Immunization of Mice with Recombinant Vaccinia Virus Expressing Authentic Dengue Virus Nonstructural Protein NS1 Protects against Lethal Dengue Virus Encephalitis

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The protective immunity conferred by a set of recombinant vaccinia viruses containing the entire coding sequence of dengue virus type 4 nonstructural glycoprotein NS1 plus various flanking sequences was evaluated by using a mouse encephalitis model. Mice immunized with recombinant vNS1-NS2a, which expresses authentic NS1, were solidly protected against intracerebral dengue virus challenge. However, mice immunized with recombinants vNS1-15%NS2a and vRSVG/NS1-15%NS2a, which express aberrant forms of NS1, were only partially protected (63 to 67% survival rate). Serologic analysis showed that mice immunized with vNS1-NS2a developed high titers of antibodies to NS1 as measured by radioimmunoprecipitation, enzyme-linked immunosorbent assay, and complement-mediated cytolytic assays. In addition, a pool of sera from these animals was protective in a passive transfer experiment. Lower titers of NS1-specific antibodies were detected in sera of animals immunized with vNS1-15%NS2a or vRSVG/NS1-15%NS2a by all three assays. These data support the view that protection against dengue virus infection in mice may be mediated at least in part by NS1-specific antibodies through a mechanism of complement-mediated lysis of infected cells. Additionally, immunization with two recombinant viruses expressing authentic NS1 of dengue virus type 2 conferred partial protection (30–50%) against dengue virus type 2 challenge.

Dengue virus poses a major threat to the public health, causing epidemics throughout tropical areas worldwide. The four serotypes of dengue virus constitute a subgroup of the flavivirus family. Many other flaviviruses are also causative agents of human disease, for example, yellow fever virus, Japanese encephalitis virus, and tick-borne encephalitis virus. These viruses are transmitted by tick or mosquito vectors; dengue virus is transmitted by mosquitoes of the genus *Aedes*. An estimated 100 million cases of dengue illness occur each year (10). Dengue virus predominantly causes a debilitating illness known as dengue fever. Occasionally, the more serious dengue hemorrhagic fever-shock syndrome results. This severe disease is most common in young children and has a high mortality rate. Following recovery from dengue virus infection, immunity to the infecting serotype (homotypic immunity) is apparently life-long (21, 24); this makes dengue virus a good candidate for immunoprophylaxis. However, despite over 40 years of effort, safe and effective dengue virus vaccines are not available. Control of flaviviruses, particularly dengue virus, is a global concern; the World Health Organization has designated dengue virus as one of five high-priority targets for vaccine development.

Humans recovering from dengue illness, especially from secondary dengue virus infections, have high titers of antibodies to nonstructural glycoprotein NS1 and to the dengue viral structural proteins, particularly the envelope (E) glycoprotein, in their sera (19, 23, 29). Antibody to E neutralizes dengue virus in vitro, and inoculation of mice with a monoclonal antibody (MAb) to E can protect against subsequent intracerebral homotypic dengue virus challenge (18). NS1 is important in protection also, since mice immunized with

purified NS1 protein (28) or inoculated with specific combinations of MAbs to NS1 (15) are protected. Protection by antibodies to NS1 is thought to involve immune recognition of NS1 at the infected-cell surface, followed by complement-mediated lysis (27, 28).

Recombinant vaccinia viruses expressing protective antigens have been tested as a vaccine strategy against a wide variety of viruses. We have recently constructed several vaccinia viruses that express E of dengue virus type 4 (DEN4), and immunization of mice with these recombinants induced resistance to dengue virus encephalitis (1, 2). However, this is legitimate concern about using E as an immunogen in humans. Antibodies to E can enhance replication of dengue virus in cells of the monocyte-macrophage lineage in vitro (11–13). This enhanced replication is thought to result from increased entry of virus-antibody complexes into cells bearing F_c receptors. This mechanism of immune enhancement of viral replication is thought to be a contributing factor in the etiology of dengue hemorrhagic fever-shock syndrome, in view of the correlation of severe disease and the presence of preexisting antibody in children, either from prior heterotypic infection or from passive transfer from a dengue virus-immune mother (reviewed in reference 10). Immunization with NS1 is an attractive possibility, since NS1 is not part of the virion, and immune enhancement can be avoided. We recently described a series of recombinant vaccinia viruses that contain the NS1 sequence of DEN4 plus various lengths of the downstream gene NS2a, with or without the upstream genes coding for capsid (C), premembrane (preM), and E (7). Recombinants that contain all of NS2a apparently express authentic NS1, whereas those that contain less than half of NS2a express an uncleaved NS1-NS2a product. In this report, we describe the construction of two additional recombinant vaccinia viruses that express

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apparently authentic NS1 of dengue virus type 2 (DEN2). We also describe the protective efficacies of these recombinant vaccinia viruses expressing NS1 of DEN4 or DEN2 in experimental immunoprophylaxis of mice.

MATERIALS AND METHODS

Cells and viruses. CV-1 and human TK⁻43 cells were grown in minimal essential medium (MEM) containing 10% fetal bovine serum. LLC-MK$_2$ cells were grown in medium 199 containing 10% fetal bovine serum. HeLa cells were obtained from N. Cooper (National Institute of Allergy and Infectious Diseases, Bethesda, Md.) and grown in suspension in Spinner MEM containing 5% horse serum. Mosquito C6/36 cells were grown at 28°C as monolayers in MEM supplemented with nonessential amino acids plus 10% fetal bovine serum. Human adenocarcinoma SW13 cells were grown in MEM containing 5% fetal bovine serum.

DEN4 strain 814669 was originally from W. Brandt (Walter Reed Army Institute of Research, Washington, D.C.) and was grown in C6/36 cells as previously described (14). DEN4 strain H241 and DEN2 strains PR159 and New Guinea C were from D. Dubois (Walter Reed Army Institute of Research).

Vaccinia virus (strain WR) and recombinant vSC8 (6) were from B. Moss (National Institute of Allergy and Infectious Diseases). Recombinant vPE11 was from P. Earl (National Institute of Allergy and Infectious Diseases). Recombinants vSC8 and vPE11 contain no dengue virus sequences and were used as negative controls in the mouse protection studies (see below). Vaccinia virus recombinants vC-M-E-NS1-NS2a, vC-M-E-NS1-15%NS2a, vNS1-NS2a, and vNS1-15%NS2a have already been described (7, 33), as has recombinant vC-M-E (2). Recombinant vRSVG/NS1-15% NS2a was made from plasmid pSC11/4INS1C (7) as previously described (6). Recombinants vDEN2(2041-4497) and vDEN2(2304-4497) were made from plasmids pSC11/DEN2 (2041-4497) and pSC11/DEN2(2304-4497), which are described in the next section. Small-scale stocks of recombinant viruses were grown on CV-1 cells. Large-scale stocks were grown in HeLa cells, and virus was purified by sucrose gradient centrifugation as previously described (17). Virus stock titers were determined on CV-1 cells.

