

1989-06-01

## Dengue virus-specific murine T-lymphocyte proliferation: serotype specificity and response to recombinant viral proteins

Alan L. Rothman  
*University of Massachusetts Medical School*

*Et al.*

Let us know how access to this document benefits you.

Follow this and additional works at: <https://escholarship.umassmed.edu/oapubs>



Part of the [Life Sciences Commons](#), and the [Medicine and Health Sciences Commons](#)

---

### Repository Citation

Rothman AL, Kurane I, Zhang Y, Lai C, Ennis FA. (1989). Dengue virus-specific murine T-lymphocyte proliferation: serotype specificity and response to recombinant viral proteins. Open Access Publications by UMass Chan Authors. Retrieved from <https://escholarship.umassmed.edu/oapubs/1582>

This material is brought to you by eScholarship@UMassChan. It has been accepted for inclusion in Open Access Publications by UMass Chan Authors by an authorized administrator of eScholarship@UMassChan. For more information, please contact [Lisa.Palmer@umassmed.edu](mailto:Lisa.Palmer@umassmed.edu).

# Dengue Virus-Specific Murine T-Lymphocyte Proliferation: Serotype Specificity and Response to Recombinant Viral Proteins

ALAN L. ROTHMAN,<sup>1</sup> ICHIRO KURANE,<sup>1</sup> YI-MING ZHANG,<sup>2</sup> CHING-JUH LAI,<sup>2</sup> AND FRANCIS A. ENNIS<sup>1\*</sup>

*Department of Medicine, University of Massachusetts Medical Center, Worcester, Massachusetts 01655,<sup>1</sup> and Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892<sup>2</sup>*

Received 7 November 1988/Accepted 21 February 1989

**Definition of the T-lymphocyte responses to dengue viruses should aid in the development of safe and effective vaccines and help to explain the pathophysiology of dengue hemorrhagic fever and dengue shock syndrome. In this study, we demonstrated that dengue virus-specific T lymphocytes were detected in spleen cells from dengue virus-immune mice using an in vitro proliferation assay. Following immunization with a single dose of infectious dengue virus, murine lymphocytes showed increased proliferation when incubated in the presence of viral antigens of the same serotype but not in the presence of control antigens. Depletion experiments with antibody and complement showed that the population of responding cells expressed the  $\text{Thy1}^+ \text{L3T4}^+ \text{Lyt2}^-$  phenotype. This indicates that the predominant proliferating cells are T lymphocytes of the helper-inducer phenotype. Dengue virus-specific memory lymphocyte responses were detectable for at least 22 weeks after immunization. The response to primary infection was primarily serotype specific, with some serotype cross-reactivity present at a low level. We demonstrated that lymphocytes from mice immunized with dengue 4 virus proliferate in response to a combination of dengue 4 virus C, pre-M, E, NS1, and NS2a proteins expressed in Sf9 cells with a recombinant baculovirus, and, to a lesser extent, to the dengue 4 virus E protein alone.**

Infection with dengue viruses continues to present a major public health problem (7). The World Health Organization has targeted dengue viruses for intensive work toward vaccine development (2). A major concern has been to prevent the occurrence of severe complications, such as hemorrhage or shock, which have been reported to be related to the presence of preexisting immunity to a heterologous dengue virus serotype (3, 8, 20). This unusual phenomenon has been speculated to be due to antibody-dependent enhancement of infection of Fc receptor-bearing cells such as monocytes based on in vitro studies (9). A role for T cells has also been postulated, as, for example, in the immune elimination of infected monocytes (6) and in elaboration of gamma interferon, which can further augment the antibody-dependent enhancement of infection (12).

There have been few studies of the T-lymphocyte responses to dengue virus. Previous studies with human lymphocytes in our laboratory have shown that a population of  $\text{CD4}^+$  T cells from dengue virus-immune individuals can proliferate after exposure to dengue virus antigens and that these cells produce gamma interferon when stimulated (14). However, these studies are limited by the long interval between natural dengue virus infection and our testing and by the inability to define previous natural exposure to dengue virus and other flaviviruses that might alter the immune responses. The study of cellular immune responses to dengue virus in laboratory animals obviates some of these concerns.

Defining the dengue virus epitopes that might be involved in protection or immunopathological phenomena or both is desirable for future vaccine development. We have begun to study the murine T-lymphocyte responses to dengue virus and to viral proteins in order to identify such epitopes. We report here the characterization of an in vitro proliferative response to dengue virus antigens following immunization

with live virus. Murine T-lymphocyte responses were predominantly serotype specific and, to a lesser extent, cross-reactive after a single immunization with live virus. A preparation containing recombinant dengue 4 virus C, pre-M, E, NS1, and NS2a proteins or a similar preparation containing dengue 4 virus E protein alone induced proliferative responses in T lymphocytes from dengue 4 virus-immune mice.

