Defining T-Cell-Mediated Immune Responses in Rotavirus-Infected Juvenile Rhesus Macaques

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The appearance of virus-specific CD4⁺ and/or CD8⁺ T lymphocytes in peripheral blood of captive juvenile rhesus macaques (*Macaca mulatta*) was observed following rotavirus infection. These cell-mediated immune responses were measured following experimental or natural infection after rotavirus was isolated from stool specimens of asymptomatic animals. The virus isolated was a new strain of simian rotavirus that we named TUCH (for Tulane University and Cincinnati Children's Hospital). Restimulation of peripheral T lymphocytes by inactivated double- or triple-layered TUCH rotavirus particles containing either VP6 or VP4 and VP7 on their respective surfaces resulted in increased quantities of interleukin-6 (IL-6) and IL-12 in cell culture supernatants. Recall responses to rotavirus by CD4⁺ and CD8⁺ T lymphocytes were associated with accumulation of intracellular IL-6 and gamma interferon. Antigen presentation of TUCH rotavirus to lymphocytes was mediated via differentiated cultures of monocyte-derived dendritic (HLA-DR⁺) cells. This is the first report demonstrating cell-mediated immune responses to rotavirus in nonhuman primates. Further exploration of rhesus macaques in vaccine trials with human rotavirus vaccine candidates is the major objective of future studies.

In the United States, rotavirus infection of 6- to 36-month-old children results in more than 50,000 hospitalizations each year (41). Throughout the world, at least 500,000 children die annually due to rotavirus-induced dehydration (25, 28, 41). Although extensive trials of novel vaccine candidates were performed with gnotobiotic piglets or genetically defined mice (28, 41, 44), none of these candidates have been evaluated in primates. Therefore, development of a nonhuman primate (NHP) model for evaluation of human rotavirus vaccine candidates is urgently needed. Although several NHP species, including chimpanzees and baboons as well as rhesus, vervet, pigtailed, and squirrel monkeys, have been reported to seroconvert to rotavirus with high, virus-specific antibody titers, no studies on cell-mediated immune responses have been conducted with an NHP model in the context of rotavirus (11, 14, 29, 36, 43). One reason is that clinical disease associated with rotavirus infection in NHPs, which has been reported to be limited to the first days or weeks of life (20, 21, 29, 36, 42), is difficult to produce experimentally.

In this study, the cell-mediated immunological responses to rotavirus infection in juvenile rhesus macaques that were housed in biosafety level 2 facilities of the Tulane National Primate Research Center (TNPRC) were evaluated. We anticipated that cell-mediated immune responses could be measured in these animals by the time passive maternally acquired immunity subsided, i.e., at between 4 and 6 months of age. Thus, we studied the T lymphocytes collected from peripheral blood of juvenile rhesus macaques after a detectable rotavirus

infection. Based on recent studies with the adult mouse model (4, 5, 24), our hypothesis was that immunological memory in rotavirus-infected rhesus macaques would be associated with increased activity of CD4⁺ T lymphocytes. We tested this hypothesis by analyzing the *in vitro* T lymphocyte (CD4⁺ and CD8⁺)-mediated cytokine production in response to both double-layered (DL) and triple-layered (TL) rotavirus particles.

MATERIALS AND METHODS

Rhesus macaques and sample collection. (i) **Conventional animals.** Sixteen juvenile rhesus macaques (*Macaca mulatta*) of either sex were prescreened for evidence of a previous rotavirus infection by the presence of virus-specific immunoglobulin G (IgG) and IgA in serum (42). The ages of animals ranged between 2.6 and 5.9 months. All animals were born at the TNPRC and were housed under biosafety level 2 conditions in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care. Only simian retrovirus (simian immunodeficiency virus, simian retrovirus, and simian T-cell leukemia virus)-negative animals were used. Investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council. Ten of the screened animals displayed no evidence of a previous rotavirus infection; i.e., they had no detectable serum rotavirus IgA and low or undetectable levels of rotavirus IgG. These 10 animals were clinically monitored on a daily basis, and stool specimens were collected each day and examined for the presence of rotavirus antigen. Blood specimens were also collected on a monthly basis and examined for evidence of seroconversion to rotavirus, i.e., a ≥ 4 -fold increase in the level of serum rotavirus IgA or IgG (35, 42). Two months following detection of rotavirus and subsequent seroconversion, mononuclear cells were isolated from the peripheral blood of three animals (EA17, ED29, and EA42) and evaluated for the presence of rotavirus-specific T cells.