Subcloning of DEN2 sequences. All enzymes were from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or New England BioLabs, Inc. (Beverly, Mass.), and were used approximately as recommended by the manufacturer. Standard recombinant DNA techniques were used throughout. The source of DEN2 sequences was clone 2 (9; provided by J. Strauss and E. Strauss, California Institute of Technology, Pasadena), which contains DEN2 cDNA of the S1 vaccine strain of DEN2 PR159, from nucleotides (nt) 2041 to 5891, cloned into the EcoRI site of pGEM1 by using linkers, oriented such that the pGEM1 BamHI site is about 20 nt from DEN2 residue 2041. The KpnI site of clone 2 at DEN2 nt 4493 to 4498 was converted to a BamHI site by insertion of a synthetic oligonucleotide, 5'-GGGATCCCGTAC-3'. The 2.5-kilobase-pair BamHI fragment of the resulting clone was inserted into the BglII site of plasmid pSC11[BglII] (7), creating pSC11/DEN2(2041-4497), in which the BamHI fragment is oriented such that the DEN2 sense strand is under control of the vaccinia virus p7.5 promoter. Translation of mRNA transcribed from this promoter should begin at the first AUG codon (which occurs at DEN2 nt 2137 to 2139, corresponding to the methionine at residue 681 of the DEN2 polyprotein) and is expected to produce a polyprotein con-

taining the C-terminal 95 amino acids of E, all of NS1 and NS2a, the N-terminal 122 amino acids (ending at DEN2 amino acid 1467) of NS2b and 6 vector-encoded residues (GIWGIL).

A second DEN2 construct, containing less E, was made as follows. The 0.6-kilobase-pair XhoII-NsiI fragment (nt 2304 to 2952) of pSC11/DEN2(2041-4497) was used to replace the 0.9-kilobase-pair XmaI-NsiI fragment of the same plasmid by using two synthetic oligonucleotides, 5'-CCGGGAGAT CTCCAT-3' and 5'-GATCATGGAGATCTC-3', to bridge between the XmaI and XhoI sites. (The XmaI site is just upstream of DEN2 nt 2041, within the pGEM1 sequences). This resulted in plasmid pSC11/DEN2(2304-4497), which encodes a polyprotein that should initiate with an AUG partially provided by the first oligonucleotide (underlined AT) and should contain DEN2 amino acids 737 to 1467 (which contain the C-terminal 39 hydrophobic amino acids of E, all of NS1 and NS2a, and the first 122 residues of NS2b), followed by 6 vector-encoded residues, as described above. The structure of the sense strand in the region of the oligonucleotide was verified by DNA sequencing by using the dideoxynucleotide chain termination method.

Analysis of infected-cell proteins. Preparation of recombinant vaccinia virus-infected CV-1 cell lysates radiolabeled with [^{35}S]methionine was done as already described (7). To prepare proteins from DEN2-infected cells, LLC-MK$_2$ cells growing in a six-well dish were infected with DEN2 PR159 or New Guinea C at a multiplicity of infection of 0.1 to 1 PFU per cell, and 3 to 5 days later, the cells were labeled for 2 to 6 h with 0.5 ml of methionine-free MEM (containing 100 μCi of [^{35}S]methionine per ml) per well. The cells were then lysed in situ with radioimmune precipitation assay buffer, and the lysates were clarified as already described (7). ^{35}S-labeled DEN4 814669-infected cell proteins were similarly prepared by using C6/36 cells. Immune precipitations using hyperimmune mouse ascitic fluid (HMAF) specific for DEN4 or DEN2 (obtained from J. McCown, Walter Reed Army Institute of Research) were done as previously described (7). The following 11 MAbs specific for DEN2 NS1 (16) were obtained from E. Henchal (Walter Reed Army Institute of Research): 16-25/3, 20-1/1, 27-12/4, 34-23, 40-21/9, 47-10/10, 63-15, 68-5/16, 82-8/3/2, 101-4/7, D7-3E9-4. These MAbs were used at a 1:5 dilution for immune precipitation. Sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis and fluorography were done as previously described (7).

Mouse protection. The protocol used for immunization of mice and subsequent challenge with dengue virus has already been described (2). Briefly, female BALB/c mice were immunized at 3 weeks of age (day 1) and again on day 14 by intraperitoneal inoculation of recombinant vaccinia virus (10^7 PFU). Immunized animals were bled from the orbit on day 20 and challenged on day 21 by intracerebral injection of 100 50% lethal doses of either DEN4 H241 or DEN2 New Guinea C. Mice were observed for 21 days for signs of encephalitis, and the number of deaths was recorded daily. Sera were also collected from some survivors for comparison with prechallenge sera. Control animals were immunized with the following recombinant viruses that contain no dengue viral sequences: vSC8, which contains only the lacZ gene, or vPE11, which contains the lacZ gene and a portion of the human immunodeficiency virus envelope gene. Serum donors for passive transfer were immunized as described above, blood was collected on days 21 and 24, and sera were pooled. Recipient mice were inoculated intraperitoneally with 0.6 ml of the pooled sera or a 1:10 dilution of DEN4
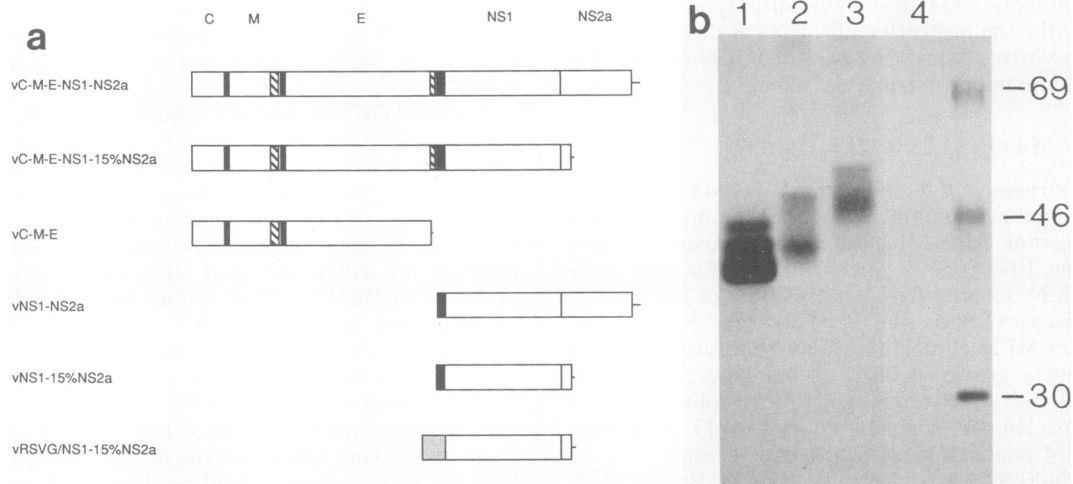
FIG. 1. (a) Polyproteins encoded by DEN4 cDNA contained in vaccinia virus recombinants. The region of the dengue virus polyprotein expressed by each recombinant is shown, with the N terminus at the left. Viral protein sequences are represented by boxes, while amino acid sequences at the C termini encoded by the vector are shown by thin horizontal lines. This vector-encoded tail is 3 residues for vC-M-E; 5 residues for vC-M-E-NS1-15% NS2a, vNS1-15%NS2a, and vRSVG/NS1-15%NS2a; and 25 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 respiratory syncytial virus G glycoprotein. (b) Forms of NS1 expressed by vNS1-NS2a, vNS1-15%NS2a, and vRSVG/NS1-15%NS2a. Equal fractions of ^{35}S-labeled infected-cell lysates were immune precipitated with HMAF, the precipitates were electrophoresed on an SDS–12% polyacrylamide gel, and the gel was fluorographed. Viruses used to infect cells: lane 1, vNS1-NS2a, which expresses authentic-size NS1; lane 2, vNS1-15%NS2a, which expresses NS1$_x$; lane 3, vRSVG/NS1-15%NS2a, which expresses RSVG/NS1$_x$; lane 4, vSC8, which expresses no dengue virus-specific protein. The lane at the far right contained ^{14}C-labeled molecular size marker proteins. The sizes are indicated in kilodaltons at the right.

