SEROTYPE-SPECIFIC TH1 RESPONSES IN RECIPIENTS OF TWO DOSES OF CANDIDATE LIVE-ATTENUATED DENGUE VIRUS VACCINES

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Abstract. As part of a larger vaccine study, peripheral blood mononuclear cells (PBMC) were collected from volunteers for analysis of vaccine-induced T cell responses. The PBMC were re-stimulated in vitro with live dengue virus and assayed for TH1 or TH2 memory cell responses. Re-stimulated PBMC from the volunteers predominantly secreted interferon-γ. Little interleukin-4 (IL-4) or IL-10 secretion was detected, indicating a TH1 type of T cell response. The interferon-γ response was primarily serotype-specific with some serotype cross-reactivity. T cell depletion studies showed that the interferon-γ was being secreted by CD4+ T lymphocytes and/or by cells other than CD8+ T lymphocytes that were being stimulated by the CD4+ T lymphocytes. CD3+ or CD8+ T cell depletion showed that granzyme B mRNA expression correlated with the presence of CD4+ T lymphocytes. However, depletion of CD4+ T cells after four days of stimulation indicated that the granzyme B mRNA was produced by cells in culture other than lymphocytes. In summary, an antigen-specific TH1 type T cell response was seen as a response to vaccination using live attenuated dengue virus.

INTRODUCTION

An effective, safe dengue vaccine is a priority of the World Health Organization and the governments of several tropical countries. Attenuated, live virus candidate vaccines have been developed to meet this need. One group developing the attenuated live virus vaccines is the Walter Reed Army Institute of Research.1–4 Several of the candidate vaccines have been produced and evaluated in human trials. The vaccines induce various degrees of effective immune response in volunteers, as well as varying degrees of fever and rash. In general, the presence of some fever and a vaccine viremia correlate positively with an anti-viral antibody response.

The T cell study described herein is part of a larger vaccine study conducted at the Walter Reed Army Institute of Research designed to determine the effectiveness of a booster dose of live vaccine. It was reasoned that if a booster response could be demonstrated, a more attenuated live virus vaccine could be used. The booster response sought was both an antibody and a T cell response.

While T cell responses to dengue vaccines have been measured, fewer measurements of T cell responses have been made than antibody responses. Therefore, the T cell response to administration of live dengue vaccine is less well characterized. One goal of this study was to determine the nature of the T cell response to the vaccines in terms of T helper cell response, serotype specificity, and cytotoxic potential.

The predominating T cell response to the vaccines examined in this study was a TH1 response. This was determined by the secretion of interferon-γ by peripheral blood mononuclear cells (PBMC) stimulated by live dengue virus in a four-day culture. The interferon-γ was secreted by CD3+CD8- T cells and/or by cells regulated by these cells. The T cell response was dengue virus serotype-specific with some cross-reactive response. An anamnestic response was noted in some of the individuals but not others.

MATERIAL AND METHODS

Subjects. Thirty-five healthy adult volunteers ages 18−50 (21 men and 14 women) participated in a phase I clinical trial conducted by the Walter Reed Army Institute of Research that involved candidate dengue virus vaccines. The partici-
Cells were separated from whole blood by centrifugation at 1,000 × g for 30 minutes. The PBMC were collected (the cell layer above the gel in the CPT tube) and washed twice in Hanks’ balanced salt solution (Life Technologies, Inc., Rockville, MD) with centrifugation at 500 × g. Isolated PBMC were resuspended in 4 mL (per CPT tube) of Cell Freezing Media/dimethylsulfoxide (Sigma, St. Louis, MO) and frozen in 1-mL aliquots overnight at -70°C using cell freezing containers. The PBMC were then transferred to vapor phase liquid nitrogen for long-term storage.

