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Immunization of Mice with Dengue Structural Proteins and Nonstructural Protein NS1 Expressed by Baculovirus Recombinant Induces Resistance to Dengue Virus Encephalitis

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We have constructed a recombinant baculovirus containing a 4.0-kilobase dengue virus cDNA sequence that codes for the three virus structural proteins, capsid (C) protein, premembrane (PreM) protein, and envelope glycoprotein (E), and nonstructural proteins NS1 and NS2a. Infection of cultured *Spodoptera frugiperda* cells with this recombinant virus resulted in the production of E and NS1 proteins that were similar in size to the corresponding viral proteins expressed in dengue virus-infected simian cells. Other dengue virus-encoded proteins such as PreM and C were also synthesized. Rabbits immunized with the dengue virus protein products of the recombinant virus developed antibodies to PreM, E, and NS1, although the titers were low, especially to PreM and E. Nevertheless, the dengue virus antigens produced by the recombinant virus induced resistance in mice to fatal dengue encephalitis.

Dengue viruses continue to cause major epidemics throughout the tropical and subtropical regions of the world. Despite many years of research effort, vaccine development still remains at the experimental stage (1, 3). The predominant disease associated with dengue virus infection is a debilitating illness known as dengue fever. Less frequently, dengue virus causes a hemorrhagic shock syndrome in young children, with a mortality rate as high as 2 to 10% (5, 7, 12). Thus, control of dengue fever and dengue hemorrhagic shock is a major global concern. Recently, the World Health Organization designated the dengue viruses one of five high-priority targets for accelerated vaccine development.

We recently cloned DNA fragments that spanned the entire length of the dengue virus type 4 RNA genome. Analysis of the complete sequence revealed that the entire genome is 10,644 nucleotides in length and codes for a single polyprotein of 3,386 amino acids (9, 19). This polyprotein is cleaved by proteolytic processing to generate individual viral proteins. The three structural proteins, C, M, and E, are located at the amino terminus, while nonstructural proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 are at the carboxy terminus of the polyprotein (9, 16, 17, 19). Intracellular virions lack the M protein, but instead contain a precursor glycoprotein, designated PreM, that is not found in mature virions (13). We initially attempted to express the three structural proteins, capsid (C), membrane (M) (or its precursor PreM), and envelope glycoprotein (E), as well as the nonstructural protein NS1 from a single 5′-terminal fragment of cloned dengue virus DNA with vaccinia virus as a vector (2, 20). In this study we observed that the three dengue virus glycoproteins, PreM, E, and NS1, were produced in recombinant vaccinia virus-infected cells and their glycosylation pattern was similar to that observed during dengue virus infection. Infection of cotton rats with the vaccinia virus recombinant induced a poor immune response to the NS1 glycoprotein, while antibodies for the other two glycoproteins were not detected at all. To achieve expression of the dengue virus structural proteins, as well as NS1, at a higher level than occurred with the vaccinia virus-dengue virus recombinant, we turned to the high-yielding baculovirus-insect cell system recently developed by M. Summers and colleagues (14, 15).

The 4.0-kilobase (kb) *Bgl*II dengue virus DNA fragment (nucleotides 88 to 4128), which encodes the three virus structural proteins and nonstructural proteins NS1 and NS2a in one open reading frame, was inserted into the unique *Bam*HI site of the baculovirus cloning vector pAC373, kindly provided by M. Summers (18). The dengue virus sequence in this construct was placed under transcriptional control of the strong baculovirus polyhedrin promoter. Also, dengue virus RNA transcripts should contain the authentic dengue virus initiation codon and produce a polyprotein that included the C, PreM (M), E, NS1, and NS2a sequence (Fig. 1). Recombinant DNA (2 μg) and wild-type baculovirus genomic DNA (10 μg) were cotransfected into *Spodoptera frugiperda* Sf9 cells to generate recombinant baculovirus through homologous recombination. Recombinant baculovirus was enriched from the progeny virus population by dot hybridization with a labeled dengue virus cDNA probe in conjunction with serial dilution passage and plaque purification in Sf9 cells. Viral plaques that lacked discernible polyhedrin protein were isolated by further plaque isolation on Sf9.