HMAF 1 day before challenge with DEN4 at 6 weeks of age as described above.

Analysis of mouse sera. Three methods were used to analyze mouse sera for anti-dengue virus antibodies: immune precipitation, enzyme-linked immunosorbent assay (ELISA), and complement-mediated immune cytolysis. For immune precipitations, 20 µl of ^{35}S-labeled proteins from DEN4-infected cells were precipitated by 5 µl of mouse serum or 1 µl of DEN4 HMAF as a positive control for at least 2 h on ice. Collection of immune complexes by using Pansorbin, washing, solubilization, and SDS-gel electrophoresis was done as previously described (7).

A standard ELISA protocol, which included use of Immulon 1 U plates (Dynatech Laboratories, Inc., Alexandria, Va.), was used. The antigen in the solid phase was DEN4 814699-infected cell lysate, and the second antibody was peroxidase-conjugated goat anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Sera were diluted from 1:10 to 1:640 in twofold steps. Positive samples were those whose A_{590} values exceeded twice the average A_{590} value for the 1:10 dilutions of the negative control sera from mice immunized with vPE11. The titer of a serum was the reciprocal of the last dilution that gave a positive result. Samples that were negative at 1:10 were assumed to contain no antibodies to NS1. Some samples had very high A_{590} values at 1:640; these were assigned a titer of 1:1,280 for calculation of group averages. This was likely an underestimate of the titers of these samples.

A modified complement-mediated cytolytic assay was performed to determine the cytolytic activity of pooled postimmunization mouse sera (26, 27). Briefly, target human adenocarcinoma SW13 cells grown to confluence in 96-well microtest plates were infected with DEN4 H241 and labeled

with Na_2^{51}CrO$_4$ at 48 h postinfection, at which time cells were uniformly infected as determined by immunofluorescence assay. Labeled infected monolayers were incubated at 37°C for 2 h with heat-inactivated serum and rabbit complement (1:20) before being harvested with a Titertek supernatant collection system. Assays were performed in quadruplicate, and percent lysis was calculated by using the following formula: (experimental ^{51}Cr release − spontaneous release) × 100/(maximal release − spontaneous release). Spontaneous release was determined with serum and heat-inactivated complement, and maximal release was the value obtained after detergent lysis of the cell monolayer with 1% Nonidet P-40. As a positive control, rabbit antiserum specific for DEN2 NS1 (28) was used. Samples which had percent lysis values that exceeded twice the value obtained with negative control sera were considered positive. The titer of a sample was the reciprocal of the last dilution which was positive.

RESULTS

Analysis of protein expression by recombinant vaccinia viruses. The structures of the polyproteins encoded by the recombinant vaccinia viruses containing DEN4 cDNA are shown in Fig. 1a. Viruses vC-M-E, vC-M-E-NS1-NS2a, vC-M-E-NS1-15%NS2a, vNS1-NS2a, and vNS1-15%NS2a have been described previously (2, 7, 33). Cells infected with vC-M-E-NS1-NS2a expressed preM, E, and NS1 proteins that comigrated on SDS-polyacrylamide gels with the authentic proteins from DEN4-infected cells (33; data not shown). Expression of C and NS2a was not detected. Similarly, cells infected with vC-M-E expressed preM and E that comigrated with DEN4 proteins, but expression of C was not detected (2). Recombinant vC-M-E-NS1-15% NS2a directed expression of authentic preM and E proteins, but

TABLE 1. Protection of mice against DEN4 encephalitis by immunization with recombinant vaccinia viruses[a]

Virus	Expression of authentic NS1	No. of survivors/total (%)[b]
vC-M-E-NS1-NS2a	+	17/18 (94)
vC-M-E-NS1-15%NS2a	−	15/15 (100)
vNS1-NS2a	+	28/28 (100)
vNS1-15%NS2a	−	20/30 (67)
vRSVG/NS1-15%NS2a	−	36/57 (63)
Negative control	−	8/52 (15)

[a] On the basis of standard chi-square analysis for pairwise comparisons, each of the first five viruses is significantly different from the negative control group ($P < 0.0005$). The vNS1-15%NS2a ($P < 0.005$) and vRSVG/NS1-15%NS2a ($P < 0.0005$) groups are each significantly different from vNS1-NS2a, but they are not significantly different from each other.