**Bulk culture of PBMC and stimulation with live virus.** Frozen vials of PBMC were removed from liquid nitrogen storage and gently thawed at 37°C. The PBMC were washed twice with RPMI 1640 medium (Life Technologies, Inc.) and resuspended in complete media containing 10% human male AB serum (Sigma) plus supplements (penicillin [100 U/mL]-streptomycin [0.1 mg/mL]-fungicin [0.25 mg/mL] [Sigma], 2 mM L-glutamine [Life Technologies, Inc.], and 0.5 mM 2-mercaptoethanol [Sigma]). For the enzyme-linked immunosorbent assay (ELISA) of bulk (non-depleted) PBMC cultures, cells were resuspended at a concentration of 2.5 × 10^6 cells/mL and 100 µL of the resuspended cells (250,000 cells) were restimulated/cultured per individual well of a 96-well V-bottom plate (Corning Costar, Acton, MA). For the analysis of T cell-depleted PBMC (for immunoassay and granzyme B reverse transcriptase–polymerase chain reaction [RT-PCR]), cells were resuspended at a concentration of 3.25 × 10^6 cells/mL and 200 µL (650,000 cells) were incubated with magnetic beads in 1.5-mL microcentrifuge tubes. The beads were then washed with 200 µL of complete media containing virus at the same PFU/100 µL as the non-depleted culture to remove any unbound cells (total volume = 400 µL). Two hundred microliters was then added per well for restimulation/culture. More cells were used as input during magnetic cell separation because some cell loss (bead sticking) is inevitable. It was critical to keep cell density/cell-cell contact consistent for effective antigen presentation (antigen-presenting cell–T cell interaction). An equal volume (100 µL) of dengue virus-1, -2, -3, or -4, which was diluted in 10% complete media at a concentration of 3,000–24,000 PFU/100 µL, was then added per well containing 100 µL of cells (final concentration = 3,000–24,000 PFU/200 µL or per well). Furthermore, an equal volume (200 µL) was added per 1.5-mL microcentrifuge tube during magnetic separation (final concentration = 3,000–24,000 PFU/200 µL or per well). Control wells received an equal volume of medium without virus. The cells were cultured at 37°C in an atmosphere of 5% CO₂ for four days.

**Immunooassay.** A chemiluminescent immunooassay was done to determine the quantity of lymphokine secreted in tissue culture supernatant at the end of four days of culture. A 96-well immunooassay plate (Microlite 2; Dynatech Laboratories, Chantilly, VA) was coated overnight with 50 µL/well of 10 µg/mL of unlabeled anti-lymphokine ( interleukin-4 [IL-4], IL-10, or interferon-γ) antibody (Pharmingen, San Diego, CA) in a 0.1 M potassium bicarbonate buffer. The plates were washed and 100 µL of I-block buffer (Tropix, Bedford, MA) was added for one hour. Standards (recombinant IL-4, IL-10, and interferon-γ; Pharmingen) were then added in I-block buffer beginning with a concentration of 10 ng/mL. Eight three-fold dilutions of the standard were made. Undiluted samples, controls (Q-Kit Control; R&D Systems, Minneapolis, MN) and standards were diluted in an equal volume of 1-block buffer. Aliquots of 50 µL were added to each assay plate. The samples were incubated for one hour at room temperature. The plates were then washed. Secondary biotinylated antibody was diluted 1:1,000 in I-block buffer and 50 µL/well was added to the assay plates. The plates were washed and 50 µL/well of avidin-alkaline phosphatase (Avidix AP; Tropix) was added to the assay plates. The plates were incubated for one hour at room temperature. The plates were then washed and incubated twice for one minute with assay buffer (Tropix). The CDP-Star substrate (Tropix) was added to each well (100 µL/well). After 10 minutes, the plates were read on a MD2250 luminometer (Dynatech Laboratories). The first specimens were assayed using a modified protocol. Instead of a detector step using avidin–alkaline phosphatase, avidin–aequorin (Sealite Sciences, Atlanta, GA) was used. This material became unavailable during the study so the protocol was modified. Results using standard and control specimens were identical for the two assay formats.

**Serotype cross-reactivity.** To examine serotype specificity, PBMC collected on days 42, 45, or 105 from selected recipients of the monovalent attenuated vaccines (see Results) were stimulated for four days at a concentration of 250,000 cells/well with each serotype of virus in independent cultures. Culture supernatants were then analyzed using the chemiluminescent lymphokine ELISA.