In order to determine whether the 4.0-kb dengue virus DNA sequence was stably integrated in the baculovirus genome, viral DNA was extracted from the recombinant virus and then digested with *Xho*I and *Sst*I or with *Xho*I and

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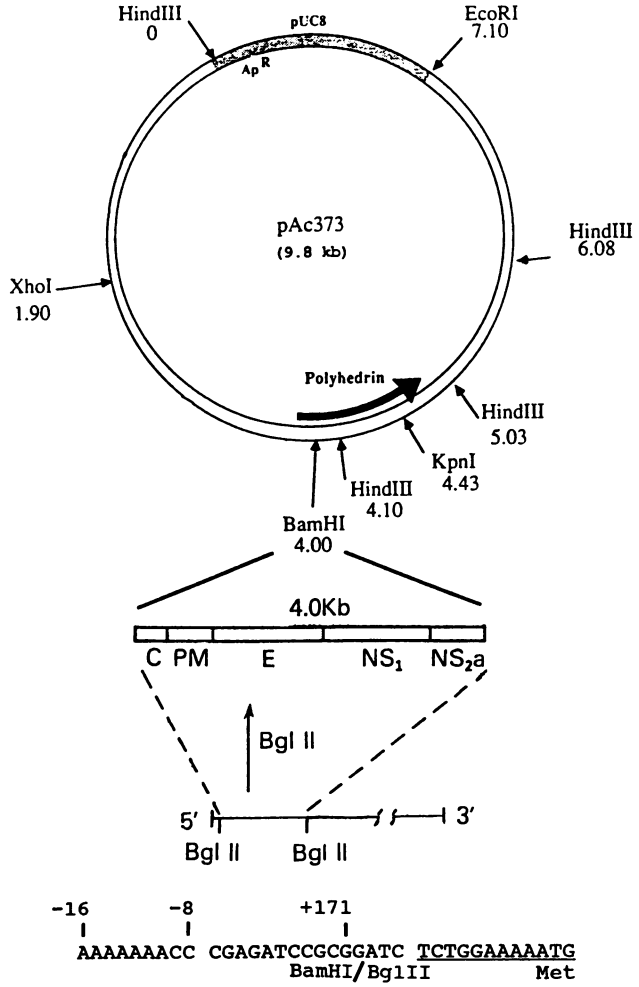

FIG. 1. Construction of recombinant dengue virus DNA in baculovirus intermediate vector. Vector pAC373 DNA, provided by M. Summers, contains a 10-nucleotide polylinker including a unique *Bam*HI site at −8 and +171 of the polyhedrin structural gene. Plasmid components (pUC8 and Ap^r) and several other marker restriction enzyme cleavage sites are shown. The *Bgl*II-cleaved dengue virus DNA fragment (nucleotides 88 to 4128) that codes for the viral structural proteins and nonstructural proteins NS1 and NS2a in one open reading frame was inserted into the *Bam*HI site of pAC373. Recombinant DNA containing the insert in the sense orientation was isolated for construction of recombinant baculovirus. The 5′ noncoding sequence depicting the juncture between the polyhedrin gene and the first dengue virus gene (underlined) is shown.

*Kpn*I. Recombinant vector DNA, which served as a control, was also digested in the same manner. The digests were separated on an agarose gel and blotted on nitrocellulose paper for probing with a ^{32}P-labeled dengue virus cDNA sequence (dengue virus nucleotides 1303 to 1326). This analysis revealed that the predicted 4.0-kb DNA fragment of the *Xho*I-*Sst*I digest and the 6.6-kb DNA fragment of the *Xho*I-*Kpn*I digest were present in the recombinant baculovirus DNA and were similar in size to the corresponding fragments present in the intermediate recombinant vector (data not shown). This indicated that the entire 4.0-kb dengue virus DNA fragment was integrated into the desired location of the baculovirus DNA genome. Also, the recombinant virus appeared to be stable.

Dengue virus-specific antibodies were used in an indirect immunofluoresence assay to detect the synthesis of dengue viral proteins in recombinant baculovirus-infected Sf9 cells. Three monoclonal antibodies, 2H2, specific for PreM; 1H10, specific for dengue virus type 4 (E); and 1G6, specific for the NS1 nonstructural protein, kindly provided by M. K. Gentry, were used to detect expression of dengue viral protein. Recombinant virus-infected cells contained antigens which were stained with each of these antibodies, whereas uninfected cells or cells infected with wild-type baculovirus were negative. This indicates that the dengue virus DNA sequence was transcribed and the resulting mRNA was translated properly to produce dengue virus proteins. The PreM, E, and NS1 proteins appeared to accumulate within infected cells but were not expressed on their surface, as indicated by failure to detect staining of the outer membrane of live infected cells (data not shown).