## MATERIALS AND METHODS

**Mice.** Male BALB/c mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and were used between 4 and 8 weeks of age.

**Viruses.** Mouse-adapted dengue 1 virus (Hawaii strain), dengue 2 virus (New Guinea C strain), dengue 3 virus (PR6 strain), and dengue 4 virus (814669 strain) were generously provided as mouse brain homogenates by Jack McCown, Walter Reed Army Institute of Research. These virus stocks had titers of  $10^7$  to  $10^8$  PFU/ml in Vero cells by standard techniques (13). Tissue culture-adapted dengue 2 virus (New Guinea C strain) was kindly provided by W. Brandt, Walter Reed Army Institute of Research. Dengue 3 virus (CH53489 strain) was kindly provided by B. Innis, Armed Forces Research Institute of Medical Sciences. Viruses were propagated in C6/36 cells as previously described (13). Culture supernatants were collected 7 days after infection; they had titers of  $10^6$  to  $10^7$  PFU/ml and were stored at  $-70^\circ\text{C}$ .

**Vero cell-derived dengue virus antigens.** Dengue virus antigens were produced as previously described (14). Vero cells were grown to confluence and infected with dengue viruses at an approximate multiplicity of infection of 1 PFU per cell. When  $>50\%$  cytopathic effect was noted (usually on day 2 or 3 for dengue 2 virus and on day 3 or 4 for dengue 1, 3, or 4 virus), the cells were collected with a cell scraper, washed with phosphate-buffered saline, and treated with 0.025% glutaraldehyde in phosphate-buffered saline for 15 min at  $4^\circ\text{C}$ . The cells were washed again and suspended in

\* Corresponding author.

RPMI medium and then sonicated with a sonic dismembrator (Artek Systems Corp., Farmingdale, N.Y.) and centrifuged at  $1,500 \times g$  for 10 min. The supernatant was then aliquoted and stored at  $-70^{\circ}\text{C}$ . Similar preparations not treated with glutaraldehyde had the following titers in Vero cells: dengue 1 virus,  $3.8 \times 10^6$  PFU/ml; dengue 2 virus,  $1.7 \times 10^7$  PFU/ml, dengue 3 virus,  $3.0 \times 10^6$  PFU/ml; and dengue 4 virus,  $2.8 \times 10^6$  PFU/ml. Control antigen was prepared in identical fashion with uninfected Vero cells.

**Recombinant baculovirus-derived dengue virus antigens.** *Spodoptera fugiperda* (Sf9) cells were infected with wild-type and recombinant baculoviruses and prepared as previously described (23). Sf9 cells infected with a recombinant baculovirus containing a 4.0-kilobase cDNA sequence of dengue 4 virus 814669 strain have been shown to express dengue 4 virus C, pre-M, E, NS1, and NS2a proteins (23). A recombinant baculovirus containing a shorter cDNA sequence for the dengue 4 virus E protein was also used to infect Sf9 cells.

**Mouse immunization and preparation of spleen cells.** Mice were immunized intraperitoneally with one dose of 0.3 ml (approximately  $5 \times 10^6$  PFU) of mouse-adapted dengue virus. Spleens were removed from mice 4 to 12 weeks after immunization except as noted. Single-cell suspensions were prepared by mincing and passing spleen tissue through sterile gauze. Erythrocytes were lysed by brief exposure to ACK lysing buffer (155 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , 190 mM EDTA). Cells were then washed, and viable cells were counted.

**Measurement of lymphocyte proliferation.** Proliferative responses to dengue virus antigens were tested by a modification of the method of Chain et al. (4). Spleen cells ( $4 \times 10^5$ ) were cultured with various dilutions of antigen in 0.1 ml of RPMI 1640 medium supplemented with glutamine (2 mM), 100 U of penicillin per ml, 100  $\mu\text{g}$  of streptomycin per ml, 2-mercaptoethanol ( $5 \times 10^{-5}$  M; Sigma Diagnostics, Sterling, Va.), and 1% BALB/c mouse serum (Biotrol, Inc., Indianapolis, Ind.) in 96-well round-bottom plates (Costar, Cambridge, Mass.). After 3 days of incubation at  $37^{\circ}\text{C}$ , the culture medium was changed as follows. For most experiments, 0.1 ml of RPMI 1640 supplemented with 5% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) was added to all wells. Cells were suspended by repeated up-and-down pipetting, and 0.1 ml of the contents of each well was transferred to a second 96-well round-bottom plate containing 0.1 ml of RPMI 1640 with 5% fetal bovine serum per well. Plates were incubated for two more days. Then the cells were pulsed with 1.25  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine (Dupont, NEN Research Products, Boston, Mass.) per well for 6 h. Cells were harvested onto glass filter paper with a semiautomatic cell harvester (Skatron, Inc., Sterling, Va.), and [ $^3\text{H}$ ]thymidine incorporation was measured in a liquid scintillation counter (Packard Instrument Co., Inc., Sterling, Va.). The stimulation index was calculated by dividing the mean counts per minute (cpm) of triplicate wells incubated with viral antigen by the mean cpm of triplicate wells incubated with control antigen. A stimulation index of 2 or greater was considered significant.

