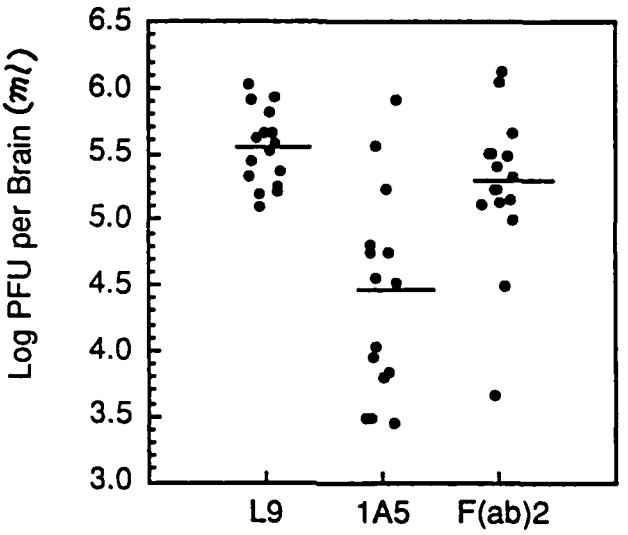


Figure 8. Effect of IgG2a or $F(ab)_2$ anti-NS1 Mab 1A5 on replication of YF in mouse brain. One day after intracerebral challenge with a uniformly lethal dose of YF, mice were passively immunized with intact (IgG) or fragmented ($F[ab]_2$) Mab 1A5. An IgG2a anti-RSV Mab (L9) served as a control. Bar indicates median for each group. Results from three experiments with 15-16 mice for each condition were pooled.


Figure 9. Acetylation of DEN2 NS1 by $^{14}C$ iodoacetamide. Panel A. Silver stain, 11% SDS polyacrylamide gel. Dithiothreitol-reduced (lane 1, "R"), or unreduced (lane 2, "NR") NS1 treated with $^{14}C$ iodoacetamide. Panel B. Radioautogram of Panel A. Alkylation occurred only after reduction of NS1. Identical results were obtained with YF NS1.

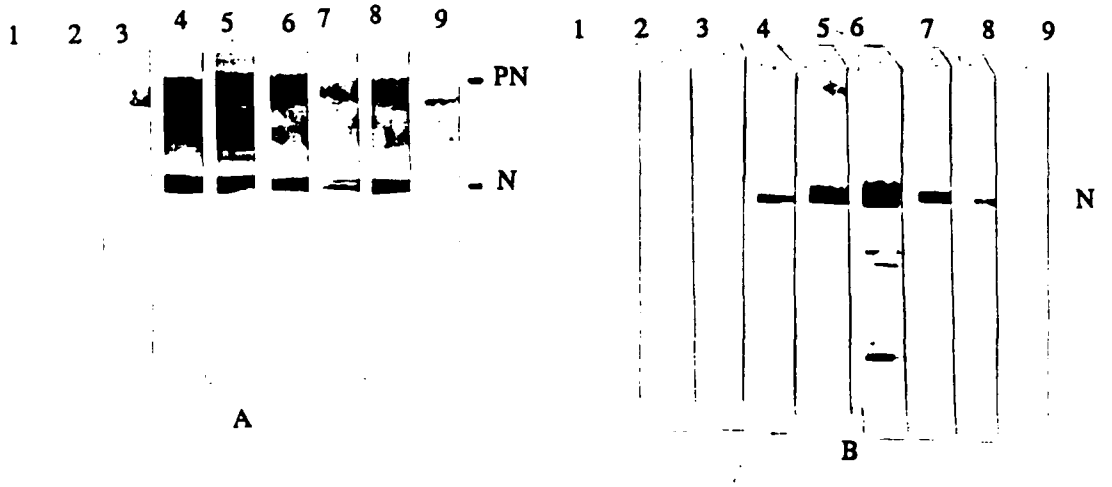


Figure 10. Western blot analysis of sera from mice immunized with native, denatured or fragmented YF NS1. Immunoaffinity-purified unheated, unreduced (A) or boiled and reduced (B) YF NS1 was subjected to SDS-PAGE transferred to Immobilon paper and probed with pooled sera from mice immunized with: 5, native NS1; 6, SDS-treated NS1; 7, dithiothreitol-reduced NS1; 8, reduced and alkylated NS1; 9, trypsin-digested NS1. Controls consisted of: 1, normal mouse srum; 2, SDS-treated ovalbumin; 3, native ovalbumin; 4, anti-YF NS1 Mab 8G4. N - monomeric NS1, PN = polymerized NS1.