[b] The data represent the pooled results of five independent experiments. Some of the data from individual experiments have been presented previously as parts of other sets of pooled data (1, 2).
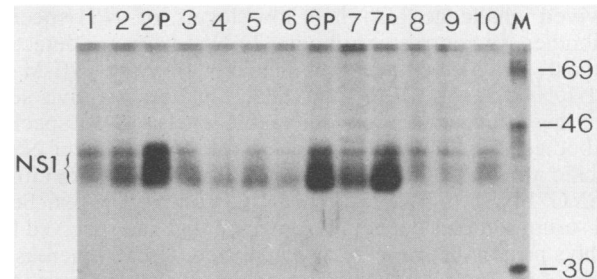

FIG. 2. Analysis of postimmunization mouse sera by radioimmunoprecipitation. Sera were collected from immunized mice 1 day before challenge with DEN4. Some animals were also bled 21 days postchallenge. Three microliters of each serum was used to immune precipitate 15 μl of [^{35}S]methionine-labeled DEN4-infected cell lysate, and the precipitates were resolved by SDS-gel electrophoresis and fluorography. The 10 individual animals used had been immunized with vNS1-NS2a. P, Serum samples taken 21 days postchallenge. The numbers on the right indicate molecular sizes in kilodaltons.

expression of C was not seen (7; data not shown). In addition, cleavage between NS1 and NS2a did not occur, which resulted in an NS1-NS2a fusion product ($NS1_x$) that was larger than the authentic protein (7; see below). The DEN4-specific proteins expressed by cells infected with vNS1-NS2a, vNS1-15%NS2a, or vRSVG/NS1-15%NS2a are shown in Fig. 1b. As reported previously (7), vNS1-NS2a directed expression of NS1 of authentic size, which appeared in the gel as a cluster of bands. Cells infected by vNS1-15%NS2a produced an NS1 species ($NS1_x$) larger than authentic NS1. $NS1_x$ results from failure to cleave between NS1 and NS2a, and this protein presumably consists of NS1 plus the N-terminal 32 amino acids of NS2a plus 5 vector-encoded residues. Recombinant vRSVG/NS1-15%NS2a is similar to vNS1-15%NS2a, but the 24-residue DEN4 NS1 signal sequence is replaced by the 71 N-terminal amino acids of the respiratory syncytial virus G glycoprotein, which acts as a signal but is not cleaved (25, 31, 32). Cells infected with vRSVG/NS1-15%NS2a expressed an even larger NS1 species ($RSVG/NS1_x$), as expected, since this product should contain the 71-amino-acid respiratory syncytial virus G glycoprotein sequence at the N terminus in addition to the uncleaved NS2a residues at the C terminus. The reduced intensities of the $NS1_x$ and $RSVG/NS1_x$ bands compared with NS1 presumably were due to decreased stability or decreased reactivity with the polyclonal antiserum used for immunoprecipitation.

Protective immunity of DEN4 NS1 expressed by recombinant vaccinia viruses. It has been established that dengue virus NS1 is a protective antigen, since immunization of mice with purified NS1 of DEN2 induces resistance to dengue encephalitis (28). We extended this observation by evaluating the protective immunity induced in mice immunized with recombinant vaccinia viruses expressing DEN4 NS1 alone or in combination with structural proteins. BALB/c mice were immunized at 3 weeks of age and again at 5 weeks by intraperitoneal inoculation of 10^7 PFU of recombinant vaccinia virus. Subsequently, immunized mice were challenged by intracerebral inoculation of DEN4 H241 at 6 weeks of age and observed for 21 days for symptoms of dengue encephalitis and death. The pooled results of several experiments are presented in Table 1. Each of the 52 mice in the negative control group that received a recombinant virus (vSC8 or vPE11) that does not contain DEN4 sequences developed symptoms of encephalitis, and only 15% of those animals survived. In contrast, mice immunized with vC-M-E-NS1-NS2a showed little or no signs of en-

cephalitis and 94% of that group survived challenge. Additionally, 15 mice that received recombinant vC-M-E-NS1-15%NS2a were fully protected. It was shown previously that immunization with a vaccinia virus recombinant expressing only E is completely protective (1, 2). Since vC-M-E-NS1-NS2a and vC-M-E-NS1-15%NS2a express E, it was not surprising to find that these viruses also induced solid protection against dengue virus challenge.

To assess the role that NS1 might play in protection of mice from encephalitis, recombinants vNS1-NS2a, vNS1-15%NS2a, and vRSVG/NS1-15%NS2a were evaluated for protective efficacy. All mice immunized with vNS1-NS2a survived DEN4 challenge (Table 1). However, most of those animals developed transient symptoms of mild encephalitis, and in two cases, illness progressed to hindleg paralysis. Groups of mice immunized with vNS1-15%NS2a or vRSVG/NS1-15%NS2a had a survival rate near 65%, significantly higher than that of the negative control group (15%). In addition, most mice vaccinated with the latter two constructs were ill after challenge. Thus, unlike the solid protection with no illness induced by immunization with recombinant vaccinia viruses expressing E, mice immunized with recombinants expressing only NS1 or NS1-15%NS2a fusion proteins exhibited morbidity. In terms of survival, vNS1-NS2a, expressing authentic NS1, was most protective, while vNS1-15%NS2a and vRSVG/NS1-15%NS2a, expressing nonauthentic NS1, were significantly less protective.

Analysis of seroresponse to NS1 by immunoprecipitation and ELISA. Sera obtained from vaccinated animals 1 day before DEN4 challenge were analyzed to determine their levels of NS1-specific antibodies. Initially, these sera were used to immunoprecipitate NS1 from a [^{35}S]methionine-labeled lysate of DEN4-infected cells. Analysis of representative sera from animals immunized with vNS1-NS2a are shown in Fig. 2; results for sera from animals immunized with other recombinants are not shown (but see reference 2). Sera from animals immunized with vC-M-E-NS1-NS2a or vNS1-NS2a had similar levels of NS1-specific antibodies, as measured by the similar intensities of labeled NS1 precipitated by these sera. Antibodies specific to other DEN4 proteins were not detected in any sera (also see reference 2). The one animal vaccinated with vC-M-E-NS1-NS2a that succumbed to dengue encephalitis had a low level of anti-NS1 antibodies, although some other mice in this group that

survived challenge also had low levels of NS1-specific antibodies. In contrast, antibodies to NS1 were not detected in most sera from mice immunized with either vC-M-E-NS1-15%NS2a or vNS1-15%NS2a. The few positive sera from these mice had barely detectable levels of NS1-specific antibodies. Immunoprecipitation also failed to detect NS1-specific antibodies in sera from mice immunized with either vRSVG71/NS1-15%NS2a or negative control viruses. Several serum samples collected from mice that had survived for 21 days postchallenge were also analyzed. Greatly increased levels of NS1-specific antibodies were present in these sera (Fig. 2), as expected for animals that had been inoculated with challenge virus.