**Antibody and complement depletion of responding cells.** Anti-Thy1.2 antibody obtained from Dupont, NEN Research products, was used at a concentration of 1:100. Anti-L3T4 and anti-Lyt2 antibodies obtained from Becton Dickinson and Co. (Mountview, Calif.) and goat anti-mouse immunoglobulin G obtained from Organon Teknica (Malvern, Pa.) were used at a concentration of 1:20. In separate experiments, nonimmune spleen cell preparations were

treated with the indicated antibody concentrations and complement. When examined by direct fluorescence microscopy, less than 4% of the corresponding cell populations remained.

In the depletion experiments, on day 3 of culture, 0.1 ml of RPMI 1640 supplemented with 10% fetal bovine serum was added to each well. After two more days, cells were collected by aspirating wells. The cells were washed with RPMI 1640 without serum, and  $5 \times 10^6$  cells were suspended in 1.0 ml of the indicated antibody and incubated for 1 h at  $4^{\circ}\text{C}$ . The cells were washed again with RPMI 1640 and then suspended in 1.0 ml of rabbit complement (Low-Tox-M; Cedarlane Laboratories, Hornby, Ontario, Canada) diluted 1:10 in RPMI 1640 for 1 h at  $37^{\circ}\text{C}$ . The cells were washed and suspended in 2.5 ml of RPMI 1640 supplemented with 10% fetal bovine serum. Equal volumes (0.2 ml) from each treated sample were added to 6 wells of a 96-well round-bottom plate, and 1.25  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine was added to each well. After 18 h of incubation, cells were harvested onto glass filter paper and [ $^3\text{H}$ ]thymidine incorporation was measured as described above.

**Statistics.** Statistical comparison of [ $^3\text{H}$ ]thymidine incorporation between groups was done by Student's *t* test (one tailed), and  $P < 0.05$  was used as the criterion for statistical significance.

## RESULTS

**Stimulation of lymphocyte proliferation by homologous dengue virus antigens.** Groups of mice were immunized with one dose of live virus of a single dengue virus serotype, and the spleen cells were incubated with antigens of the homologous dengue virus serotype. Spleen cells from mice immunized with any of the serotypes proliferated significantly when incubated in the presence of viral antigen (Fig. 1a to d). The response was dengue virus specific, as demonstrated by the failure of the control Vero cell antigen to stimulate as high a response. Spleen cells of nonimmune mice did not show a specific response to the dengue virus or control antigens (Fig. 1). The response depended on the concentration of antigen present in the culture medium, but significant stimulation was seen at antigen dilutions of 1:200 or higher.

To determine the phenotypes of the proliferating cells, we treated the 5-day-cultured spleen cells with antibody and complement prior to pulsing with [ $^3\text{H}$ ]thymidine. The increase in [ $^3\text{H}$ ]thymidine incorporation was eliminated by treatment with anti-Thy1 antibody and complement (Fig. 2), demonstrating that the responder cell was a T lymphocyte. Treatment with anti-L3T4 and complement but not anti-Lyt2 and complement also reduced the level of [ $^3\text{H}$ ]thymidine incorporation. This indicates that T lymphocytes of the helper-inducer phenotype made up the predominant responding population.

Mice were tested at various times after immunization to determine the duration of the immune response. Dengue virus-specific T-lymphocyte responses were detected as early as 2 weeks after immunization (Fig. 3). This response remained for at least 10 weeks. Significant stimulation was still detected 22 weeks after immunization. In separate experiments, dengue virus-specific lymphocyte proliferation (stimulation index, 2.2 to 2.8) was detected over 1 year after immunization (data not shown).

