INTRODUCTION

Currently, epidemics of dengue fever/dengue hemorrhagic fever (DF/DHF) are one of the most important emergent global public health problems in the tropical world, and DF/DHF is considered the most important viral arthropod-borne disease in terms of morbidity and mortality. Approximately two-thirds of the world’s population live in areas infested with disease vectors. The majority of these areas. Dengue is the second most important tropical disease after malaria, with approximately 50–100 million cases of DF/DHF each year.1,2

There is evidence that some structural and nonstructural proteins of dengue virus, such as premembrane (prM) protein, membrane (M) protein, envelope (E) glycoprotein, and nonstructural (NS) proteins NS1 and NS3 may elicit a protective immune response.3,4 Specifically, the 60-kD E protein is considered the most important antigen involved in virus attachment to cells and virus-specific membrane fusion. It also elicits formation of virus-neutralizing antibody, hemagglutination-inhibiting antibody, and virus-enhancing antibody, and is involved in the cell-mediated immune response to dengue virus.5

Presently, prevention of dengue infections is dependent upon control of *Aedes aegypti*. However, with few exceptions, most countries have been unable to control the mosquito and disease transmission. Therefore, the development of safe and effective tetravalent vaccines against all four dengue viruses has been designated as a priority by the World Health Organization.6

Conventional and recombinant strategies have been followed to develop a dengue vaccine.6 Recombinant vaccines include chimeric, subunit, and nucleic acid vaccines. Dengue proteins have been intensively studied as recombinant proteins in *Escherichia coli*, baculoviruses, vaccinia viruses, and less frequently, in yeast.7–15

The E protein contains the major epitopes responsible for eliciting formation of neutralizing antibodies. However, to serve as a potent antigen, it must be properly folded in a conformation that maintains the integrity of neutralizing antibodies.16 Yeast represents an alternative eukaryotic expression system for the generation of flaviviral glycoproteins. Well-established fermentation methods that can generate high cell densities and the strong, tightly regulated methanol-inducible alcohol oxidase (AOX1) promoter make the yeast *Pichia pastoris* a very useful host for heterologous protein expression.17

For these reasons, we expressed a cDNA encoding a recombinant the dengue 4 virus E protein (E4rec) in *P. pastoris*. The E gene was truncated at its carboxy terminus to remove the hydrophobic membrane anchor segment to maximize the secretion of the protein, as recommended by others.18 In previous studies, we have shown that E4rec has a molecular mass of 60 kD, is glycosylated, and induces both humoral and cellular immune responses in immunized mice (Sariol C and others, unpublished data). Finally, challenge with dengue 4 virus indicated good protection (85%; *P* < 0.0005) against lethal encephalitis.19 This study represents the first attempt to evaluate a recombinant dengue protein expressed in yeast as a potential vaccine in small animals (mice) and non-human primates.

MATERIALS AND METHODS

Cells and viruses. African monkey kidney cells (Vero cells) and baby hamster kidney (BHK)21 cells were grown at 37°C in medium 199 and Eagle’s minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (HFBS). The *Ae. albopictus* cell line C6/36-HT was grown at 33°C in MEM supplemented with 10% HFBS, 1% non-
essential amino acids, and 1% (200 mM) glutamine. The Vero cell line was used for virus production, the BHK21 cell line was used for virus titration and neutralization studies, and the C6/36-HT mosquito cell line was used for virus isolation.

The Dominica strain of dengue 4 virus, which was provided by World Health Organization, was propagated in Vero cells, aliquoted, and stored at -80°C. It had been previously passaged twice in the C6/36-HT cell line and once in the Vero cell line. The A15 strain of dengue virus 2, which was isolated during the 1981 Cuban DHF epidemic, was propagated in mice, aliquoted, stored at -80°C, and used at the fourth passage level in mice.