**T cell subset depletions.** To examine the specific cellular source of lymphokine production, PBMC were depleted of CD3+ or CD8+ T lymphocytes prior to stimulation. Selected PBMC were washed twice with RPMI 1640 medium and resuspended at a concentration of 3.25 × 10^6 cells/mL in 5% complete media (30% more PBMC were used as input to compensate for cell loss during the depletion procedure). For the negative depletion, cells (200 µL, 6.5 × 10^5 PBMC) were incubated with washed antibody-coated magnetic beads in 1.5-mL microcentrifuge tubes. Two types of beads were used: M-450 anti-CD3 and anti-CD8 Dynabeads (Dynal, Oslo, Norway). The anti-CD3 beads were used at a concentration of 5.2 × 10^6 particles/tube (200 µL) giving an approximate 20:1 bead-to-target cell ratio. The anti-CD8 beads were used at a concentration of 4.0 × 10^6 particles/tube (200 µL) giving an approximate 31:1 bead-to-target cell ratio. The cells were incubated with beads at 4°C for 30 minutes with moderate agitation. Non-depleted PBMC were used as controls. Using an MPC-2 magnetic particle concentrator (Dynal), labeled cells were removed from the cell mixture. The CD3+ and CD8+ negatively selected PBMC were transferred to fresh 1.5-mL microcentrifuge tubes. To remove any residual unbound cells, the concentrated Dynabeads were gently washed once with 200 µL of complete medium containing the appropriate virus (concentration = 3,000–24,000 PFU/100 µL). After transfer, the final volume (400 µL) was divided equally into two wells of a 96-well V-bottom culture plate. Depleted and control PBMC culture supernatants were analyzed after four days of re-stimulation using the chemiluminescent lymphokine ELISA. In addition, the cultured PBMC were assayed for intracellular granzyme B mRNA. The CD4+ depletion was performed similarly, but the separation was done after stimulation using M-450 CD4+ Dynabeads. The anti-CD4 beads were used at a concentration of 4.004 × 10^6 particles per tube (200 µL) giving an approximate 31:1 bead-to-target cell ratio. The CD4+ negatively selected PBMC were assayed only for intracellular granzyme B mRNA.
Flow cytometry. Depletion efficiency (measured as % depletion) was determined using fluorescence-activated cell sorting (FACS) analysis after dual staining of a randomly selected, unstimulated PBMC population (both non-depleted control and CD3+ or CD8+ depleted sets). The cells were incubated with phycoerythrin-labeled anti-CD4+ or anti-CD8+ and fluorescein isothiocyanate–labeled anti-CD3+ antibodies (Becton-Dickinson) for 30 minutes at 4°C. Labeled PBMC were then washed three times with fluorescence buffer (phosphate-buffered saline [Sigma], 0.05% sodium azide, 1% fetal bovine serum [Summit Biotechnology, Boulder, CO]) and preserved in fluorescence fixative (phosphate-buffered saline, 1% formalin, 0.05% sodium azide) prior to analysis. Depletion efficiency, using the CD4+ Dynabeads, was not measured.

**Granzyme B assay.** Non-depleted control PBMC and T cell subset–depleted PBMC were assayed for intracellular granzyme B mRNA after four days of stimulation with wild-type virus. An RT-PCR assay in a 96-well plate format was used.

The mRNA purification was done using the “Straight A’s” mRNA Isolation System (Novagen, Madison, WI). After centrifugation and removal of PBMC culture supernatants for lymphokine ELISA analysis, pelleted PBMC were lysed using 200 μL/well of lysis buffer containing 10 mM dithiothreitol and then incubated with 200 μg/well of washed oligo-dT magnetic beads for 30 minutes at room temperature. After thoroughly washing the beads with eight volumes of wash buffer using an MPC-96 magnetic particle concentrator (Dynal) to remove DNA, proteins, and cellular debris, mRNA was eluted at 70°C for 20 minutes with 200 μL/well of water. The eluate was transferred to a 1.5-mL microcentrifuge tube and a second round elution was performed with an additional 200 μL/well of water. The 400 μL of eluate was then precipitated using 50 μL of 3 M sodium acetate (pH 5.2), 20 μg of glycogen (Novagen), and 300 μL of isopropanol. After a final wash with 70% cold ethanol, the mRNA pellet was suspended in 30 μL of water. Analysis of glyceraldehyde-3-phosphate dehydrogenase RNA by RT-PCR showed consistent yields among extractions.