Western blot (immunoblot) analysis was used to identify individual dengue viral proteins in order to determine whether the polyprotein was proteolytically cleaved and properly glycosylated in Sf9 cells (Fig. 2A). When lysates from recombinant virus-infected, wild-type baculovirus-infected, and uninfected Sf9 cells were analyzed with dengue virus E-specific antibodies and ^{125}I-labeled protein A, a labeled band was observed which had a mobility similar to that of E produced during dengue virus infection. The recombinant baculovirus also appeared to produce authentic NS1, as indicated by radioimmunoprecipitation of an infected cell lysate with hyperimmune antiserum or NS1-specific monoclonal antibodies (Fig. 2B). PreM was not clearly detected by these analyses. Thus, the expression and processing of dengue virus E and NS1 in insect cells appears to be similar to that observed during dengue virus infection of primate cells.

The level of dengue virus proteins expressed in recombinant baculovirus-infected cells was compared with the dengue virus proteins produced in dengue virus-infected primate cells by Western blotting with polyvalent sera (hyperimmune mouse ascitic fluid) or monoclonal antibody 1H10, specific for E, or monoclonal antibody 1G6, specific for NS1. Western blot analyses showed that the amount of E or NS1 produced in insect cells was approximately 25% of that produced in dengue virus-infected primate cells at a multiplicity of infection of 1. Recently, several in-phase vectors that contain the entire noncoding sequence of the polyhedrin gene plus a variable length of the polyhedrin N-terminal sequence have been developed (8). These vectors express foreign genes as fusion proteins with unusually high efficiency (K. L. Coelingh, personal communication). The use of these new vectors may allow us to achieve even higher levels of dengue virus gene expression.

The experimental mouse model of dengue disease was used to determine whether dengue viral proteins produced by the baculovirus recombinant could induce resistance to the development of central nervous system symptoms and death caused by this virus. Mice were immunized intramuscularly with a cell lysate (0.3×10^6 cells) with or without Freund adjuvant. The same material was inoculated again after 3 days and again at 2 weeks. The immunized animals were bled at 3 weeks and were challenged the next day intracerebrally with 10^2 50% lethal doses (LD_{50}) of a homotypic dengue type 4 virus (strain H241). Following challenge, animals were observed for 3 weeks for signs of central nervous system disease and death. Most mice immunized with the lysate of recombinant baculovirus-infected cells were protected against illness and death (Table 1). On the