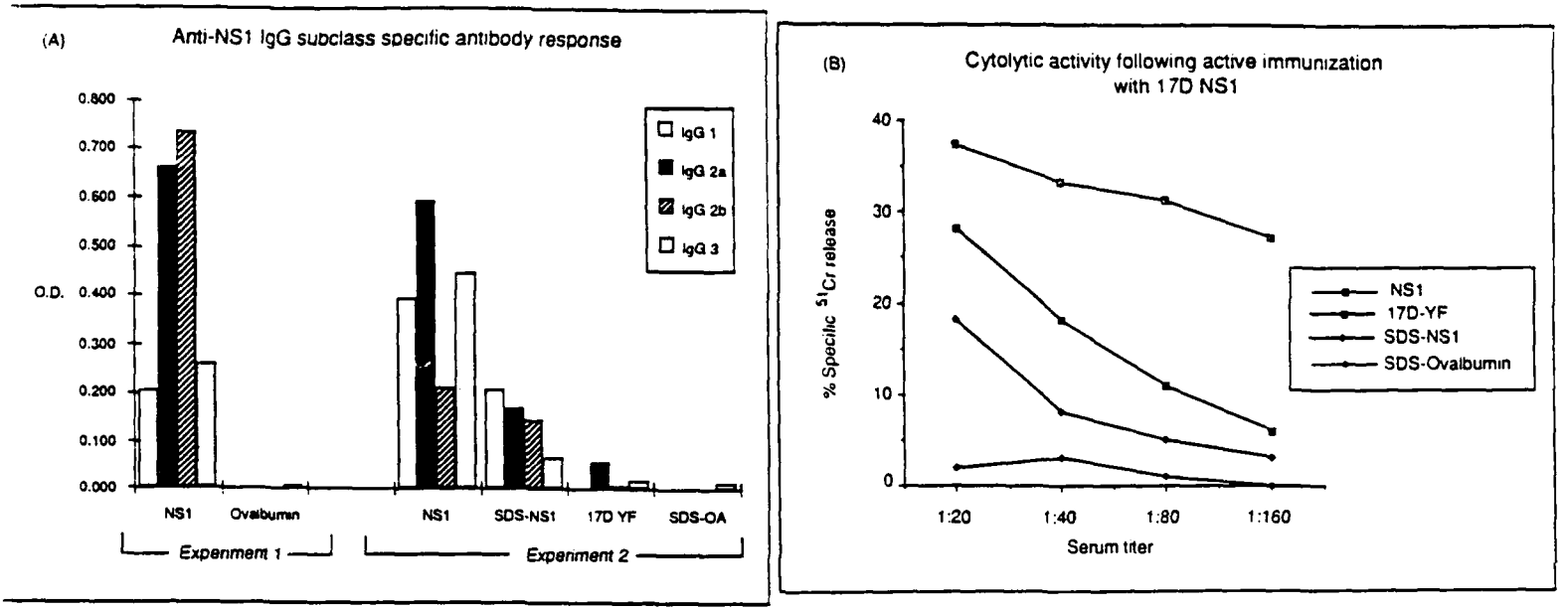


Figure 11. Anti-YF NS1 IgG subclass distribution and cytolytic activity of sera from mice immunized with purified 17D YF NS1. Three-week old white Swiss or BALB/c mice were inoculated intraperitoneally (i.p.) with ca . 10 ug native or SDS-denatured NS1 in complete Freund's adjuvant followed 2 weeks later by a 5 ug i.p. dose in PBS. SDS-denatured ovalbumin (OA) given in the same schedule or a single i.p. dose of $10^6$ pfu active 17D YF served as controls. Mice were exsanguinated at 6 weeks and sera from 3 mice, corresponding to each condition,were pooled before assay.

(A) Subclass distribution of anti-NS1 IgG in sera from 2 experiments. Experiment 1 used Swiss white CD1 mice; Experiment 2, BALB/c mice. Relative contributions of each subclass to the total IgG response to NS1 were measured using rabbit IgG anti-mouse IgG2a, 2b, 1, or 3 with NS1 in the solid phase. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used to develop the assay. Previously described anti-YF NS1 Mab (Schlesinger et al., 1985) representing each subclass were used as standards.