**Serotype specificity of dengue virus-specific lymphocyte proliferation.** To determine the specificity of the proliferative responses, spleen cells from mice immunized with a single dengue virus serotype were incubated with antigens of all

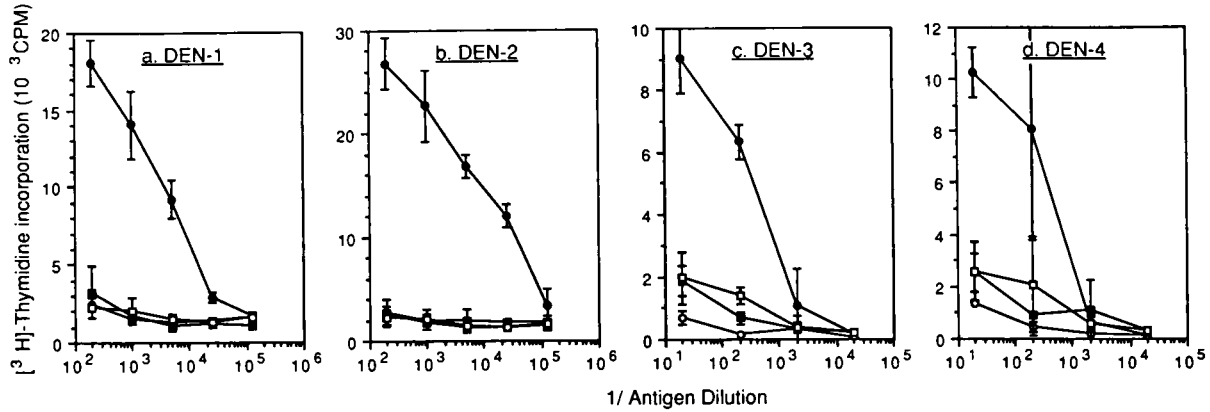


FIG. 1. Proliferative responses to homologous dengue virus antigens. Mice were immunized with one of the four dengue virus serotypes, and spleen cells were later tested with antigens from the same serotype. Symbols: ●, ■, responses to viral antigens; ○, □, responses to control antigens. ●, ○, mice immunized with the indicated virus; ■, □, unimmunized mice.

four dengue virus serotypes and yellow fever virus. Following immunization with a single serotype of dengue virus, lymphocyte proliferation was greatest in response to incubation with the homologous dengue virus serotype (Table 1). There was some response to the heterologous dengue virus serotypes and to yellow fever virus. Using our criterion of a stimulation index of >2.0, we noted significant proliferative responses with dengue 1 virus-immune spleen cells incubated with dengue 3 virus antigen (experiment 1), with dengue 2 virus-immune spleen cells incubated with dengue 3 virus and yellow fever virus antigens (experiment 1), and with dengue 4 virus-immune cells incubated with dengue 2 virus antigen (experiment 2).

**Stimulation of lymphocyte proliferation by baculovirus-dengue virus recombinants.** Spleen cells from mice immunized with a single dose of live dengue 4 virus were also tested for the ability to proliferate after incubation with dengue virus antigens expressed by using a recombinant baculovirus vector. Dengue 4 virus-immune spleen cells were stimulated by a lysate of Sf9 cells infected with a

recombinant baculovirus expressing dengue 4 virus C, pre-M, E, NS1, and NS2a proteins (Fig. 4a). The T-cell responses were specific for the dengue 4 virus proteins expressed, e.g., the stimulation index was 7.8 (at an antigen dilution of 1:2,560) compared with the control preparation of wild-type baculovirus-infected Sf9 cells. Spleen cells from nonimmune mice did not show a dengue virus-specific response (Fig. 4a).

We also detected dengue virus-specific stimulation by dengue 4 virus E protein alone using a recombinant baculovirus containing a shorter dengue 4 virus genome sequence (Fig. 4b). The response to the E protein-containing preparation was lower than the response to the preparation containing multiple dengue 4 virus proteins. Nonimmune mice did not show a response to this subunit preparation (data not shown).