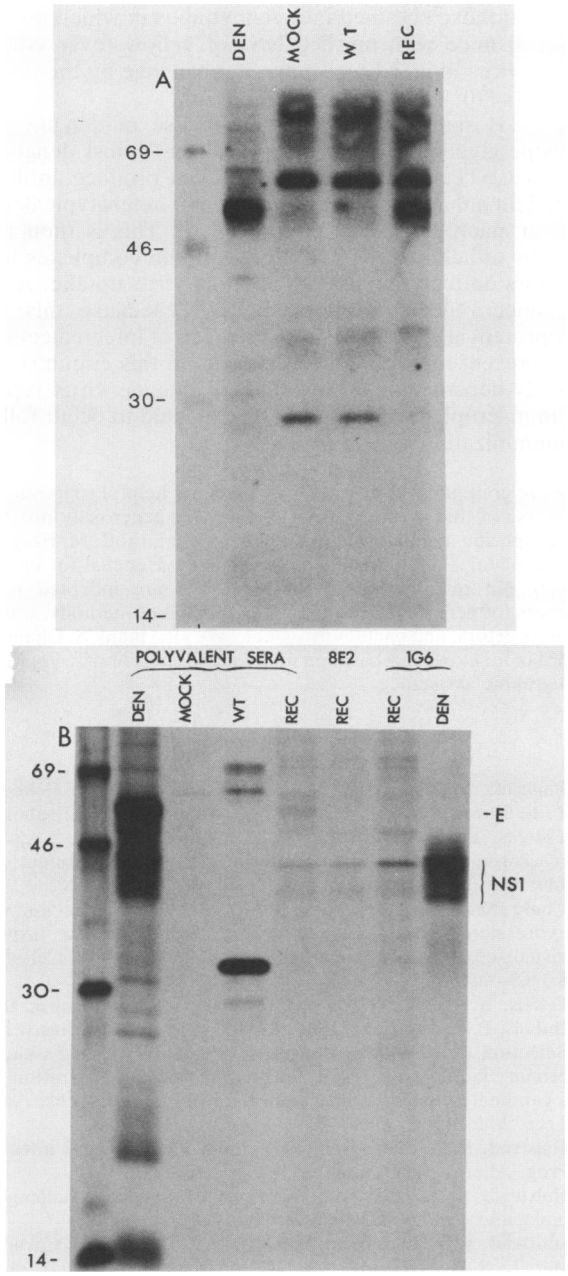

FIG. 2. Analysis of dengue virus envelope glycoprotein E and nonstructural protein NS1 produced by baculovirus recombinant. (A) *S. frugiperda* Sf9 cells were grown in Grace insect cell medium supplemented with 0.3% lactalbumin hydrolysate, 0.3% yeastolate, and 10% fetal calf serum. Confluent Sf9 cells were infected with 10 PFU of recombinant (REC) or wild-type (WT) baculovirus per cell. Three days after infection, 10^6 Sf9 cells were lysed in 0.5 ml of RIPA buffer (0.01 M Tris, pH 7.5, 1% deoxycholate, 1% Triton, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). Control lysate was similarly prepared from dengue (DEN) type 4 virus-infected $LLCMK_2$ cells. Cell lysates (60 μl) were separated on a 12% SDS–polyacrylamide gel (acrylamide-bisacrylamide, 60:1.6) for 16 h at 80 V in buffer containing 25 mM Tris, pH 8.0, 0.2 M glycine, and 0.1% SDS. Protein bands in the polyacrylamide gel were transferred electrophoretically onto nitrocellulose paper. The nitrocellulose blot was then reacted with a 1:200 dilution of dengue virus E-specific antiserum raised in rabbits against a synthetic E oligopeptide (amino acid numbers 260 to 273). Radioactive ^{125}I-labeled protein A was then used for detection of the dengue virus E glycoprotein. (B) Metabolic labeling of wild-type virus-, recombinant baculovirus-infected, and uninfected Sf9 cells with [^{35}S]methionine (100 μCi/ml, specific activity, 1,100 Ci/mmol) was carried out in methionine-free medium for 2 h. The labeled lysates were then prepared in RIPA buffer for use in immunoprecipitation with dengue virus-specific mouse hyperimmune polyvalent antiserum or NS1-specific monoclonal antibodies (1G6 and 8E2), kindly provided by M. K. Gentry. Protein size markers (in kilodaltons) are shown on the left. Note: monoclonal antibodies did not immunoprecipitate a 40- to 42-kb protein from uninfected Sf9 cells or wild-type baculovirus-infected cells (data not shown).

TABLE 1. Dengue type 4 virus structural proteins and nonstructural protein NS1 expressed by recombinant baculovirus protect mice against dengue encephalitis

Sf9 cell lysate used for immunization	Adjuvant	Response to challenge with 100 LD_{50} of dengue type 4 virus	
		Mortality rate (no. of dead/ no. tested)	Morbidity[a] rate (no. sick/no. tested)
Uninfected	+	10/10	10/10
	−	10/10	10/10
Baculovirus	+	8/10	10/10
	−	7/10	10/10
Recombinant virus infected[b]	+	1/10	2/10
	−	0/10	1/10
None (unimmunized control)	−	5/5	5/5

[a] Animals showed symptoms of central nervous system disease.
[b] Recombinant dengue virus-baculovirus produced dengue type 4 virus structural proteins and nonstructural protein NS1.

other hand, all animals immunized with a lysate of uninfected cells or wild-type baculovirus virus-infected cells developed signs of neurologic illness following challenge, and most died. This indicates that dengue virus antigens produced by the recombinant baculovirus induced resistance to dengue viral encephalitis. Protection was observed whether mice were immunized with a lysate of baculovirus recombinant-infected cells administered alone or emulsified in Freund adjuvant.

Seroresponse of immunized mice to individual dengue virus proteins was analyzed by radioimmunoprecipitation of [^{35}S]methionine-labeled dengue virus antigens (Fig. 3A). Sera from mice immunized with the recombinant virus-infected cell lysate precipitated dengue virus NS1 with high efficiency; in contrast, serum antibodies specific for E or PreM were not readily detected. The low level or lack of a significant antibody response to E was confirmed by our failure to detect neutralizing antibodies in the serum of immunized mice by the plaque reduction technique.