(B) Relative cytolytic activities among sera from mice of Experiment 2. A previously described complement-mediated cytolytic assay was used to determine the cytolytic capacity of monospecific antisera prepared to YFV NS1 or E (Schlesinger et al., 1985, 1990). Briefly, SW13 target cells grown to confluence in 96-well microtest plates were infected with 17D YFV and labeled with $Na_2{}^{51}CrO_4$ 24 or 48 h later, at which time SW13 cells were uniformly infected. Labeled infected monolayers were incubated at 37°C for 2 h with heat-inactivated antibody and rabbit complement (1:20) before harvesting with a Titertek supernatant collection system. Assays were performed in quadruplicate and the standard error usually did not exceed 5%. Percentage of specific lysis was calculated using the formula (experimental $^{51}Cr$ release-spontaneous release)/(maximal release- spontaneous release) x 100 in which spontaneous release was determined with antibody and heat-inactivated complement and maximal release was the value obtained after detergent lysis of the cell monolayer.

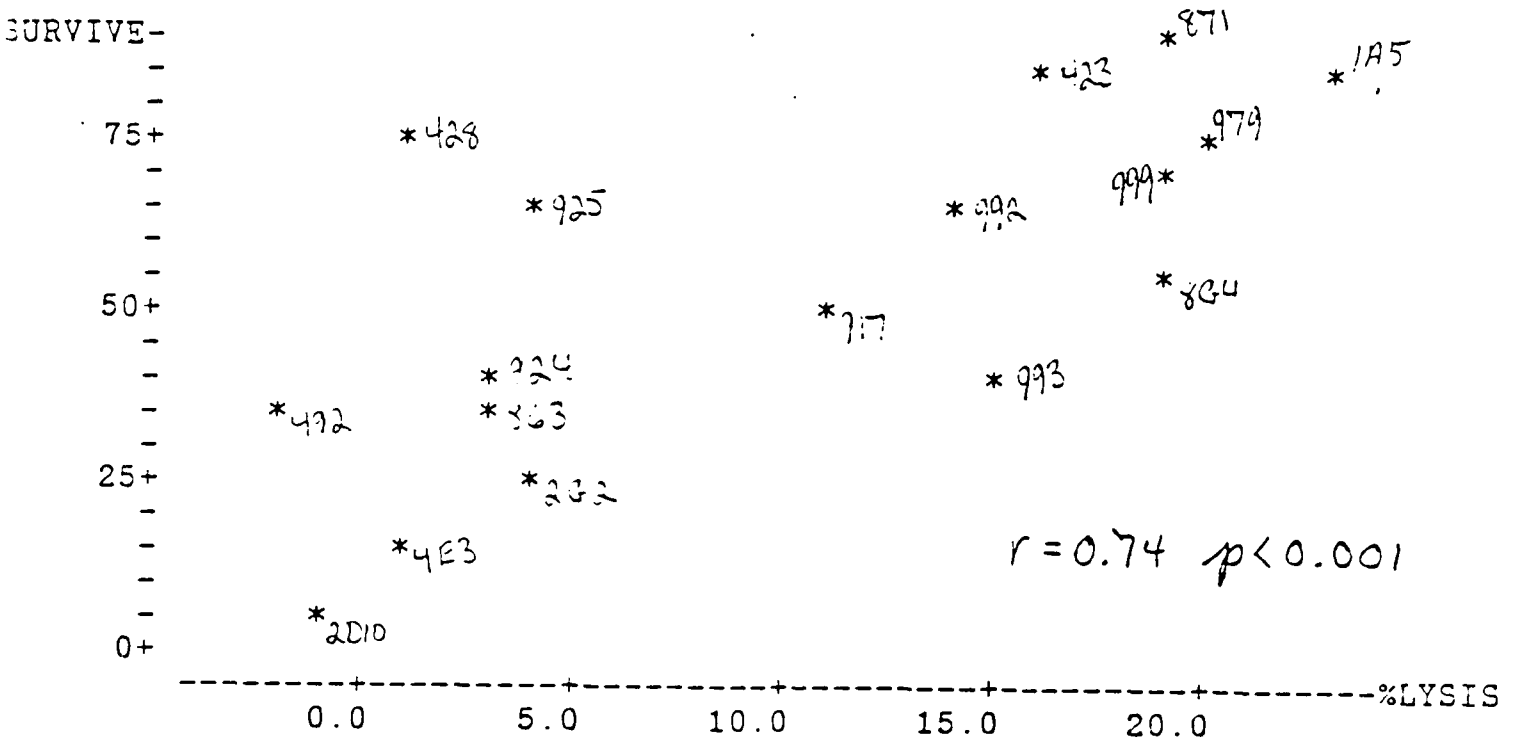


Figure 12. Lytic versus protective capacities among anti-YF NS1 Mab. Mab (Table 7) were ordered by protective capacity as determined by reported (Schlesinger et al., 1985; Gould et al., 1986) survival in intracerebral mouse protection tests and cytolytic activity measured by per cent specific $^{51}Cr$ release from 17D YF-infected mouse neuroblastoma cells (Neuro 2a).

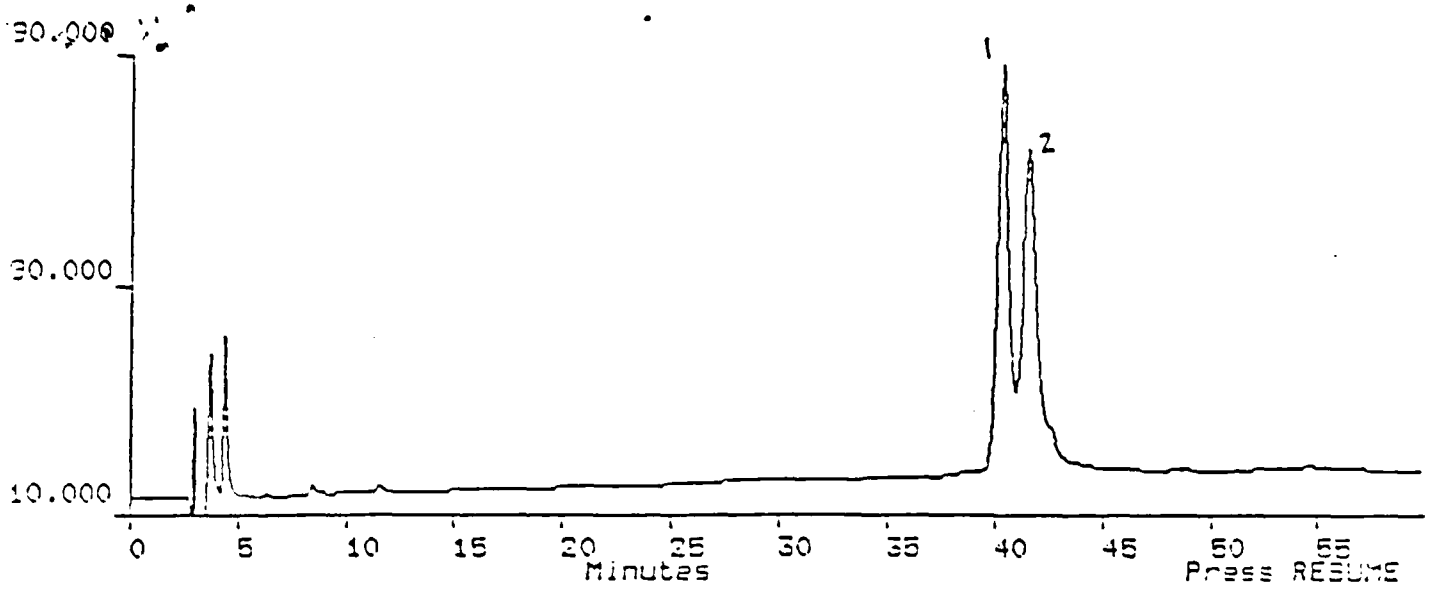


Figure 13A. Fractionation of affinity-purified DEN2 NS1. Approximately 10 umol DEN2 NS1 loaded onto Vydac C4 column and separated with a linear 30-80% Buffer B gradient at 1 ml/min. Buffer A, 0.1% TFA. Buffer B, 80% acetonitrile in Buffer A. Peaks 1 and 2 reacted with anti-DEN2 NS1 Mab (Western blot) and represent 46 kd (1) and 30 kd (2) moieties of DEN NS1.