**Dengue 4 envelope recombinant protein (E4rec).** Cloning and expression of truncated dengue 4 E protein has been previously reported. Briefly, the gene encoding the truncated dengue 4 E protein was inserted into the yeast expression vector pfaO at the EcoRI site so that the recombinant gene was under the control of the alcohol oxidase AOX promoter. A termination codon was introduced after amino acid 442 resulting in a protein lacking the C-terminal 53 amino acids. The recombinant vector was linearized by digestion with PstI and used to transformed the *P. pastoris* MP-36, producing a DNA fragment that integrated into the promoter (AOX) region of the yeast chromosome by a double crossover event. Stable transformants, expressing truncated E protein sequences were obtained. Analysis of the recombinant protein (E4rec) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting showed the presence of a 64-kD antigenic band. The presence of the recombinant protein was again confirmed by Western blotting and an enzyme-linked immunosorbent assay (ELISA) using anti-dengue 4 hyperimmune mouse ascitic fluid (HMAF) and dengue 4 virus-specific monoclonal antibody (MAb).

The growth of *P. pastoris* strain MP36 containing E4rec and purification of the recombinant protein using immobilized metal ion adsorption chromatography have been previously described by Hermida and others. The purity of the protein was 70%. The control protein preparation was prepared in a similar manner using *P. pastoris* strain MP36 transformed with pFao vector without the E4rec insert.

**Immunization and monkey infection.** Six male Macaca fascicularis monkeys (weight = 4–6 kg) seronegative for antibodies to dengue virus were used in the study. Before each procedure, animals were anesthetized intramuscularly with ketamine HCl (5 mg/kg). Prior to each immunization, weight, cardiac and respiratory frequencies, and temperature were recorded. The animals were immunized four times at intervals of 25 days by subcutaneous injections with 0.5–1 mL of E4rec or control protein diluted in 20 mM Tris, 6 mM EDTA, pH 7.5, using alum as an adjuvant. Three animals were immunized with 100 μg of E4rec and the other three were injected with 150 μg of the control protein. The monkeys were challenged 98 days after the first immunization by inoculation with 10^5 plaque-forming units (PFU) of dengue 4 virus in a volume of 1 mL equally distributed in the upper legs. Blood samples were taken for analysis at regular intervals (15 days after immunization). Sera from clotted blood were stored at -80°C until used for detection of antibody to dengue virus, virus isolation, and a polymerase chain reaction (PCR). Samples were tested simultaneously. The maintenance and care of experimental animal complied with the Cuban Institute of Health Guidelines for the human use of laboratory animals.

**Virus titration.** Virus titer was determined by a plaque assay on BHK21 cells in 24-well polystyrene plates.

**Serologic tests.** Antibodies to dengue virus in immunized animals were detected by an ELISA, a hemagglutination-inhibition (HI) assay, and a plaque reduction neutralization technique (PRNT). Serum samples were tested for dengue virus-specific IgM antibody by an IgM capture ELISA as previously described.

An inhibition ELISA was performed in 96-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) coated with 10 μg/mL of protein A–Sepharose-purified immunoglobulins from human sera with a high titer of antibodies to dengue virus in the presence of 0.1 M carbonate buffer, pH 9.6. After overnight incubation at 4°C, plates were blocked for one hour at 37°C with 1% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween 20. The same buffer was used for serum and antigen dilutions and for washes between incubations. Fifty microliters of serum dilutions and 50 μL of dengue 4 virus (H241 strain) antigen from infected suckling mice (prepared by the sucrose-acetone method) were added simultaneously to each well and incubated for one hour at 37°C. The antigen dilution was previously adjusted to give optical density (OD) values of 1.2–1.5 in the presence of a human serum seronegative for dengue virus. To determine whether inhibition or reaction of the antigen occurred, anti-dengue 4 virus HMAF or MAb 4B622 diluted 1:4,000 was added. After incubation for one hour at 37°C, reactivity was detected using peroxidase-conjugated goat anti-mouse IgG (Amersham-Pharmacia, Buckinghamshire, United Kingdom), hydrogen peroxide, and o-phenylenediamine. The reaction was stopped by addition of 12% H₂SO₄ and the color intensity was read at 490 nm in an ELISA reader. Sera dilutions with half or less of the OD obtained with the negative control serum were considered positive.

The HI assay was performed as previously described, using goose erythrocytes, and acetone was used to remove serum inhibitors.