(ii) **Cesarean-section derived animals.** In addition, three Cesarean-section (C-section)-derived, lactogenic immunity-deprived, rotavirus-free animals (FB82, FB88, and FB97) were experimentally inoculated at the age of 3 weeks with TUCH rotavirus. The oral challenge dose (3×10^4 focus-forming units) was determined based on studies published elsewhere (35, 42). Stool collections and clinical observations were performed daily between postinoculation days (p.i.d.) 0 and 14. Mononuclear cells were derived from peripheral blood at p.i.d. 0, 3, and 10 to evaluate the T-cell responses to rotavirus.

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Quantification of rotavirus in stools. The collected stools were frozen at -80°C until analysis. Twenty percent suspensions of thawed stool samples were made in Earle's balanced salt solution, homogenized, and centrifuged ($1,500 \times g$, 5 min, 4°C) to remove debris. Quantities of rotavirus antigen in the fecal samples were determined by using an enzyme-linked immunosorbent assay (ELISA) protocol developed for quantifying macaque rotavirus. The protocol was modified from the one used for quantifying mouse rotavirus as described elsewhere (23, 24, 42). The measured amounts of rotavirus antigen were expressed in nanograms per milliliter of 20% stool suspension.

Characterization of the simian rotavirus strain TUCH obtained from juvenile rhesus macaques. Six of the juvenile rhesus macaques (older, naturally exposed, conventional animals) monitored in this study shed detectable quantities of rotavirus in their stools. The electropherotypes of genomic RNAs obtained from all six animals were identical. The isolated rotavirus, characterized as reported elsewhere (42), was determined to be a new, previously unidentified strain that we have named TUCH (for Tulane University and Cincinnati Children's Hospital).

Preparation of DL and TL TUCH rotavirus particles. The TUCH rotavirus was adapted to grow in MA-104 cells as described elsewhere (42). The third passage of this virus was expanded for cesium chloride (CsCl) purification as previously described (23). In brief, viral lysate was treated with 1,1,2-trichlorotrifluoroethane (Sigma, St. Louis, Mo.) to remove cellular debris. The collected viral suspension was then layered over a cushion of 1.4-g/ml CsCl and centrifuged (SW28 rotor, 25,000 rpm, 90 min). The viral bands in the cushion were collected, and the density of CsCl was adjusted to 1.37 g/ml. The virus was further purified by centrifugation (SW 39.1 rotor, 35,000 rpm, 18 h). Intact TL particles (1.36 g/ml) and DL particles (1.38 g/ml) lacking VP4 and VP7 were collected from the same gradients. After purification, particles were dialyzed against phosphate-buffered saline (PBS) containing 20% glycerol and frozen at -80°C . Inactivation of purified rotavirus was performed by UV irradiation as described elsewhere (23). Protein concentrations were determined by using the Bradford reagent as described by the manufacturer (Amresco, Salton, Ohio).