DISCUSSION

We demonstrated the presence of dengue virus-specific memory T lymphocytes following immunization with a single dose of live dengue virus. We used a modification of the usual lymphocyte proliferation assay to provide a superior signal-to-noise ratio by diminishing the level of nonspecific

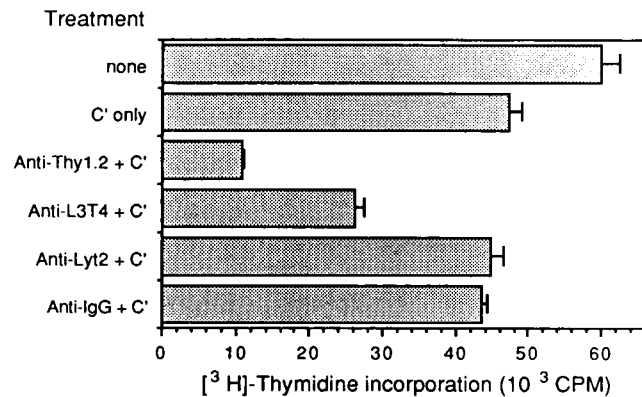


FIG. 2. Effect of depletion with antibody and complement (c') on the proliferative response. Mice were immunized with dengue 2 virus, and 4 weeks later the spleen cells were harvested and incubated with a 1:200 dilution of dengue 2 virus antigen as described in Materials and Methods. Depletions were carried out at the end of the 5-day incubation period. The mean cpm of [<sup>3</sup>H]thymidine incorporation of cells incubated with control antigen in this experiment were 17,909 with no treatment and 13,986 after treatment with complement alone.

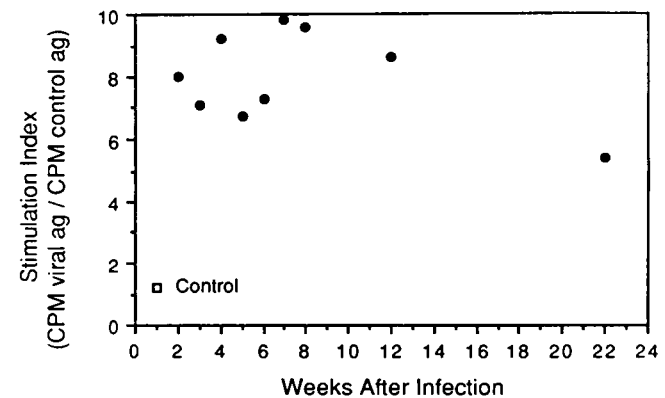


FIG. 3. Persistence of memory T-lymphocyte responses to dengue virus after immunization. Individual mice were immunized with dengue 2 virus at various times before testing. Spleen cells from all mice were stimulated in vitro in the same assay. Dengue 2 virus and control antigens (ag) were added to wells at 1:2,000 dilution.

TABLE 1. Dengue virus serotype specificity of lymphocyte proliferation

Expt no.	Immunizing dengue virus	Response to viral antigen <sup>a</sup>				
		Dengue 1	Dengue 2	Dengue 3	Dengue 4	Yellow fever
1	1	<u>7.3</u>	1.9	2.1		1.3
	2	1.7	<u>10.6</u>	2.5		2.4
	None	1.5	1.3	0.9		1.2
2	2	0.9	<u>3.9</u>	0.8	0.9	0.8
	3	1.7	1.9	<u>4.7</u>	1.4	1.3
	4	1.0	4.2	1.6	<u>7.7</u>	1.2
	None	0.5	1.2	1.3	1.1	1.5

<sup>a</sup> Values expressed as stimulation index calculated as cpm of cells incubated with viral antigen divided by cpm of cells incubated with control antigen. Values representing the responses to homologous viral antigens are underlined. Antigens were used as 1:200 dilution in experiment 1 and at 1:2,000 dilution in experiment 2. [<sup>3</sup>H]thymidine incorporation (cpm) of spleen cells incubated with the control antigen preparation was as follows: experiment 1, dengue 1 virus immune, 2,466; dengue 2 virus immune, 2,530; nonimmune, 2,198; experiment 2, dengue 2 virus immune, 2,545; dengue 3 virus immune, 2,174; dengue 4 virus immune, 1,117; nonimmune, 231.

proliferation. Preliminary studies had shown that the method used in these experiments was optimal for revealing dengue virus-specific lymphoproliferative responses (A. L. Rothman, I. Kurane, and F. A. Ennis, manuscript in preparation).

We found the predominant proliferating population of lymphocytes to be Thy1<sup>+</sup> L3T4<sup>+</sup> Lyt2<sup>-</sup> cells. However, we cannot exclude the presence of some dengue virus-specific Lyt2<sup>+</sup> cytotoxic T cells. We used inactivated cell-associated viral antigen as a stimulus, which may preferentially induce the proliferation of L3T4<sup>+</sup> cells (17). We have not yet

characterized the functions of these stimulated cells. It has been reported that cells of the helper-inducer phenotype are involved in the delayed-type hypersensitivity response to dengue 4 virus as measured by footpad swelling in cyclophosphamide-treated mice (18). Alternatively, these cells may provide help for antibody production or the generation of cytotoxic T cells and produce gamma interferon, which may increase the number of dengue virus-infected Fc receptor-bearing cells and contribute to the immunopathological processes which have been observed during secondary dengue virus infections (12, 14).