In order to determine whether the disparity of immune response to different dengue virus antigens is host specific, two rabbits were inoculated with a lysate of baculovirus recombinant-infected cells (10^6) emulsified in complete Freund adjuvant followed by a booster inoculation of the same antigen in incomplete adjuvant 2 weeks later. Control animals received a lysate prepared from wild-type baculovirus-infected cells. Four weeks after the initial immunization, serum samples were tested for the presence of dengue virus-specific antibodies. Immunoprecipitation of radiolabeled dengue virus antigens was performed, and the precipitates were analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Sera from rabbits immunized with the recombinant virus-infected cell lysate specifically precipitated dengue virus E, NS1, and PreM glycoproteins; the

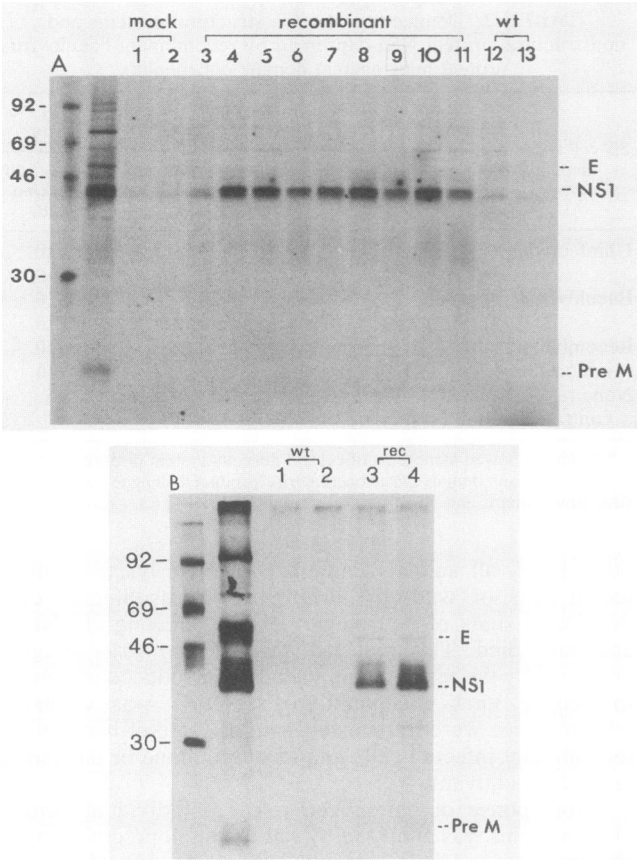

FIG. 3. Immune response to recombinant baculovirus-infected cell lysate analyzed by radioimmunoprecipitation. (A) Three-week-old mice were inoculated with a lysate of recombinant baculovirus-infected Sf9 cells (lanes 3 to 11). Control mice received a lysate of wild-type baculovirus-infected cells (lanes 12 and 13) or a lysate of uninfected cells (lanes 1 and 2). Serum was collected 3 weeks after initial immunization and tested by immunoprecipitation of [^{35}S] methionine-labeled dengue viral proteins. Dengue virus protein markers and standard protein size markers are shown (in kilodaltons). (B) Two rabbits were immunized with a lysate of wild-type baculovirus-infected Sf9 cells (lanes 1 and 2) or a lysate of recombinant baculovirus-infected Sf9 cells (lanes 3 and 4) emulsified in Freund complete adjuvant, followed by a second inoculation of the lysate in Freund incomplete adjuvant 2 weeks later. Serum obtained 4 weeks after primary immunization was tested for dengue virus-specific antibodies by immunoprecipitation of [^{35}S]methionine-labeled dengue viral proteins. Radioimmunoprecipitation of dengue viral proteins by mouse hyperimmune antiserum is shown on the right, while protein size markers (in kilodaltons) are shown on the left.

largest concentration of antibodies appeared to be directed against NS1 (Fig. 3B). The development of antibodies to dengue virus structural proteins was confirmed by an enzyme immunoassay with purified dengue 4 virus; the two rabbits had a titer of 1:80 or 1:160. Other serological assays, such as virus neutralization measured by plaque reduction, hemagglutination-inhibition, and complement fixation, were negative.

The observation that little or no virus-neutralizing activity was detected in mouse sera prior to virus challenge raises the possibility that mechanisms other than viral neutralization were responsible for the observed protection. This suggests that the active protective antigen present in the baculovirus recombinant lysate was NS1. In this regard, our results are similar to those obtained in recent studies in which immunization of mice with purified NS1 of yellow fever virus or dengue virus induced resistance to challenge by the respective virus (10, 11).

There is some concern about the use of dengue virus envelope glycoprotein for immunization against dengue virus disease because antibodies to E can produce antibody-dependent enhancement of replication of heterotypic dengue virus in macrophage cell culture (4, 6). This is thought to occur by attachment of E-immunoglobulin complexes to Fc receptors on macrophages, facilitating virus uptake. A similar concern does not apply to NS1 because this viral glycoprotein is expressed on the surface of infected cells but is not present in or on dengue virions. In this circumstance, antibody-dependent enhancement of dengue virus replication in macrophages would not be expected to occur following immunization with NS1.

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