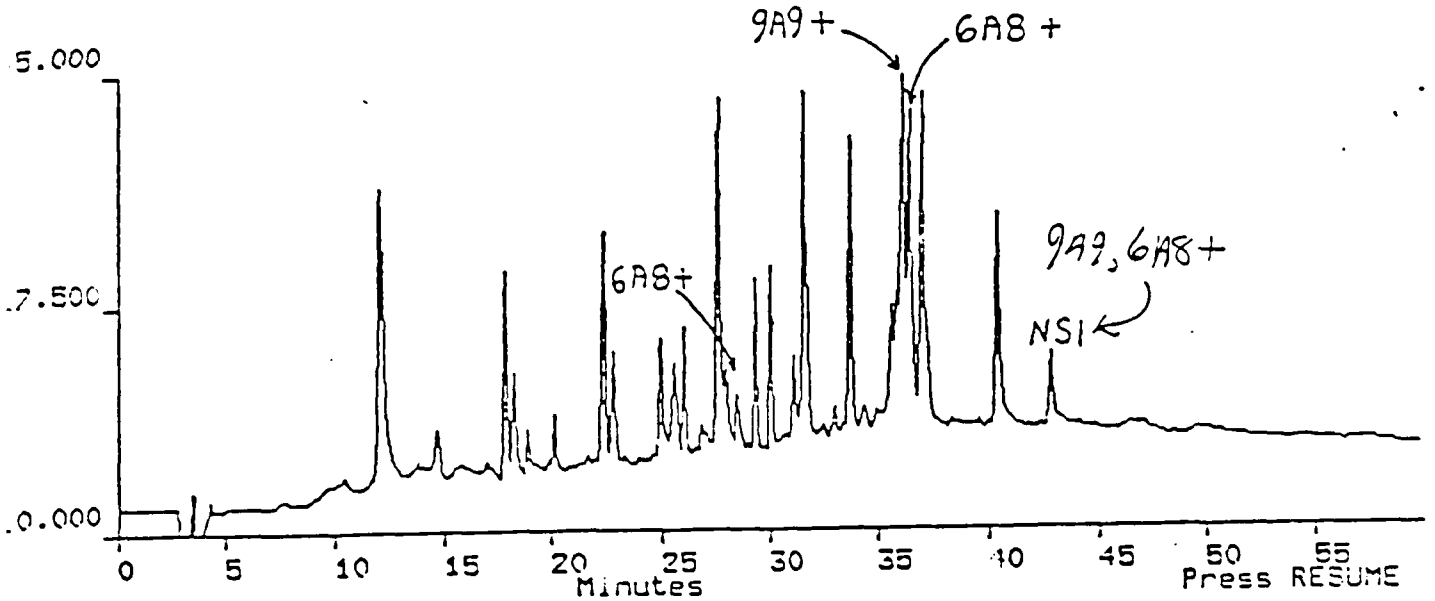


Figure 13B. Fractionation and immunoassay of tryptic peptides derived from dithiothreitol-reduced DEN2 NS1 (46 Kd). Ca 100 ug HPLC-purified NS1 in 50 mM ammonium bicarbonate pH 8.0 was digested with HPLC-purified trypsin (protein: enzyme 50:1) for 2 h at 37°C and loaded onto a Vydac C18 column followed by separation with a 0-100% buffer B gradient. 1 ml/min and absorbance at 314 nm. Individual peptide peaks were lyophilized using a Savant Speed-Vac drier, reconstituted in phosphate buffered saline containing 0.1% Triton-X and tested by Elisa for reactivity (+) with "protective" anti-DEN NS1 Mab 6A8 or nonprotective Mab 9A9.