We observed that the memory T-cell responses after primary infection with dengue viruses are directed predominantly at serotype-specific determinants. We detected some cross-reactive responses to other dengue virus serotypes and to yellow fever virus, generally in those animals which showed the highest levels of proliferation in response to the homologous serotype. These cross-reactive responses were generally only 25 to 30% as strong as the response to the homologous serotype. We did detect a high level of cross-reactive response to dengue 2 virus antigen in dengue 4 virus-immune mice, although there was no reciprocal level of cross-reactivity in dengue 2 virus-immune mice. This might be explained partly by the higher viral content in our dengue 2 virus antigen or may indicate homology in the T-cell epitopes of these two dengue virus serotypes. A previous study on the delayed-type hypersensitivity response to dengue 4 virus in mice had also noted a predominantly serotype-specific response, with some cross-reactivity to dengue 2 and dengue 1 viruses (19).

Our studies with human lymphocytes have shown a greater degree of cross-reactivity of the proliferative response among the four dengue virus serotypes (14). This

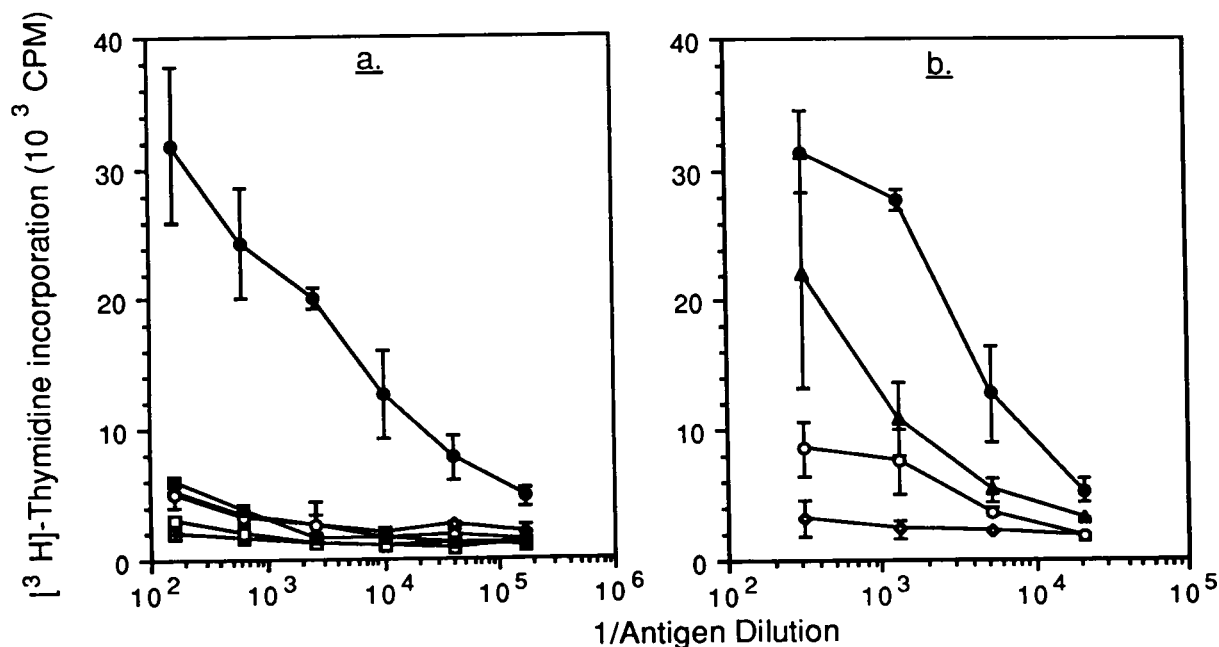


FIG. 4. Proliferative responses to recombinant baculovirus preparations. (a) Spleen cells from dengue 4 virus-immunized ( $\diamond$ ,  $\circ$ ,  $\bullet$ ) and control ( $\square$ ,  $\square$ ,  $\blacksquare$ ) mice were incubated with lysates from uninfected Sf9 cells ( $\diamond$ ,  $\square$ ), wild-type baculovirus-infected Sf9 cells ( $\circ$ ,  $\square$ ), or recombinant baculovirus-infected Sf9 cells (expressing dengue 4 virus C, pre-M, E, NS1, and NS2a proteins) ( $\bullet$ ,  $\blacksquare$ ). The standard deviations of values with spleen cells from dengue virus-immune mice are the only ones shown, for clarity. (b) Spleen cells from dengue 4 virus-immunized mice were incubated with lysates from uninfected Sf9 cells ( $\diamond$ ), wild-type baculovirus-infected Sf9 cells ( $\circ$ ), or recombinant baculovirus-infected Sf9 cells expressing either multiple dengue 4 virus proteins ( $\bullet$ ) or only the dengue 4 virus E protein ( $\blacktriangle$ ).

difference may be due to the fact that some of the individuals we studied had been immunized previously with yellow fever virus vaccine or had experienced more than one natural flavivirus infection, which might have augmented their cross-reactive T-lymphocyte clones.