Lymphocyte in vitro cell cultures and antigen presentation. Peripheral blood mononuclear cells (PBMCs) from three experimentally and three naturally infected animals with a history of rotavirus (TUCH) rectal shedding and/or seroconversion (42) were used to study the in vitro cell-mediated immune responses to rotavirus. The PBMCs were resuspended in lymphocyte culture medium (RPMI; Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Gibco) and seeded in 24-well cell culture plates coated with collagen (BD Biosciences, San Diego, Calif.) at a concentration of 2×10^7 cells per well. The PBMCs were then allowed to adhere (2 h, 37°C , 5% CO_2). Nonadherent cells were removed and frozen in 1 ml of RPMI medium containing 40% fetal bovine serum and 10% dimethyl sulfoxide (17). Fresh medium containing 100 ng of granulocyte-macrophage colony-stimulating factor (clone 215-GM) per ml and an equal amount of interleukin-4 (IL-4) (MP4-25D2; R&D Systems, Minneapolis, Minn.) was added to adherent cell cultures. Adherent, monocyte-like cells were left in vitro for 5 days to differentiate into dendritic cells (DCs) as described previously (26). On day 5, purified rotavirus (DL or TL) particles were added to the cultures (1 $\mu\text{g}/\text{ml}$, 16 h, 37°C). The nonadherent cells were then thawed, and the B and NK cells were partially depleted by using commercially available magnetic separation columns loaded with anti-human CD19 and CD56 antibodies (Miltenyi Biotec, Auburn, Calif.) to yield purified T cells according to the manufacturer's instructions. Adherent cells (DCs) and nonadherent cells (T cells) were cocultured at a 1:2 ratio for 4 to 5 days. Following incubation, cell culture supernatants were harvested and screened for the presence of IL-6 (BD Biosciences) and IL-12 (Biosource, Camarillo, Calif.) according to the manufacturer's protocols. In addition, the $\text{CD}4^{+}$ and $\text{CD}8^{+}$ T cells were tested by flow cytometry for the production of intracellular IL-6 and gamma interferon (IFN- γ). A mixture of the mitogens phytohemagglutinin (50 ng/ml) (Gibco, Carlsbad, Calif.), calcium ionophore (250 ng/ml) (Sigma), and lipopolysaccharide (10 ng/ml) (Sigma) was used for overnight stimulation of T cells as positive controls. The DCs without rotavirus were used as mock or negative controls.

Confocal microscopy. DCs loaded with rotavirus (DL or TL) particles were tested for the presence of viral antigens by confocal microscopy as previously described (10). In addition, the phenotypes of these cells were corroborated by the use of CD11c (S-HCL-3), CD14 (M5E5), and HLA-DR (G46-6) antibodies (BD PharMingen, San Diego, Calif.) and flow cytometry. Colocalization of rotavirus antigens with simian class II antigen (which is known to cross-react with its human analog) of the major histocompatibility complex (HLA-DR) was tested. Polyclonal guinea pig antirotavirus (SA11 strain) antiserum was used in conjunction with goat anti-guinea pig-fluorescein isothiocyanate conjugate (ICN Biomedicals, Aurora, Ohio). Nuclear DNA was stained with ToPro-3 according to the instructions of manufacturer (Molecular Probes, Eugene, Oreg.). Imaging

was done with a True confocal laser scanning microscope SP2 (Leica Microsystems, Wetzlar, Germany).

Lymphocyte proliferation. Serially diluted DCs (0.6×10^3 to 5×10^3 per well of a 96-well plate) loaded with TUCH rotavirus (TL particles at 0.1 μg per 10^6 cells) were cultured in vitro with 10^5 of lymphocytes per well for 5 days in RPMI medium supplemented with 10% fetal bovine serum (Gibco) as previously described (26). To assay autologous T-cell proliferation, DCs without rotavirus (mock or negative control) were used. During the last 16 h, cells were pulsed with 0.5 μCi of [^3H]thymidine (Perkin-Elmer, Wellesley, Mass.) per well. Results for each sample were expressed as mean counts per minute of [^3H]thymidine incorporation for cells stimulated with DCs loaded with or without rotavirus. Due to the limited number of available cells (T cells and DC), only the samples from two animals (ED29 and EA42) were assayed.