The RT-PCR steps were performed in 96-well PCR plates. Oligonucleotide primers (22 basepairs), which correspond to exons of the human granzyme B (CTLA-1) gene and amplify a 120-basepair region, were synthesized by Dr. Stuart Cohen at the Walter Reed Army Institute of Research. The primers had the following sequences: grb2a (sense) 5′-AgC CgA CCC AgC AgT TTA TCC C-3′, grb2b (anti-sense) 5′-C TCT ggT Ccg CTT ggC CTT CTT-3′.

For each RT reaction, the total reaction volume was 40 μL and included the following: 5 mM MgCl₂, 10× buffer II (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1 mM of each dNTP, 40 units of RNase inhibitor (Perkin-Elmer, Norwalk, CT), 10 units of avian myeloblastosis virus reverse transcriptase (Siekagaku America, Falmouth, MA), 3 pmoles of grb2a primer, water, and 4 μL of mRNA template. The RT incubation steps were done in a 9600 Thermocycler (Perkin-Elmer) at 42°C for 90 minutes, 99°C for five minutes, and 4°C indefinitely. For each PCR, the total reaction volume was 50 μL and included the following: 2 mM MgCl₂, 10× buffer II (same as in the RT reaction), 0.4 mM of each dNTP, 1.25 units of AmpliTaq, 1 pmole each of grb2a and grb2b primers, water, and 5 μL of cDNA template. The PCR incubation steps were also done in a 9600 Thermocycler with an initial denaturation/annealing step at 95°C for 10 minutes; 30 cycles of denaturation at 95°C for 30 seconds with a 10-second ramp, annealing at 60°C for 30 seconds with a 30-second ramp, and extension at 72°C for 30 seconds with a 30-second ramp; a final extension at 72°C for seven minutes; and an incubation step at 4°C indefinitely.

Final amplified PCR products (10 μL) were separated by electrophoresis on ethidium bromide–stained 2% agarose (FMC Bioproducts, Rockland ME)/1× Tris-acetate-EDTA gels and analyzed using a digital camera (Scientific Imaging Systems, New Haven CT).

**RESULTS**

**Lymphokine secretion by dengue virus–stimulated cells.** Live dengue virus was used to stimulate PBMC cultures. The serotype of stimulating virus used in culture was the same as the serotype of the vaccine virus. After four days, the tissue culture supernatants were assayed for the presence of interferon-γ, IL-4, and IL-10. In all cultures, test results for IL-4 and IL-10 were consistently negative. Two controls were used to insure that the assay was working properly. First, the standard curve used recombinant lymphokine and second, a control sample was used to insure that the lymphokines could be detected in the presence of tissue culture supernatant.

In contrast to the negative expression of IL-4 and IL-10, high levels of interferon-γ were detected in several of the culture supernatants. Figure 1 shows the kinetics of interferon-γ expression in cells collected from volunteers receiving monovalent vaccines. Overall, the highest interferon-γ responses were by PBMC collected from recipients of dengue-1 and dengue-2 candidate vaccines, although there were a few high responses in dengue-3 and dengue-4 recipients. Interferon-γ was occasionally detected by the 14th day after the first inoculation, but often the expression was not detected until just prior to or just after administration of the second dose. The kinetics of secretion was therefore much slower than expected. In regard to booster responses for the monovalent recipients in this study, there were no consistent patterns. Depending on the individual, interferon-γ levels either increased or decreased after administration of the second dose.

Unstimulated PBMC from all volunteers at all collection points showed undetectable levels of interferon-γ. The mean level of expression from stimulated day 0 cells was 127 pg/mL with a standard deviation of 230 pg/mL.

For the monovalent vaccine recipients, there were 16 positive and 14 negative interferon-γ responders (mean ± 3 SD). Sixteen of 30 monovalent vaccine recipients had PBMC cultures with interferon-γ levels ≥ 1,000 pg/mL for at least one time point. Twelve had sustained interferon-γ secretion ≥ 1,000 pg/mL for two or more consecutive time points. Also, 12 of 30 had secretion ≥ 1,000 pg/mL at the last time point assayed.