The ability of antibodies to dengue 4 virus to neutralize infective virus was measured by a PRNT as previously described. Briefly two-fold serial dilutions of sera were incubated for one hour at 37°C with a working dilution of virus calculated to give 20–25 PFU/mL in the final volume of virus-serum mixture. After incubation, 50 μL of virus-serum mixture was added to 0.5 mL of a BHK21 cell suspension (1.5 × 10^6 cells) grown in 24-well polystyrene plates. After incubation for four hours at 37°C in an atmosphere of 5% CO₂, 0.5 mL of 3% medium-viscosity carboxymethylcellulose in Earle's MEM containing 10% heat-inactivated FBS was added to each well. Plates were incubated for six days before the addition of stain. The serum dilution that resulted in a 50% reduction in plaque count (PRNT₅₀), as determined by probit analysis, was considered the end point titer.

**Detection of viremia.** Animals were bled every two days for 14 days and viremia was assayed by cell culture inoculation followed by an indirect immunofluorescence assay (IFA) and a reverse transcriptase–PCR (RT-PCR).

Virus isolation was conducted by adding 0.2 mL of a 1:30 dilution of serum onto monolayers of C6/36-HT cells grown in 24-well plastic plates according to the method of Rodriguez and others. Following centrifugation for 30 minutes at 2,500
Development of IgG antibody response in macaques detected by inhibition enzyme-linked immunosorbent assay and hemagglutination inhibition with minor modifications. Amplification of DNA was done samples using the acid-guanidine isothiocyanate procedure,iated cells were incubated for 10 days at 33°C medium supplemented with 2% HFBS was added. The inocu-

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* Values are reciprocal antibody titers by IgG enzyme-linked immunosorbent assay/hemagglutination inhibition. In the first column, 1–4 = number of immunizations, 0 = day of challenge, and 2–30 = days post-challenge. D1, D2, and D5 = immunized animals; D8, D9, and D10 = control animals; GMT = geometric mean antibody titer; NT = not tested.

RESULTS

Antibody responses prior to virus challenge. The immunogenicity of partially purified E4rec (100 μg) was tested in M. fascicularis monkeys. Four doses at intervals of 25 days were applied. Inhibition ELISA studies showed that all animals that received E4rec seroconverted after the second dose and antibody titers ranged between 1:20 and 1:10. Antibody titers are shown in Table 1.

Low levels of HI antibodies (titers = 1:20 and 1:10) were detected in two immunized animals after the third dose. Neutralizing antibodies to dengue 4 virus with a titer of 1:30 were detected after four immunizations in all immunized monkeys prior to challenge. No dengue 2 virus–neutralizing antibodies were detected. No IgM antibodies were detected in any animal after immunizations.

Virus challenge assay. E4rec was evaluated for protective efficacy against challenge with dengue 4 virus by RT-PCR and virus isolation. No virus isolate was obtained in any monkeys after three blind passages in the C6/36-HT cell line.

Using the PCR, we detected circulating nucleic acid that was assumed to represent viremia. One vaccine-immunized monkey was protected from viremia, while the other two were partially protected, with viremia lasting three and seven days. This latter animal showed very weak DNA amplification at days 6 and 8. Monkeys in the control group developed viremia lasting 5, 7, and 13 days as detected by the RT/PCR. The mean duration of viremia in naive animals was 9.33 days, while viremia in immunized animals lasted 4 days (Table 2). None of the animals developed sign of illness.

Serologic response after challenge. Post-challenge sera were studied to examine if virus challenge induced an anamnestic response in monkeys immunized with E4rec. After challenge, all immunized monkeys showed IgG titers higher than the controls at days 6, 8, and 10 (at days 6 and 10 by the inhibition ELISA and at day 10 by HI; $P < 0.05$). The reciprocal antibody titers and the geometric mean antibody titers (GMTs) as detected by the ELISA and HI are shown in Table 1. The reciprocal GMTs as detected by the ELISA and HI are shown in Figures 1 and 2. The virus neutralizing antibody
titers and GMTs are shown in Table 3. Monkeys immunized with E4rec elicited the highest neutralizing antibody titers ($P < 0.05$) ranging from 1:85 to 1:640 at day 30. One monkey showed 2.8-fold increase in neutralizing antibody titer, while the others showed 7.3-fold and 21.3-fold increases. All animals showed neutralizing antibody titers (> 1:20) to dengue 2 virus after 30 days of challenge.