Intracellular cytokine staining and flow cytometry. Intracellular staining was performed as described previously (9) with modifications. Briefly, T cells (10^6 cells per sample) were stimulated with either DL or TL rotavirus particles or controls as described above under "Lymphocyte in vitro cell cultures and antigen presentation" and then washed with 10 ml of ice-cold PBS (pH 7.4) and centrifuged ($300 \times g$, 20 min) following 4 h of incubation with Golgi block (BD PharMingen) at 37°C . The pelleted cells were then resuspended in 100 μl of PBS with 0.1% sodium azide and 0.2% fetal bovine serum. Fluorochrome-conjugated reagents specific to human and rhesus macaque CD3 (SP34), CD4 (SK3), and CD8 (SK1) surface molecules, including appropriate isotype controls (BD PharMingen), were added to cell suspensions and incubated in the dark for 40 min at 4°C . The cells were then fixed in 100 μl of 2% paraformaldehyde overnight at 4°C . Following fixation, cells were permeabilized in 100 μl of 0.1% saponin for 1 min. The cells were then pelleted and resuspended in 100 μl of PBS. Fluorochrome-conjugated reagents specific to human and rhesus macaque IL-6 (MQ2-13A5) and IFN- γ (4S.B3) antigens, including appropriate isotype controls (BD PharMingen), were added to cell suspensions and incubated in the dark for 40 min at 4°C . Following staining, cells were centrifuged and resuspended in a 200 μl of 1% paraformaldehyde in PBS. The cells were then transferred to a 12- by 75-mm polystyrene round-bottom tube (Becton Dickinson, Franklin Lakes, N.J.) containing 300 μl of 1% paraformaldehyde (final volume of 500 μl). Vials containing the labeled cells were stored in the dark at 4°C for up to 4 days until tested by flow cytometry. A FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson) was used to perform the quantitation of stained lymphocyte populations. Gating was set based on the $\text{CD}3^{+}$ "bright" T lymphocyte population (see Fig. 4B). Proportions of IL-6^{+} or $\text{IFN-}\gamma^{+}$ cells in $\text{CD}4^{+}$ or $\text{CD}8^{+}$ cells (T helper or cytotoxic lymphocytes, respectively) were assessed by four-color analysis.

Statistical analysis. Cytokines measured in cell culture supernatants and $\text{CD}4^{+}$ and $\text{CD}8^{+}$ T lymphocytes in the mock-stimulated and rotavirus DL or TL particle-stimulated cells were compared by using a paired-sample Student *t* test with a two-tailed distribution.

RESULTS

Selection of juvenile rhesus macaques. The juvenile rhesus macaques used in this study were selected based on their clinical histories of rotavirus infection and age. Conventional animals were born as a part of timed-breeding program, and all were born within a 3-month period during the spring of 2002. The primary selection criterion for study enrollment was the absence of serum IgA to rotavirus, which is a known correlate of infection (44). A secondary criterion was the presence of low or undetectable quantities of serum rotavirus IgG. All adult rhesus macaques in our center that were evaluated were found to have high levels of rotavirus IgG, and this antibody is transferred transplacentally from mother to infant, where its levels in the blood gradually decay over the first 4 to 6 months of life. At the time of study enrollment, all 10 conventional animals exhibited undetectable levels of serum rotavirus IgA and low or undetectable levels of serum rotavirus IgG (42). Within 2 months after study enrollment, 8 out of 10 animals seroconverted to rotavirus and 6 displayed rectal shedding of the virus (42). Animals that shed rotavirus were the ones that also seroconverted. The cell-mediated immune responses to rotavirus

TABLE 1. TUCH rotavirus rectal shedding in stool samples of three experimentally inoculated, C-section-derived juvenile rhesus macaques as demonstrated by ELISA