Four volunteers received tetravalent vaccines (an equal mixture of all four monovalent strains). Figure 2 shows the interferon-γ production by PBMC collected from these tetravalent recipients. The PBMC were stimulated in separate cultures using one of each of the four serotypes of dengue virus. The PBMC from volunteers 3 and 36 secreted high amounts of interferon-γ, ≥ 1,000 pg/mL, for at least one time
FIGURE 1. Interferon-γ production by peripheral blood mononuclear cells collected from vaccine volunteers and stimulated with serotypespecific virus. All volunteers received only one serotype of vaccine. The graphs on the left show results of volunteers who were given the second dose around day 32. The graphs on the right show results from volunteers who received the second dose around day 92. A response greater than 1,000 pg/mL was seen just prior to the second dose in most volunteers. Only four volunteers had a response greater than 1,000 pg/mL within the first 15 days of receiving the first vaccine dose. Vertical error bars show the mean ± SD.
point after stimulation with each of the four of the serotypes. The PBMC from volunteer 35 secreted high amounts of interferon-\(\gamma\) in response to three of the four serotypes (not dengue-3). The PBMC from volunteer 34 secreted interferon-\(\gamma\) only in response to dengue-2 virus. Highest responses were predominantly to DEN-1 and DEN-2. The kinetics of interferon-\(\gamma\) production was delayed in the tetravalent vaccine volunteers, as it was in the monovalent volunteers. High levels of interferon-\(\gamma\) were detected just prior to and just after inoculation of the second dose. In regard to booster responses, as with the monovalent recipients, there were no consistent interferon-\(\gamma\) secretion patterns after administration of the second dose.

In aggregate, these results indicate that the predominant T lymphocyte response in both monovalent and tetravalent vaccine recipients was an antigen specific T\(_{H1}\) response.

**Serotype cross-reactivity.** The PBMC from 12 of the monovalent vaccine recipients were examined for the presence of dengue serotype-specific and cross-reactive responses. Based on kinetics, those individuals who secreted \(\geq 1,000\) pg/mL of interferon-\(\gamma\) in PBMC culture supernatants at the last time point (second to last collection day) were chosen. The PBMC were stimulated in independent cultures for four days with each dengue serotype, followed by analysis of secreted interferon-\(\gamma\) in culture supernatants. Although there was some serotype cross-reactivity, the highest response was always seen in PBMC stimulated with the same serotype as the original vaccination (Table 1). Thus, the interferon-\(\gamma\) responses seen in PBMC from these selected monovalent vaccine recipients were dengue serotype specific.

Cross-reactive responses were half (or less) of the serotype-specific response. For dengue-2 vaccine recipients, the highest cross-reactive response was with dengue-4 virus. For dengue-4 vaccine recipients, the highest cross-reactive response was with dengue-2 virus. For dengue-1 vaccine recipients, the cross-reactive responses varied. There was only one dengue-3 vaccine recipient in this group and that response was serotype specific.

**T cell subset depletions.** To verify that this was a T\(_{H1}\) response, the identity of the cells secreting interferon-\(\gamma\) was
determined. This was done by depleting T cells or T cell subsets prior to culture. The cells used in this study were mixed PBMC separated from whole blood using density gradient centrifugation. The predominant cells in PBMC populations include T cells, B cells, monocytes, and natural killer (NK) cells. For this assessment, we chose the time point of the highest interferon-response based on kinetics in 13 monovalent and three tetravalent volunteers.

Cells were removed from PBMC using immunomagnetic cell separation. The depletion efficiency was assessed using flow cytometry in test depletions. The FACs analysis of the cultured PBMC was not done due to the small number available. In the test depletions, removal of CD3+ cells using a CD3 monoclonal antibody resulted in a 92% reduction of CD3+ cells relative to non-depleted PBMC controls. The CD3 depletion was monitored using dual labels for CD3 and CD4, dual labels for CD3 and CD8, and single label for CD3. The CD3 depletion was more thorough for CD4+ cells than CD8+ cells, with 98% of the CD3/CD4 T cells being depleted and 90% of the CD3/CD8 cells being depleted in the CD3-depleted groups. Removal of CD8+ cells using a CD8 monoclonal antibody resulted in a 99.9% reduction of CD8+ cells.

Selected PBMC were depleted of CD3+ or CD8+ T lymphocytes, stimulated in culture with dengue virus for four days, and then examined for secreted interferon-γ. Results were compared with those obtained from non-depleted PBMC controls cultured at the same time. The CD4+ T lymphocytes were not depleted prior to stimulation because other cell populations need help from CD4+ T cells for the production of interferon-γ.