Additional analysis of the seroresponse to NS1 before challenge was performed by using ELISA. Forty-eight individual sera were assayed, eight each from groups of mice that had been immunized with vPE11, vC-M-E-NS1-NS2a, vC-M-E-NS1-15%NS2a, vNS1-NS2a, vNS1-15%NS2a, or vRSVG/NS1-15%NS2a. Serial dilutions of sera were incubated with DEN4-infected-cell lysate in the solid phase, and peroxidase-conjugated goat anti-mouse immunoglobulin G was used as the second antibody. Sera from animals immunized with vC-M-E-NS1-NS2a or vNS1-NS2a had the highest mean titers, a finding which was in agreement with the immunoprecipitation results. It was shown previously that animals immunized with vC-M-E-NS1-NS2a developed a low or undetectable level of virion-specific antibodies as measured by ELISA (2). Furthermore, the group average titer of sera from mice immunized with vC-M-E-NS1-15%NS2a was less than 5% of the average for the vC-M-E-NS1-NS2a-immunized group. Also, the mean ELISA titer of sera from the vC-M-E-NS1-15%NS2a group was not higher than the mean titer of sera from the vNS1-15%NS2a group (Fig. 3). Taken together, these results suggest that the antibodies detected by ELISA are specific for NS1 and/or NS2a, not for structural proteins. Since these sera immunoprecipitated NSA1 but not NS2a, it is likely that the antibodies detected by ELISA were directed primarily against NS1. The mean ELISA titers of sera from animals immunized with vC-M-E-NS1-15%NS2a, vNS1-15%NS2a, or vRSVG/NS1-15%NS2a were considerably lower than the mean titers of sera from the vNS1-NS2a and vC-M-E-NS1-NS2a groups, in agreement with the results of the immunoprecipitation experiments. Thus, only recombinants expressing authentic NS1 elicited a high-titer antibody response to NS1 in serum.

Functional characterization of sera. Current evidence suggests that protective immunity induced by NS1 is mediated by antibodies which sensitize infected cells for complement-mediated lysis (27). Pooled sera from groups of immunized animals were analyzed for the ability to lyse ^{51}Cr-labeled DEN4-infected target cells in the presence of complement (Table 2). Sera from animals immunized with vC-M-E-NS1-NS2a or vNS1-NS2a had the highest titers by this assay, 1:160 and 1:320, respectively. In contrast, pool sera from vC-M-E-immunized mice had a titer of less than 1:10, suggesting that the antibodies detected by this assay are not directed against structural proteins. Sera from animals immunized with vNS1-15%NS2a or vRSVG/NS1-15%NS2a had low titers, approximately 1:40. Thus, the cytolytic antibody titer of sera from immunized animals correlated with both the level of antibody detected by immunoprecipitation and ELISA and the degree of resistance to dengue virus challenge. This supports the view that NS1-induced protection is mediated by complement-dependent cytolytic antibodies.

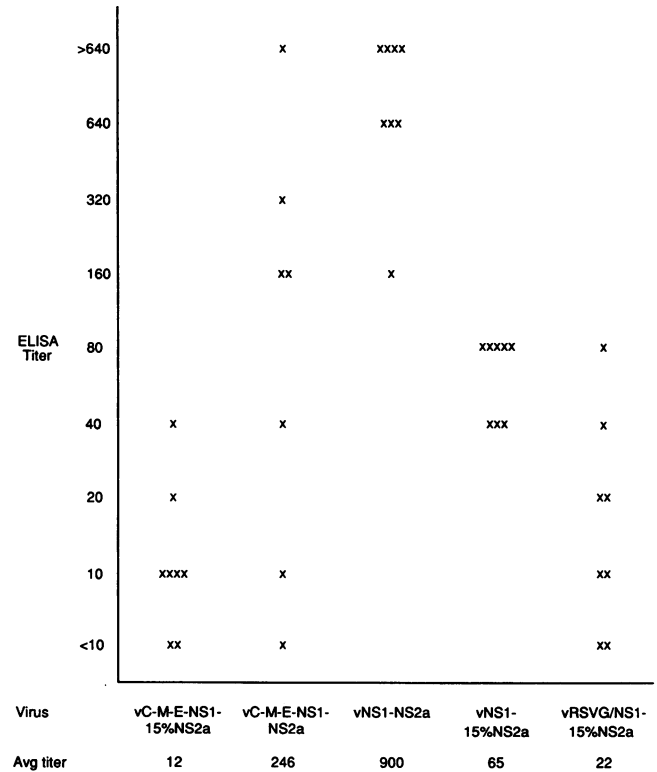| ELISA Titer	vC-M-E-NS1-15%NS2a	vC-M-E-NS1-NS2a	vNS1-NS2a	vNS1-15%NS2a	vRSVG/NS1-15%NS2a
>640		x	xxxx		
640			xxx		
320		x			
160		xx	x		
80				xxxxx	x
40	x	x		xxx	x
20	x				xx
10	xxxx	x			xx
<10	xx	x			xx
Avg titer	12	246	900	65	22

FIG. 3. ELISA titers of mouse sera. Sera were collected 1 day before challenge from mice immunized with the recombinant viruses shown and also from mice immunized with vPE11 to serve as the negative control. Eight serum samples from each group were assayed by ELISA by using DEN4-infected cell lysate as the antigen. The ELISA titer is the reciprocal of the highest dilution of a sample which was positive. Samples which were negative at a 1:10 dilution are shown as having a titer of <10 and were assumed to contain no antibodies to NS1. Samples with very high A_{590} values at a 1:640 dilution are shown as having a titer of >640; these samples were assigned a titer of 1,280 for calculation of the geometric mean titer.

To demonstrate that antibodies to NS1 in serum were responsible for protection against dengue encephalitis, we performed passive transfer experiments. Unimmunized recipient mice were inoculated with pooled postimmunization sera from donor mice that had been immunized with vNS1-NS2a or vSC8. The recipients were challenged with DEN4 1 day later and observed for 21 days for symptoms of dengue encephalitis. Mice inoculated with DEN4 hyperimmune mouse ascitic fluid or sera from vNS1-NS2a-immunized animals exhibited significant resistance to challenge compared with mice inoculated with phosphate-buffered saline or sera from mice immunized with vSC8 (Table 2). These observations indicate that humoral immunity played a role in the resistance induced by immunization with NS1. In mice immunized with vNS1-NS2a, it is likely that antibodies against NS1 were responsible for the protection observed.