The presence of IgM antibodies to dengue virus was detected after challenge in two immunized animals (days 6 and 8 until day 30) and in all control animals (day 6 to day 60). The intensity of the response according to OD was relatively similar in the three control animals and one of the vaccinated monkeys. The other animals showed a lower and late response. The relationship between the presence of viremia and development of IgM antibodies to dengue virus in immunized and controls animals is shown in Figure 3. One immunized animal did not develop either viremia or IgM antibodies to dengue virus. The animal with the longest duration of viremia had the highest IgM antibody response. In both immunized and control animals, the longest duration of viremia correlated with the earliest development and highest level of IgM antibodies to dengue virus.

**Laboratory investigations.** Differential blood counts and platelet counts were normal in all animals.

**DISCUSSION**

In the present report, the efficacy of E4rec as a potential vaccine against infection with dengue virus was evaluated in monkeys. This evaluation of a dengue virus recombinant protein expressed in *P. pastoris* was extended from mice to non-human primates. E4rec was immunogenic in *M. fascicularis* monkeys, protecting one of three immunized animals and reducing viremia in the others. No clinical signs or abnormal results in hematologic tests were observed during immunization and challenge.

The vaccinated animals showed anamnestic antibody responses upon virus challenge, indicating successful priming by the recombinant vaccine. The fully protected monkey developed the lowest antibody titers as analyzed by ELISA, HI assay, and PRNT after challenge, and showed the smallest increase in neutralizing antibodies. Furthermore, no IgM antibodies to dengue virus were detected. The animal with the longest duration of viremia showed the highest antibody titers (both IgG and IgM). An intermediate situation was observed for the monkey with reduced viremia. As expected, primed animals with a secondary antibody response to challenge showed a higher neutralizing antibody response.

Since the ELISA used in this study is based on a competi-
It has been demonstrated that neutralizing antibody is important for protection against infection with dengue virus. Similar to previous reports,10,28 the presence of neutralizing antibody in our animals did not correlate with protection from viremia. All vaccinated monkeys had the same neutralizing antibody titer (1:30) at the time of challenge. However, they were not protected in a uniform manner. Men and others reported protective immunity in monkeys immunized with E recombinant protein expressed in vaccinia virus when the neutralizing antibody titer was ≥ 1:70.9 Perhaps the humoral or cellular immune responses were not sufficient despite the rapid post-challenge increase in neutralizing antibody observed in our study. A cytotoxic T lymphocyte (CTL) response is important in most viral infections for clearing virus-infecting cells. We can not exclude that E4rec may not have stimulated a CTL response.

An anamnestic antibody, although important, may not be sufficient for protection from infection with dengue virus. This antibody response suggests that there was virus replication in the vaccinated monkeys, even when viremia was shorter. Conversely, the IgM antibody response following challenge of vaccinated monkeys may reflect a primary antibody response against some (other) proteins that are not included in the vaccine.29

It was remarkable that no heterotypic neutralizing antibodies were detected after immunization. In addition, no antibody-dependent enhancement to dengue 2 virus was observed at a low dilution of sera (1:10) from immunized monkeys. Conversely, at day 30 after challenge, both heterotypic neutralizing antibodies and antibody-dependent enhancement to dengue 2 virus were observed. A compromise between the level of the humoral and cellular immune responses and their specificity and long duration could be the key for an effective vaccine against dengue.

Encouraged by the results of studies in mice, we extended our evaluation of protective efficacy to monkeys immunized with E4rec because the response in monkeys to dengue infection is the closest to that in humans. Although monkeys develop viremia, infected animals do not become symptomatic. Although the E recombinant protein expressed in P.
pastoris provided partial protection against viremia, the results were not effective enough to recommend it as a vaccine candidate. Further work is required to improve the ability of the immunogen to prevent disease. New genetic constructions are being developed to improve the folding of the recombinant protein. Longer intervals between vaccine doses and the viral challenge will be included in future studies. Finally, other proteins that contain CTL epitopes should be included in the vaccine.

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