Removal of CD3+ cells prior to culture substantially reduced the production of interferon-γ (Table 2). The range for reduction in interferon-γ after CD3+ depletion was 59–100%. Reduced but detectable production of interferon-γ was seen in some cultures depleting of CD3+ cells. This residual production indicates that either the small amount of residual CD3+ cells remaining after immunomagnetic cell separation are secreting interferon-γ and/or another population of cells is also secreting interferon-γ.

Except in one individual, removal of CD8+ cells prior to culture did not reduce the production of interferon-γ. In nine of the 16 cultures, removal of CD8+ cells actually increased its production, possibly due to removal of suppression by these cells or by reducing the killing of infected antigen-presenting cells by CD8+ cytotoxic lymphocytes.

Together, these results indicate that the interferon-γ seen in these PBMC cultures is either secreted by CD3+CD4+ T lymphocytes and/or by cells regulated downstream by CD4+ T lymphocytes. This supports the finding of a Th1 response.

**Granzyme B.** A Th1 response is associated with, among other things, a cytotoxic lymphocyte response. In an effort to see if cells capable of cell-mediated killing were present in these vaccine volunteers, granzyme B mRNA was measured in the PBMC cultured for the depletion experiments. After removal of culture supernatants for lymphokine analysis, the cells were pelleted and lysed for extraction of mRNA. Granzyme B-specific primers were used in an RT-PCR and the PCR products were analyzed by agarose gel electrophoresis. Gel band intensity was converted into a +, - scale using a reference photograph for comparison. Extra cells from seven of the volunteers were cultured without virus. The unstimulated PBMC from these seven volunteers had little (- or +) expression of granzyme B mRNA. With antigen-specific stimulation, expression was substantially up-regulated in all 16 of the selected vaccine recipients (Figure 3). The T cell subset depletion using CD8 monoclonal antibody did not significantly reduce granzyme B expression relative to control PBMC. There were three individuals (16, 22, and 33) whose granzyme B expression was reduced in the CD8-depleted group. In one (33), the decrease was substantial. In contrast, T cell subset depletion using CD3 monoclonal antibody reduced expression in 14 of the volunteers. In eight of the monovalent volunteers and in all three tetravalent volunteers, the decrease was substantial. Four of the interferon-γ non-responders were also examined for granzyme B mRNA. All showed low levels of expression.
In cells from seven of the volunteers, T cell subset depletion using CD4 monoclonal antibody was done after the four days of culture. The depletion was done after culture to provide T helper cell activity to all cells needing T cell help during culture. Removal of CD4+ cells after stimulation did not affect granzyme B expression relative to non-depleted controls in the seven volunteers analyzed. Thus, although there is an antigen-dependent production of granzyme B mediated by CD3+CD4+ T\textsubscript{H1} cells, the actual cells that produce the granzyme B appear to be cells other than T cells. Whether this is production by NK cells or macrophages remains to be determined.

**DISCUSSION**

Two objectives of this study were to determine if there was a measurable T cell response in the monovalent and tetravalent vaccine recipients and if a cell-mediated immune response to the second dose of vaccine could be seen. For those objectives, T cell response kinetics were measured by re-stimulating cells collected at intervals around the two doses. The re-stimulation was done with live virus in bulk cultures of PBMC collected during the study.

A third objective of this study was to determine the nature of the T cell response in terms of 1) cell type defined by lymphokine repertoire, 2) dengue serotype-specific and cross-reactive responses, and 3) a measure of cytotoxic potential and granzyme B expression. These responses were measured in PBMC from both monovalent and tetravalent vaccine recipients. In regard to the tetravalent vaccine recipients, it was important to determine if a response could be detected to all four serotypes of dengue virus.

Human and mouse T helper responses can be divided into two groups based upon their pattern of lymphokine expression. T helper 1 (T\textsubscript{H1}) cells are characterized by the secretion of IL-2 and interferon-\(\gamma\). Of those two lymphokines, interferon-\(\gamma\) is the most important in terms of identifying T\textsubscript{H1} cells. T helper 2 (T\textsubscript{H2}) cells are characterized by the secretion of IL-4, IL-5, IL-6, and IL-10. In mixed populations of cells or PBMC bulk culture, one of the two secretion patterns usually predominates.