p.i.d.	Mean ELISA shedding titer $\pm$ SEM (ng/ml) ^a		
	RM 1	RM 2	RM 3
0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
1	9 $\pm$ 13	443 $\pm$ 595	0 $\pm$ 0
2	4,304 $\pm$ 5,837	16,657 $\pm$ 9,678	38,989 $\pm$ 28,211
3	39,774 $\pm$ 6,159	6,835 $\pm$ 3,817	47,209 $\pm$ 31,601
4	49,886 $\pm$ 12,623	7,854 $\pm$ 1,374	58,131 $\pm$ 15,548
5	55,810 $\pm$ 6,837	6,918 $\pm$ 714	63,555 $\pm$ 11,887
6	43,297 $\pm$ 2,099	1,274 $\pm$ 919	43,965 $\pm$ 22,101
7	17,360 $\pm$ 6,961	77 $\pm$ 33	3,603 $\pm$ 2,030
8	1,256 $\pm$ 258	1,577 $\pm$ 1,027	1,156 $\pm$ 1,597
9	764 $\pm$ 319	697 $\pm$ 682	352 $\pm$ 327
10	113 $\pm$ 132	222 $\pm$ 271	507 $\pm$ 198
11	27 $\pm$ 38	36 $\pm$ 8	477 $\pm$ 88
12	0 $\pm$ 0	37 $\pm$ 6	396 $\pm$ 55
13	0 $\pm$ 0	5 $\pm$ 9	87 $\pm$ 102
14	0 $\pm$ 0	0 $\pm$ 0	6 $\pm$ 0

^a The average value of rotavirus antigen shedding (nanograms per milliliter of a 20% stool suspension) was calculated based on values generated with three or four stool samples collected daily from each animal. RM, = rhesus macaque.

were studied in three animals with histories of both seroconversion and shedding. In order to also include negative and early stages of the cell-mediated immune response, three C-section-derived, lactogenic-immunity-deprived animals were used. In contrast to conventional juveniles, these animals were housed in a rotavirus-free environment, where they were experimentally inoculated with TUCH rotavirus at 3 weeks of age. Within 2 days after experimental inoculation, all three juveniles shed high levels of rotavirus antigens in their stools as detected by ELISA (Table 1). These high shedding levels persisted for 5 to 7 days in each animal, indicating acute infection

and replication of rotavirus in gastrointestinal tract. Interestingly, no clinical symptoms of diarrhea and/or dehydration were seen in any of the animals. The shedding gradually dropped to undetectable (two animals) or to a very low level (one animal) within 2 weeks postinoculation (Table 1).

Expansion of a new simian rotavirus (TUCH) and its in vitro antigen presentation. The TUCH rotavirus, isolated from a fecal specimen of a naturally infected macaque, was expanded in vitro in order to harvest and purify DL and TL particles. Purified and concentrated particles were used to restimulate peripheral T lymphocytes from convalescent animals. Antigen presentation was conducted in vitro by the use of monocyte-derived HLA-DR⁺-differentiated DCs (Fig. 1A). DCs were derived from monocytes in the presence of granulocyte-macrophage colony-stimulating factor (100 ng/ml) and human IL-4 (50 to 100 ng/ml). Aliquots of cultured DCs were harvested at day 5 and phenotyped by fluorescence-activated cell sorting. These cells were CD14 negative, CD11c bright, and HLA-DR bright positive (not shown). The differentiated DCs were then treated with purified, inactivated rotavirus particles for 12 h. Subsequently, treated and untreated DCs were harvested, washed, and cocultured with lymphocytes for 4 to 5 days. T-cell proliferation was assayed by thymidine incorporation. The presence of rotavirus antigen in DCs was visualized by confocal microscopy (Fig. 1A). The colocalization of rotavirus antigens and HLA-DR cell surface antigens was detected by spectral overlap of the two respective fluorochromes and was observed in virtually all rotavirus-positive cells (Fig. 1A). This indicated that DCs were involved not only in trapping but also in active antigen presentation of rotavirus particles to T cells (Fig. 1B). No differences were seen between the cultures restimulated by DL or TL particles. No virus replication and/or cytopathic effect was noted by visual inspection of the in vitro

FIG. 1. Confocal microscopy of rotavirus antigen-presenting DCs. (A) Spectral overlap of viral (green, TUCH rotavirus) and cell surface (red, HLA-DR) antigens suggested that DCs were involved in not only virus trapping but also in antigen presentation. Nuclear DNA (ToPro3) appears blue. Magnification, $\times 200$. (B) Cocultures of DCs loaded with rotavirus antigens (small arrow) and purified T lymphocytes (large arrow). No cytopathic effect was noted in any of the cultures of DCs. Magnification, $\times 200$.