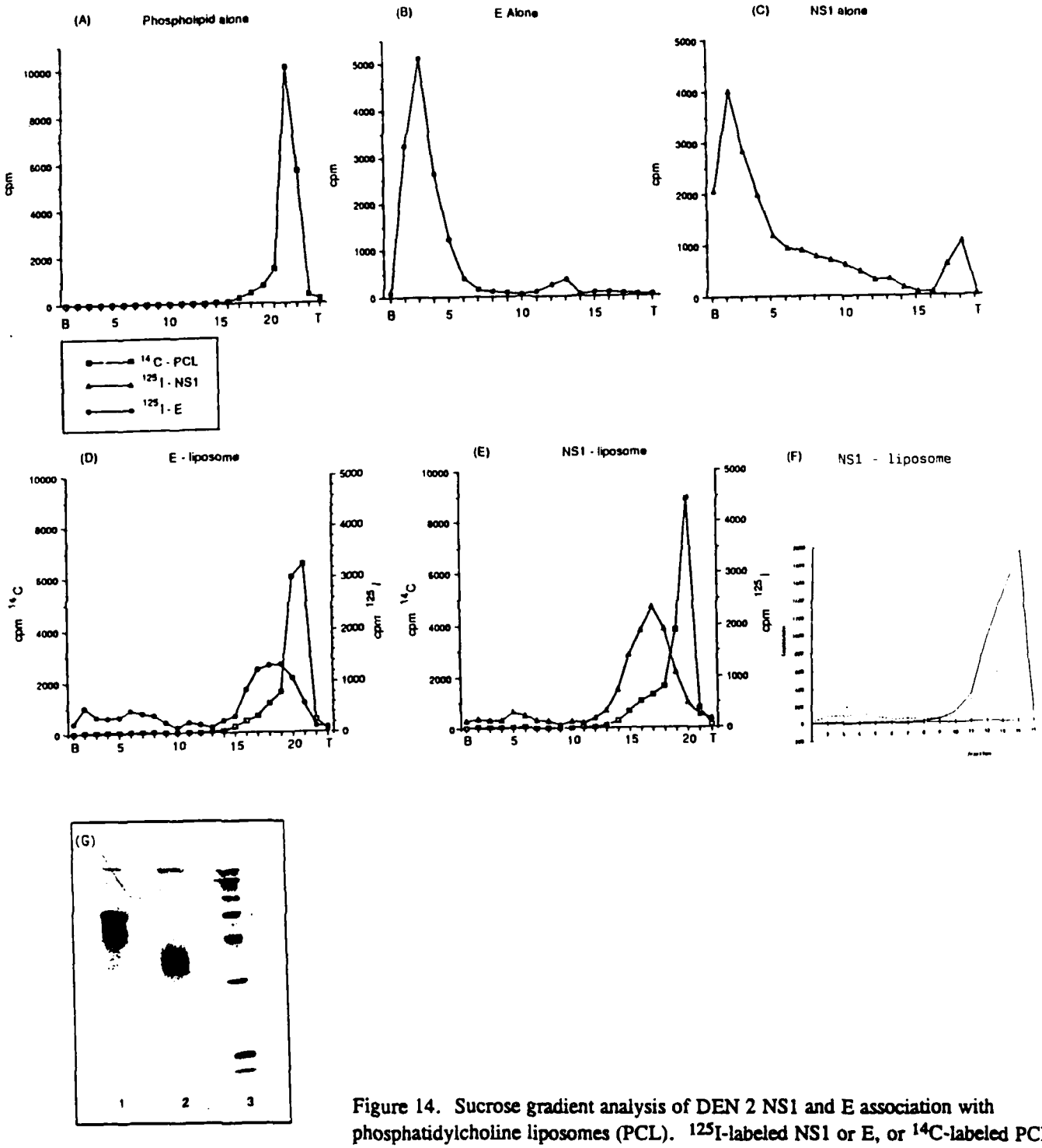


Figure 14. Sucrose gradient analysis of DEN 2 NS1 and E association with phosphatidylcholine liposomes (PCL). $^{125}$I-labeled NS1 or E, or $^{14}$C-labeled PCL in Tris-NaCl, 40% sucrose was added onto a 60% sucrose cushion followed by addition of a 0-40% linear sucrose gradient. In parallel tubes $^{14}$C-PCL formed with either $^{125}$I E or NS1 were similarly positioned. Gradients were centrifuged 21 h at 127,000 g in a SW 14 rotor after which 0.5 ml fractions were collected for $^{125}$I and $^{14}$C counting.

A-E: results of an experiment with E and NS1 liposomes prepared in parallel: (A) $^{14}$C-PCL alone; (B) $^{125}$I-E alone; (C) $^{125}$I-NS1 alone; (D) $^{14}$C-PCL formed with $^{125}$I-E; (E) $^{14}$C-PCL formed with $^{125}$I-NS1; (F) $^{14}$C-PCL formed with $^{125}$I-NS1 in a second experiment: $^{14}$C-liposome (——); $^{125}$I-NS1 (- - - -); (G) SDS-PAGE electrophoresis of radioiodinated E and NS1 used for incorporation into $^{14}$C-labeled PCL.

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