Protection of mice against DEN2. Two recombinant viruses that contain DEN2 sequences, vDEN2(2041-4497) and vDEN2(2304-4497) (Fig. 4a), were constructed for expression of authentic DEN2 NS1 and for evaluation of their protective efficacies in mice. Both viruses encode all of NS1 and NS2a and the N-terminal 84% of NS2b, but they differ in the extent of E sequences present. vDEN2(2041-4497) is expected to produce a polyprotein containing 95 amino acid

TABLE 2. Functional characterization of sera
from immunized mice

Inoculum for passive transfer	Cytolytic antibody titer (reciprocal)[a]	Passive transfer: no. of survivors/total
Sera from animals immunized with:		
vSC8	Negative control	2/17
vC-M-E-NS1-NS2a	160	ND
vNS1-NS2a	320	12/18[b]
vNS1-15%NS2a	40[c]	ND
vRSVG/NS1-15%NS2a	50[d]	ND
vC-M-E	<10	ND
HMAF[e]	ND	4/4
PBS[f]	ND	1/8

[a] The inverse of the last serum dilution which was positive in the complement-mediated cytolysis assay is shown. ND, Not determined. <10, The 1:10 dilution (the least dilute sample tested) was negative.

[b] $P < 0.005$, comparing vNS1-NS2a with vSC8 by standard chi-square analysis.

[c] The average of separate titrations of serum pools from mice which survived challenge and from mice that died, each of which had a titer of 40, is shown.

[d] The average of separate titrations of serum pools from mice which survived challenge (titer, 20) and from mice that died (titer, 80) is shown.

[e] DEN4 HMAF was used as a positive control for the passive transfer experiment.

[f] PBS, Phosphate-buffered saline, was used as a negative control for the passive transfer experiment.

residues of E, including the 39 C-terminal residues, which are composed of two blocks of hydrophobic amino acids (14 and 24 residues long) separated by a single arginine. These hydrophobic blocks are thought to act as a stop transfer signal for E and as a signal sequence for NS1. The predicted polyprotein of vDEN2(2304-4497) contains only the 39 C-terminal amino acids of E. The DEN2-specific proteins produced in cells infected by these two recombinants are shown in Fig. 4b. Each of these recombinants directed expression of a protein that comigrated with authentic DEN2 NS1. The level of NS1 expression in vDEN2(2304-4497)-infected cells was higher than that found in vDEN2(2041-4497)-infected cells. These NS1 products were further characterized by immunoprecipitation with MAbs specific for DEN2 NS1. Eleven MAbs were tested (16; see Materials and Methods), and the results are shown for only one of them, 27-12/4. Nine of the other MAbs also immunoprecipitated recombinant DEN2 NS1, albeit most of them did so less efficiently than 27-12/4. Only 47-10/10 failed to precipitate recombinant NS1, and this MAb was very inefficient at precipitating authentic DEN2 PR159 NS1. These results indicate that antigenic epitopes of DEN2 NS1 defined by these MAbs are present in the recombinant NS1 product.

The two recombinants expressing apparently authentic DEN2 NS1 were tested for the ability to protect mice against DEN2 challenge. Two separate experiments were conducted, and the results were combined. All 20 mice in the negative control group immunized with vSC8 developed encephalitis and died after DEN2 challenge. In contrast, 50% (12 of 24) of the mice immunized with vDEN2(2041-4497) or 29% (4 of 14) of the mice immunized with vDEN2(2304-4497) survived challenge after recovery from symptoms of encephalitis. These protection rates were each significantly higher than that of the negative control group [$P < 0.005$ for vDEN2(2041-4497), $P < 0.025$ for vDEN2(2304-4497)], but they were not significantly different from each other. In addition, these survival rates were less than the
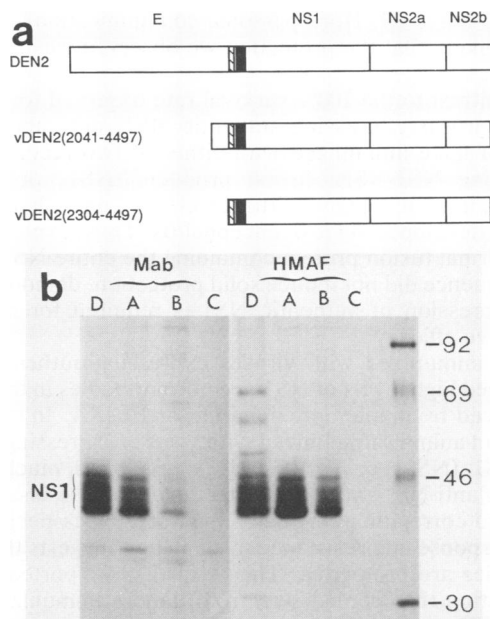

FIG. 4. (a) Polyproteins encoded by recombinants containing DEN2 cDNA. The top box shows the region of the DEN2 polyprotein from the N terminus of E to the C terminus of NS2b. The striped region is thought to act as a stop-transfer signal, while the black region is thought to act as a signal sequence for NS1. The regions of the polyprotein encoded by the two recombinant viruses are also shown. The thin horizontal lines at the C terminus represent six vector-encoded amino acid residues. (b) Proteins expressed by DEN2 recombinants. ^{35}S-labeled lysates of CV-1 cells infected with vSC8 (lanes C), vDEN2(2041-4497) (lanes B), or vDEN2(2304-4497) (lanes A) were immune precipitated with DEN2 HMAF or a DEN2 NS1 MAb, and the precipitates were resolved by SDS-gel electrophoresis and fluorography. A ^{35}S-labeled lysate of DEN2-infected cells was also precipitated as a control (lanes D). The position of NS1 is indicated. Molecular size markers (in kilodaltons) are shown at the far right.

88% survival that was reported when purified DEN2 NS1 protein was used as the immunogen (28).

DISCUSSION

We used recombinant vaccinia viruses that express DEN4 NS1 to protect mice against lethal dengue encephalitis. Viruses that express NS1 in combination with structural genes were fully protective. This was expected, since it has been shown previously (1, 2) that a virus expressing only E was fully protective. All of the mice immunized with a virus expressing authentic NS1 (vNS1-NS2a) survived challenge, although morbidity was seen in many of these animals. This virus encodes the C-terminal 24 hydrophobic residues of E and all of NS1 and NS2a. Since vRSVG/NS1-15%NS2a, which lacks the E sequences, and vNS1-15%NS2a, which contains the E sequences, induced the same level of resistance, it is likely that the C-terminal 24 hydrophobic amino acids of E present in vNS1-15%NS2a (which serves as a signal sequence for NS1) did not directly contribute to the protection observed. We cannot completely rule out the possibility that NS2a contributes to protection. However, purified NS1 protein (28) and MAbs to NS1 (15) have been shown to be protective. Furthermore, another member of our laboratory has recently demonstrated that a vaccinia virus recombinant expressing only NS1 is just as protective

as vNS1-NS2a (H. Hori, personal communication). Thus, it is very likely that the protection we observed was mediated by NS1.