FIG. 2. Lymphocyte proliferation upon stimulation with TUCH rotavirus-loaded DCs. Dose-dependent incorporation of [3 H]thymidine is shown for ED29 (A) and EA42 (B) lymphocyte cultures, derived from two conventional juvenile rhesus macaques with a history of rotavirus infection. Lymphocytes (a constant number) were stimulated with serially diluted DCs containing either negative control (DC) protein or rotavirus particles (DC + TUCH). Minimal or no T-cell proliferation was detected in control cultures in which cells were stimulated with rotavirus-free DCs.

cultures prior to or after addition of nonadherent cells (purified autologous T lymphocytes).

Lymphocyte proliferation activity. Lymphocyte proliferation was measured upon stimulation with TUCH rotavirus in two animals (ED29 and EA42) with the largest amounts of available T lymphocytes and differentiated TUCH rotavirus (TL)-loaded DCs (Fig. 2). When rotavirus-loaded, serially diluted DCs were cocultured in vitro with equal amounts of nonadherent cells, T-cell proliferation was observed (Fig. 2). By increasing the number of rotavirus-loaded DCs as well as the ratio between antigen-presenting cells (DCs) and T lymphocytes, an increase in T-cell proliferation was measured in both animals by incorporation of [3 H]thymidine (Fig. 2). Minimal or no T-cell proliferation was detected following the coculture of rotavirus-negative control DCs and autologous T lymphocytes (Fig. 2).

Detection of IL-6 and IL-12 cytokines in cell culture supernatants. We next measured the presence of the common inflammatory mediator IL-6, which is known to exert multiple functions, including differentiation of B cells as well as growth, activation, and differentiation of CD8 $^{+}$ cytotoxic T cells and activation of CD4 $^{+}$ T cells. In addition, we looked for the presence of IL-12 as a representative of cell-mediated immune response and inflammation. The supernatants harvested from the proliferating cell cultures contained measurable levels of both IL-6 (Fig. 3A) and IL-12 (Fig. 3B) cytokines. Differences between negative (DCs without rotavirus) mock control and rotavirus-stimulated cultures were significant ($P < 0.05$). Additionally, a trend ($P > 0.05$) toward higher cytokine levels (both IL-6 and IL-12) in cultures stimulated with TL compared to DL particles was observed (Fig. 3).

FIG. 3. Detection of liquid-phase IL-6 and IL-12 from TUCH rotavirus-stimulated lymphocyte cultures. IL-6 levels (A) and IL-12 levels (B) detected in lymphocyte cultures that were stimulated with DCs containing either negative control (DC) protein or DL or TL TUCH rotavirus particles are shown. Values are averages and standard errors of the means of results for three conventional juvenile macaques.

Detection of IL-6 and IFN- γ cytokines in CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes. The relatively high levels of extracellular IL-6 were corroborated by intracellular staining of CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes. In addition, IFN- γ -secreting T lymphocytes were examined by the same method. The two major T-cell populations were identified among the cells harvested from negative (mock) control, positive (mitogen) control, and TUCH (DL or TL) rotavirus-stimulated cultures by gating through CD3 $^{+}$ lymphocytes (Fig. 4).

No IFN- γ - and only very few IL-6-secreting T lymphocytes (predominantly CD4 $^{+}$) were detected at p.i.d. 3 and 10 in three rotavirus-inoculated, C-section-derived animals (Fig. 4C). No cytokine production was detected prior to rotavirus exposure at p.i.d. 0 (Fig. 4C).

Both the CD4 $^{+}$ and CD8 $^{+}$ cells collected from all three conventional juvenile animals with prior histories of rotavirus infection showed small fractions of IFN- γ $^{+}$ and IL-6 $^{+}$ cells

FIG. 4. Detection of intracellular IL-6 in CD4 $^{+}$ T cells from TUCH rotavirus-stimulated lymphocyte cultures. (A and B) Gating through lymphocytes (A) followed by CD3 $^{+}$ T cells (B). (C) Flow cytometry dot plots showing the proportions CD4 $^{+}$ IL-6 $^{+}$ T cells from C-section-derived juvenile rhesus macaques (RM1 to -3) at p.i.d. 0, 3, and 10 as measured by flow cytometry in cultures of T cells that were stimulated with DCs containing TUCH rotavirus.