Uren et al. (22) have reported cross-reactive proliferation of murine L3T4<sup>+</sup> T-cell clones among the Kunjin, West Nile, and Murray Valley encephalitis flaviviruses. Several differences between that study and ours may explain the difference in findings. They used C57BL/6J (*H-2<sup>b</sup>*) mice and stimulated spleen cells with UV-irradiated mouse brain homogenates. Furthermore, they report results for T-cell clones isolated after repeated *in vitro* restimulation with antigen. Such manipulation might favor the growth of a cross-reactive T-cell population. We are establishing T-cell clones to further define the specificity of murine lymphocytes to dengue virus.

It should be noted that our finding of relative serotype specificity of the lymphocyte response is confined to the L3T4<sup>+</sup> Lyt2<sup>-</sup> population that responded in our system. It is plausible that an L3T4<sup>-</sup> Lyt2<sup>+</sup> lymphocyte population might respond to serotype-cross-reactive epitopes and might be capable of lysing infected monocytes during secondary dengue virus infections. Another possibility is that glutaraldehyde fixation, employed in preparation of our dengue virus antigen preparation, selectively altered cross-reactive epitopes. However, we have not seen a higher level of cross-reactivity in experiments with infectious cell-free virus preparations (A. L. Rothman, I. Kurane, and F. A. Ennis, unpublished observations). It is also conceivable that virus strains associated with a higher incidence of hemorrhagic fever or shock might be more cross-reactive. Morens and Halstead (16) reported that strains of dengue 2 virus associated with more severe disease were more likely to demonstrate *in vitro* enhancement of infection by a panel of monoclonal antibodies to dengue 4 virus.

We also showed that immune spleen cells proliferate in response to dengue 4 virus proteins expressed by a baculovirus vector. This preparation, which contained dengue 4 virus C, pre-M, E, NS1, and NS2a proteins, was protective to mice against subsequent intracerebral challenge with dengue 4 virus (23). Using a dengue 4 virus subunit preparation expressed by the recombinant baculovirus vector, we demonstrated that dengue 4 virus-immune lymphocytes recognize the E protein of dengue 4 virus. Previous studies in mice have shown that monoclonal antibodies directed against the E glycoprotein of dengue 2 virus protected recipient mice against later intracerebral challenge with the same virus (11). Antibody directed against shared epitopes on the E glycoprotein protected against challenge with yellow fever virus as well (1). However, anti-E antibodies can enhance infection of Fc receptor-bearing cells *in vitro* at higher dilutions (10), and immunization with dengue 2 virus E protein expressed by a recombinant vaccinia virus failed to protect monkeys against virus challenge (5). Therefore, the E protein might not be ideal for vaccine development.

Schlesinger et al. (21) have demonstrated that immunization of mice with the NS1 glycoprotein of dengue 2 virus induced protection against later intracerebral challenge. Immunization did not result in the development of antibodies to structural proteins, making it unlikely that enhancement of subsequent infection would occur. Although the NS1 protein is highly conserved among flaviviruses (15) and the antibody response to NS1 immunization was cross-reactive in complement fixation assays, cross-protection against a subsequent challenge with dengue 1 virus was not seen (21). We

postulate that a failure to stimulate cross-reactive memory T lymphocytes may explain this shortcoming. We will test this possibility using purified or recombinant NS1 protein in experiments employing the same approaches reported here.

Future studies will be directed toward examining the effector functions of the memory T-lymphocyte population, including a search for their possible cytotoxic role. In addition, other recombinant viruses expressing structural and nonstructural dengue virus proteins will be used to identify the location of the predominant T-cell epitopes which may be involved in protective or immunopathological functions.

#### ACKNOWLEDGMENTS

This work was supported by grant DAMD 17-86-C-6208 from the U.S. Army Medical Research and Development Command and Public Health Service grant NIH-T32-AI07272 from the National Institutes of Health.