In contrast to the 100% survival rate observed for recombinant viruses expressing authentic NS1, only about two-thirds of mice immunized with either of two recombinants expressing NS1-NS2a fusion products (NS1$_x$ or RSVG/NS1$_x$) survived dengue virus challenge, and all of these animals developed signs of encephalitis. Thus, expression of an abnormal fusion protein containing the entire NS1 amino acid sequence did not induce solid protection, demonstrating that expression of authentic NS1 is required for maximal protective efficacy.

Mice immunized with viruses expressing authentic NS1 developed high levels of NS1-specific antibodies in their sera as assayed by immunoprecipitation or ELISA. In contrast, sera from animals immunized with viruses expressing abnormal NS1 (NS1$_x$ or RSVG-NS1$_x$) developed much lower levels of anti-NS1 antibodies detectable by these assays. The observed correlation between a high-titer NS1-specific antibody response and resistance to challenge suggests that NS1 antibodies are protective. This is further supported by the observation that pooled sera from donors immunized with vNS1-NS2a were able to protect naive recipients against DEN4 challenge. By what mechanism do antibodies to NS1 protect mice from dengue virus challenge? Since NS1 is not present in the virion, virus neutralization mediated by NS1-specific antibodies is probably not responsible for resistance. It was suggested previously (27) that antibodies to NS1 protect mice by the mechanism of complement-mediated lysis of infected cells. This suggestion is based on three lines of evidence. (i) NS1 is detectable at the surface of infected cells (5, 26, 30), (ii) antibodies to NS1 can fix complement and promote lysis of infected cells in vitro in the presence of complement (26, 27), and (iii) mice were protected against dengue encephalitis by prior inoculation of a mixture of two NS1-specific MAbs, both of which had high complement fixation titers (15). We observed that, consistent with this model, the mean complement-mediated cytolytic titer of sera from groups of mice immunized with vaccinia virus recombinants expressing various forms of NS1 correlated with the protective efficacy of those viruses. However, other protective mechanisms may operate as well, since some animals immunized with vRSVG/NS1-15%NS2a or vNS1-15%NS2a that survived challenge had lower prechallenge titers of anti-NS1 antibody by ELISA than did others that succumbed to challenge, and the complement-mediated cytolytic titers of prechallenge sera of survivors immunized with vRSVG/NS1-15%NS2a or vNS1-15%NS2a were not higher than the titers of nonsurvivors. A possible explanation of these observations is that antibody to NS1 may also be able to protect mice by the mechanism of antibody-dependent cytotoxic cell killing. It is also possible that immunization with NS1 or viruses expressing NS1 induces a cytotoxic T-lymphocyte response targeted against NS1, which could explain how some animals with a low titer of NS1-specific antibodies survive challenge. It is interesting that a recent study using an in vitro stimulation assay has identified at least one T-cell epitope on purified DEN2 NS1 that is recognized by BALB/c mice (22); however, the effector functions of T cells that recognize DEN2 NS1 were not characterized. However, a cytotoxic T-lymphocyte response targeted to NS1 was not identified during a study of human T-cell responses to dengue virus (3, 19).

It was also possible to protect mice against DEN2 challenge by immunization with vaccinia virus recombinants

expressing authentic DEN2 NS1. In this case, 30 to 50% of the immunized animals survived challenge, which represented significant resistance, since none of the control mice survived. The protective efficacy observed was lower than that seen following immunization with purified DEN2 NS1 (28), in which 88% of the immunized mice survived challenge versus 59% of the negative controls. The difference in survival rate between these two experiments may be due to the use of different mouse strains. Nonetheless, in the present study, in which the same strategy of vaccination with recombinant vaccinia viruses expressing authentic NS1 and the same strain of mice were used, greater resistance to DEN4 than to DEN2 was induced. A possible explanation is that DEN2 virus challenge is more virulent than DEN4 virus challenge. Others have noted previously with yellow fever virus that the degree of protection afforded by immunization with NS1 is inversely related to the virulence of the challenge virus strain (4, 8). Those researchers suggested that the more virulent strains overcome the immune response.

The present results showing protection of mice against dengue virus infection in the absence of potentially infection-enhancing antivirion antibodies suggest the advisability of further testing of these vaccinia virus recombinants in an experimental animal model whose response to infection more closely resembles the course of dengue virus infection in humans. Like humans, but unlike mice, rhesus monkeys develop viremia when dengue virus is inoculated parenterally. Unfortunately, infected monkeys do not develop illness. Nonetheless, the response of monkeys to peripheral inoculation of dengue virus more closely resembles the response of humans. In a recent preliminary experiment, we immunized three rhesus monkeys with vC-M-E-NS1-NS2a and then challenged them with dengue virus (20). Although these animals developed NS1-specific antibodies after immunization, the levels were low and the duration and peak titers of dengue viremia in these monkeys were similar to those of unimmunized controls. It is possible that the level of dengue virus proteins expressed by vC-M-E-NS1-NS2a was insufficient to induce a protective response in monkeys. Perhaps a second generation of recombinant vaccinia viruses which express larger amounts of protective antigens would result in improved immunogenicity and protective efficacy in monkeys.

LITERATURE CITED

1. **Bray, M., B. Falgout, B. Zhao, L. Markoff, B. Moss, R. M. Chanock, and C.-J. Lai.** 1989. Mice immunized with recombinant vaccinia virus expressing dengue-4 structural proteins and/or nonstructural protein NS1 are protected against fatal dengue encephalitis, p. 357–362. *In* R. A. Lerner, H. Ginsberg, R. M. Chanock, and F. Brown (ed.), Vaccines 89: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Bray, M., B. Zhao, L. Markoff, K. H. Eckels, R. M. Chanock, and C.-J. Lai.** 1989. Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without nonstructural protein NS1 are protected against fatal dengue encephalitis. J. Virol. **63:**2853–2856.

3. Bukowski, J. F., I. Kurane, C.-J. Lai, M. Bray, B. Falgout, and F. A. Ennis. 1989. Dengue virus-specific cross-reactive CD8+ human cytotoxic T lymphocytes. J. Virol. 63:5086–5091.