One factor influencing the T\textsubscript{H1} versus T\textsubscript{H2} response is the nature of the assaulting infection. Viral infections and some bacterial infections such as *Listeria* and *Mycobacterium*\(\textsuperscript{b}\) often induce a T\textsubscript{H1} response, while some parasitic infections will induce a T\textsubscript{H2} response.\(\textsuperscript{7}\) The proportion of the two responses may vary during the course of the infection. For instance, even though a viral infection usually begins with a T\textsubscript{H1} response, a T\textsubscript{H2} response can be produced later in the infection. The initial T\textsubscript{H1} response may augment cytotoxic T lymphocyte responses and direct immunoglobulin isotype switching, while the following T\textsubscript{H2} response may augment antibody production by B cells.

In natural dengue infection, one study showed a T\textsubscript{H1} response in most individuals.\(\textsuperscript{8}\) The T\textsubscript{H1} response was associated with an effective immune response without associated severe pathogenesis. In contrast, some individuals developed a T\textsubscript{H2} response that was associated with greater pathogenesis.

In spite of the association of a T\textsubscript{H1} response with an effective anti-dengue immune response, the key lymphokine of a T\textsubscript{H1} response, interferon-\(\gamma\), has both positive and negative influences on the immune response. In Thailand, Kurane and others found high levels of interferon-\(\gamma\) in the serum of patients with dengue hemorrhagic fever in comparison with lower levels in the serum of patients with dengue fever.\(\textsuperscript{9}\) The increased amount of interferon-\(\gamma\) may be a measure of immune activation. Interferon-\(\gamma\) is needed to activate and maintain activation of cytotoxic cells (CD4\textsuperscript{+} cells, CD8\textsuperscript{+} cells, and NK cells). While this mechanism may contribute to pathogenesis in severe infections, the same response may be identified in milder infections by reducing the number of virally infected cells through antigen-specific cytolysis. The positive role of interferon-\(\gamma\) in controlling dengue virus infection is demonstrated in a recent mouse knockout model deficient in interferons-\(\alpha\), -\(\beta\), and -\(\gamma\). The knockout mice were susceptible to lethal infection by dengue viruses in contrast to normal adult controls that were resistant to infection.\(\textsuperscript{10}\)

Alternatively, interferon-\(\gamma\) may contribute to the pathogenesis of dengue virus infection. One mechanism for the pathogenesis may be by immune enhancement due to increasing the infectivity of one major target cell, the macrophage. In culture, interferon-\(\gamma\) increased the antibody-mediated infection of macrophage cell line U037 by increasing the number of Fc receptors on the surface of the cells,\(\textsuperscript{11}\) although another study using normal cultured macrophages showed the opposite effect of decreasing the infection.\(\textsuperscript{12}\) Given these conflicting results, it is unclear whether interferon-\(\gamma\) contributes to increased infection of macrophages.

In this study, a T\textsubscript{H1} response was the predominant response. Results of assays for IL-4 and IL-10 were consistently negative, indicating a lack of a T\textsubscript{H2} response. High levels of interferon-\(\gamma\) were detected in the supernatants of many of the PBMC cultures, indicating the presence of a T\textsubscript{H1} response in those cultures.

Since the stimulated were whole PBMC, the cells respon-
sible for secretion of the interferon-γ needed to be determined. This was done by depleting T cells or T cell subsets using an immunomagnetic procedure. Negative depletion was done prior to culture with antibodies recognizing either CD3 or CD8. Since depletion of CD3 resulted in abrogation of interferon-γ secretion and CD8 depletion did not, it was concluded that CD3+CD8+ lymphocytes were the cell population secreting the interferon-γ or at least controlling the secretion of interferon-γ. Serial dilutions of sample were not analyzed by a PCR. Therefore, a mild reduction of signal in the CD8+ population might not have been detected because of signal saturation. Therefore, this result does not rule out some contribution of secretion by CD8+ cells; however, the predominant response was from CD8-CD3+ cells. This confirms that the interferon-γ was the result of a Th1 response. Residual interferon-γ in some cultures after depletion may have been due to some remaining CD3+CD4+ lymphocytes after depletion and/or other cells in the culture, possibly NK cells or macrophages.