#### LITERATURE CITED

1. Brandriss, M. W., J. J. Schlesinger, E. E. Walsh, and M. Briselli. 1986. Lethal 17D yellow fever encephalitis in mice. I. Passive protection by monoclonal antibodies to the envelope proteins of 17D yellow fever and dengue 2 viruses. *J. Gen. Virol.* **67**: 229-234.
2. Brandt, W. E. 1988. Current approaches to the development of dengue vaccines and related aspects of the molecular biology of flaviviruses. *J. Infect. Dis.* **157**:1105-1111.
3. Burke, D. S., A. Nisalak, D. E. Johnson, and R. M. Scott. 1988. A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.* **38**:172-180.
4. Chain, B., I. McCafferty, G. Wallace, and P. W. Askenase. 1987. Improvement of the *in vitro* T cell proliferation assay by a modified method that separates the antigen recognition and IL-2-dependent steps. *J. Immunol. Methods* **99**:221-228.
5. Deubel, V., R. M. Kinney, J. J. Esposito, C. B. Cropp, A. V. Vorndam, T. P. Monath, and D. W. Trent. 1988. Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. *J. Gen. Virol.* **69**: 1921-1929.
6. Halstead, S. B. 1980. Dengue hemorrhagic fever—a public health problem and a field for research. *Bull. W.H.O.* **58**:1-21.
7. Halstead, S. B. 1988. Pathogenesis of dengue: challenges to molecular biology. *Science* **239**:476-481.
8. Halstead, S. B., S. Nimmannitya, and S. N. Cohen. 1970. Observations related to the pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. *Yale J. Biol. Med.* **42**:311-328.
9. Halstead, S. B. and E. J. O'Rourke. 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J. Exp. Med.* **146**:201-217.
10. Henchal, E. A., J. M. McCown, D. S. Burke, M. C. Seguin, and W. E. Brandt. 1985. Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies. *Am. J. Trop. Med. Hyg.* **34**:162-169.
11. 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. Med. Hyg.* **36**:427-434.
12. Kontny, U., I. Kurane, and F. A. Ennis. 1988. Gamma interferon augments Fc receptor-mediated dengue virus infection of human monocytic cells. *J. Virol.* **62**:3928-3933.
13. Kurane, I., D. Hebblewaite, W. E. Brandt, and F. A. Ennis. 1984. Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J. Virol.* **52**:223-230.
14. Kurane, I., B. L. Innis, A. Nisalak, C. Hoke, S. Nimmannitya, A. Meager, and F. A. Ennis. 1989. Human T cell responses to dengue virus antigens: proliferative responses and interferon gamma production. *J. Clin. Invest.* **83**:506-513.
15. Mackow, E., Y. Makino, B. Zhao, Y. M. Zhang, L. Markoff, A.

- Buckler-White, M. Guiler, R. Chanock, and C. J. Lai.** 1987. The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. *Virology* **159**:217-228.
16. **Morens, D. M., and S. B. Halstead.** 1987. Disease severity-related antigenic differences in dengue 2 strains detected by dengue 4 monoclonal antibodies. *J. Med. Virol.* **22**:169-174.
17. **Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan, and T. J. Braciale.** 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* **163**:903-921.
18. **Pang, T., S. Devi, W. P. Yeen, I. F. C. McKenzie, and Y. K. Leong.** 1984. Lyt phenotype and H-2 compatibility requirements of effector cells in the delayed-type hypersensitivity response to dengue virus infection. *Infect. Immun.* **43**:429-431.
19. **Pang, T., P. Y. Wong, and R. Pathmanathan.** 1982. Induction and characterization of delayed-type hypersensitivity to dengue virus in mice. *J. Infect. Dis.* **146**:235-242.
20. **Sangdawibha, N., S. Rojanasuphot, S. Ahandrik, S. Viriyapongse, S. Jatanasen, V. Salitul, B. Phanthumachinda, and S. B. Halstead.** 1984. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. *Am. J. Epidemiol.* **120**:653-669.
21. **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 non-structural glycoprotein NS1. *J. Gen. Virol.* **68**:853-857.
22. **Uren, M. F., P. C. Doherty, and J. E. Allan.** 1987. Flavivirus-specific murine L3T4+ T cell clones: induction, characterization, and cross-reactivity. *J. Gen. Virol.* **68**:2655-2663.
23. **Zhang, Y. M., E. P. Hayes, T. C. McCarty, D. R. Dubois, P. L. Summers, K. H. Eckels, R. M. Chanock, and C. J. Lai.** 1988. Immunization of mice with dengue structural proteins and nonstructural protein NS1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis. *J. Virol.* **62**:3027-3031.