4. Cane, P. A., and E. A. Gould. 1988. Reduction of yellow fever virus neurovirulence by immunization with a bacterially synthesized nonstructural protein (NS1) fragment. J. Gen. Virol. 69:1241–1246.

5. Cardiff, R. D., and J. K. Lund. 1976. Distribution of dengue-2 antigens by electron immunocytochemistry. Infect. Immun. 13:1699–1709.

6. Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vector: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. 5:3403–3409.

7. Falgout, B., R. Chanock, and C.-J. Lai. 1989. Proper processing of dengue virus nonstructural protein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J. Virol. 63:1852–1860.

8. Gould, E. A., A. Buckley, A. D. T. Barrett, and N. Cammack. 1986. Neutralizing (54K) and non-neutralizing (54K and 48K) monoclonal antibodies against structural and nonstructural yellow fever virus proteins confer immunity in mice. J. Gen. Virol. 67:591–595.

9. Hahn, Y. S., R. Galler, T. Hunkapiller, J. M. Dalrymple, J. H. Strauss, and E. G. Strauss. 1988. Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. Virology 162:167–180.

10. Halstead, S. B. 1988. Pathogenesis of dengue: challenges to molecular biology. Science 239:476–481.

11. Halstead, S. B., and E. J. O'Rourke. 1977. Antibody-enhanced dengue virus infection in primate leukocytes. Nature (London) 265:739–741.

12. Halstead, S. B., and E. J. O'Rourke. 1977. Dengue viruses and mononuclear phagocytes I: infection enhancement and non-neutralizing antibody. J. Exp. Med. 146:201–217.

13. Halstead, S. B., E. J. O'Rourke, and A. C. Allison. 1977. Dengue viruses and mononuclear phagocytes II: identity of blood and tissue leukocytes supporting in vitro infection. J. Exp. Med. 146:218–229.

14. Henchal, E. A., M. K. Gentry, J. M. McCown, and W. E. Brandt. 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. 31:830–836.

15. Henchal, E. A., L. S. Henchal, and J. J. Schlesinger. 1988. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J. Gen. Virol. 69:2101–2107.

16. Henchal, E. A., L. S. Henchal, and B. K. Thaisomboonsuk. 1987. Topological mapping of unique epitopes on the dengue-2 virus NS1 protein using monoclonal antibodies. J. Gen. Virol. 68:845–851.

17. Joklik, W. K. 1962. The preparation and characterization of highly purified radioactively labelled poxvirus. Biochim. Biophys. Acta 61:290–301.

18. Kaufman, B. M., P. L. Summers, D. R. Dubois, and K. H. Eckels. 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am. J. Trop. Hyg. 36:427–434.

19. Kurane, I., R. Russell, A. L. Rothman, J. F. Bukowski, F. A. Ennis, C.-J. Lai, M. Bray, B. Falgout, B. Zhao, Y.-M. Zhang, and M. Brinton. 1990. Human T lymphocyte responses to dengue viruses: recognition of dengue virus proteins by T lymphocytes, p. 131–134. In F. Brown, R. M. Chanock, H.

Ginsberg, and R. A. Lerner (ed.), Vaccines 90: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

20. Lai, C.-J., Y.-M. Zhang, M. Bray, R. M. Chanock, D. R. Dubois, and K. H. Eckels. 1990. Immunization of monkeys with baculovirus recombinant-expressed dengue envelope and NS1 glycoproteins induces partial resistance to challenge with homotypic dengue virus, p. 119–124. In F. Brown, R. M. Chanock, H. Ginsberg, and R. A. Lerner (ed.), Vaccines 90: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

21. Monath, T. P. 1985. Flaviviruses, p. 955–1004. In B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. Roizman, and R. E. Shope (ed.), Virology. Raven Press, New York.

22. Rothman, A. L., I. Kurane, F. A. Ennis, C.-J. Lai, Y.-M. Zhang, M. Bray, B. Falgout, B. Zhao, and J. J. Schlesinger. 1990. Lymphocyte proliferative responses to dengue virus E and NS1 proteins in BALB/c mice, p. 135–138. In F. Brown, R. M. Chanock, H. Ginsberg, and R. A. Lerner (ed.), Vaccines 90: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

23. Russell, P. K. 1970. Pathogenesis of the dengue shock syndrome: evidence for an immunologic mechanism, p. 426–435. In P. A. Meischer (ed.), Immunology, 6th International Symposium. Schwabe & Co., Basel.

24. Sabin, A. B. 1952. Research on dengue fever during World War II. Am. J. Trop. Med. Hyg. 1:30–50.

25. Satake, M., J. E. Coligan, N. Elango, E. Norrby, and S. Venkatesan. 1985. Respiratory syncytial virus envelope glycoprotein G has a novel structure. Nucleic Acids Res. 13:7795–7812.

26. Schlesinger, J. J., M. W. Brandriss, J. R. Putnak, and E. E. Walsh. 1990. Cell surface expression of yellow fever virus non-structural glycoprotein NS1: consequences of interaction with antibody. J. Gen. Virol. 71:593–599.

27. Schlesinger, J. J., M. W. Brandriss, and E. E. Walsh. 1985. Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and by active immunization with gp48. J. Immunol. 135:2805–2809.

28. Schlesinger, J. J., M. W. Brandriss, and E. E. Walsh. 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus nonstructural glycoprotein NS1. J. Gen. Virol. 68:853–857.

29. Scott, R. M., J. M. McCown, and P. K. Russell. 1972. Human immunoglobulin specificity after group B arbovirus infections. Infect. Immun. 6:277–281.

30. Smith, G. W., and P. J. Wright. 1986. Synthesis of proteins and glycoproteins in dengue type 2 virus-infected Vero and Aedes albopictus cells. J. Gen. Virol. 66:559–571.

31. Vijaya, S., N. Elango, F. Zavala, and B. Moss. 1988. Transport to the cell surface of a peptide sequence attached to the truncated C terminus of an N-terminally anchored integral membrane protein. Mol. Cell. Biol. 8:1709–1714.

32. Wertz, G. W., P. L. Collins, Y. Huang, C. Gruber, S. Levine, and L. A. Ball. 1985. Nucleotide sequence of the human respiratory syncytial virus reveals an unusual type of viral membrane protein. Proc. Natl. Acad. Sci. USA 82:4075–4079.

33. Zhao, B., G. Prince, R. Horswood, K. Eckels, P. Summers, R. Chanock, and C.-J. Lai. 1987. Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus. J. Virol. 61:4019–4022.