Dengue-specific CD8+ and CD4+ lymphocytes have been cloned from dengue-infected individuals, and both have been implicated in pathogenesis and natural immunity to dengue viruses. While this study showed that CD4+ lymphocytes were involved in the regulation of interferon-γ production, the role of CD8+ lymphocytes in protective immunity induced by live virus vaccines could not be ruled out. Additional studies examining intracellular cytokines by flow cytometry are needed to determine in more detail which immune system cells respond to dengue vaccines.

The peak interferon-γ response was serotype specific. When cells from monovalent vaccine recipients were stimulated separately by each of the four serotypes of dengue virus, peak interferon-γ production was in response to stimulation by dengue virus homologous to the vaccine virus. Lesser, cross-reactive responses to other dengue serotypes were noted in several of the cultures. This is similar to the results obtained by other investigators using a different measurement, lymphocyte proliferation. In one study, cells from individuals receiving a dengue-2 virus vaccine exhibited the greatest response to dengue-2 virus, but cross-reactive response was observed. This was confirmed at the clonal level in which a majority of clones obtained from a dengue-3 virus vaccine recipient responded best to dengue-3 antigen, but cross-reactive responses to the other three dengue virus antigens were observed. The conclusion of the latter study was that primary dengue virus infection produces predominantly cross-reactive CD4+ lymphocyte responses (proliferation and interferon-γ production).

In this study, cross-reactive responses of the PBMC of monovalent vaccine recipients were usually half or less of the serotype-specific response. In the tetravalent vaccine recipients, secretion of interferon-γ in response to individual serotypes of dengue virus was detected in three of four tetravalent vaccine recipients. The responses varied within individual vaccine recipients enough that it was not possible to determine if the lower responses were serotype-specific responses or cross-reactive responses.

The kinetics of T cell activation as indicated by interferon-γ secretion was slower than expected. In a few instances, responses could be detected by day 14. However, in most cases, responses were not detected until just prior to administration of the second vaccine dose. It is unclear what the reason is for the delayed kinetics. One explanation could be that viral antigen production by virally infected cells is slow and persistent after vaccination. However, it is equally possible that the methods preferentially detected memory responses rather than acute responses. For instance, if active CD8+ cells were inhibiting a CD4+ response in PBMC collected during early infection, a measurable response may be attenuated. In cultures in which the CD8+ T lymphocytes were depleted, secretion of interferon-γ by the remaining was increased in more than half of the cultures. This inhibition may have been greater during early infection.

Other investigators have observed more acute lymphokine production kinetics. Serum lymphokines, including serum interferon-γ, were measured for 17 days after inoculation with an attenuated dengue vaccine. An acute response was observed in that study that peaked during the time of viremia.

The response to the second dose was mixed. Some individuals showed an increase in interferon-γ production, while others showed a decrease. The production of interferon-γ by cells collected from vaccine recipients just prior to the second dose was high enough that it may have masked any anamnestic response to the second dose. In addition, the late interferon-γ response may have made the measurement of an anamnestic T cell response more difficult. It is clear that some individuals responded to the second dose. This may indicate that there is some localized vaccine virus growth in the presence of an active immune response.

The intensity of response to either dose varied considerably among the vaccine recipients. There are many possible explanations regarding the variability and these were not addressed in the experimental design of this study. Variations in HLA phenotype are one possible explanation. Different dengue epitopes may be presented in individual with different HLA phenotypes and this different antigen presentation may explain the variation. Other immunologic differences among individuals may also contribute to the variations seen. Because of individual variability, it would be useful it include a measure of T cell response, as well as HLA phenotyping, in other vaccine studies. In the event of vaccine failure in some individuals, it may help determine why specific individuals did not respond.

In summary, the predominant T cell response to administration of these live-attenuated dengue viruses was an antigen-specific Th1 response. This was demonstrated by the secretion of interferon-γ by re-stimulated PBMC collected from monovalent and tetravalent vaccine recipients. None of the PBMC cultures from cells of vaccine recipients had significant secretion of IL-4 or IL-10 into the culture supernatant after re-stimulation.

The Th1 response was verified by showing that CD3+CD8+ lymphocytes and/or cells regulated by these T cells were secreting interferon-γ. Furthermore, vaccination up-regulated the expression of granzyme B, a marker of cell-mediated cytotoxicity. The Th1 response was predominantly dengue serotype specific, but lesser cross-reactive responses were observed.

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