TABLE 2. Cytokine production by CD4⁺ (T-helper) and CD8⁺ (cytotoxic T) lymphocytes in PBMCs from three conventional juvenile rhesus macaques with a history of rotavirus infection

Animal	Antigen ^a	% ^b			
		T-helper Lymphocytes		Cytotoxic T lymphocytes	
		CD4 ⁺ IFN- γ ⁺	CD4 ⁺ IL-6 ⁺	CD8 ⁺ IFN- γ ⁺	CD8 ⁺ IL-6 ⁺
1	N	0.1	0.4	0.0	0.0
	P	4.5	15.2	8.6	32.8
	DL	1.2	3.0	1.9	2.5
	TL	3.8	3.8	1.8	3.4
2	N	0.05	0.6	0.01	0.2
	P	3.7	12.1	7.3	25.0
	DL	0.6	4.2	0.6	3.9
	TL	0.9	4.7	0.7	4.2
3	N	0.1	0.7	0.01	0.3
	P	3.3	9.2	9.0	7.9
	DL	2.0	5.1	0.3	1.7
	TL	1.8	4.9	0.8	2.0
Mean $\pm$ SEM	N	0.1 $\pm$ 0.02	0.6 $\pm$ 0.1	0.01 $\pm$ 0.01	0.2 $\pm$ 0.1
	P	3.8 $\pm$ 0.6	12.2 $\pm$ 3.0	8.3 $\pm$ 0.9	21.9 $\pm$ 12.7
	DL	1.3 $\pm$ 0.6	4.1 $\pm$ 0.9	0.9 $\pm$ 0.7	2.7 $\pm$ 0.9
	TL	2.2 $\pm$ 1.2	4.5 $\pm$ 0.5	1.1 $\pm$ 0.5	3.2 $\pm$ 0.9

^a N, negative control; P, positive control, DL, DL TUCH rotavirus particle; TL, TL TUCH rotavirus particle.

^b Values are representatives those for of three animals from which PBMCs were collected and stimulated in vitro with the indicated antigen.

(Table 2). While average numbers of IFN- γ ⁺ and IL-6⁺ cells in both T-cell subsets did not surpass 5% (Table 2), the increase in the number of rotavirus-stimulated, cytokine-positive cells over negative (mock) control stimulated cells was significant for both cytokines ($P < 0.05$) and T-cell subsets (Table 2). No statistical difference was found between the T lymphocytes stimulated by DL or TL rotavirus particles, although cells stimulated by TL particles exhibited a slightly higher proportion of cytokine-positive cells (Table 2). When rotavirus particles were used for stimulation of cultured T lymphocytes directly, without being presented via differentiated DCs, fewer IFN- γ ⁺ or IL-6⁺ cells were detected (results not shown).

DISCUSSION

An extensive body of evidence on cross-reactivities and functional similarities of numerous human immunological markers and their rhesus macaque analogues has accumulated during recent years (7, 19, 30). It was noted that remarkably rapid development of both CD4⁺ and CD8⁺ T-cell subsets toward immunological memory occurs in rhesus macaques during the first few months of postnatal life (30). Since rhesus macaques are now being extensively used as models for vaccine development, it is important to elucidate the relatedness between immune responses in macaques and humans. This study is the first to examine cell-mediated immune responses in any NHP following rotavirus infection.

We recently reported that in a majority of conventionally reared, captive adult rhesus macaques with symptoms of chronic diarrhea, rotaviruses were not associated with clinical disease (35). Although a high incidence of recurring diarrhea has been observed in captive rhesus macaques (8, 27, 33, 35), it has been linked to the presence and cumulative effect of a

number of bacterial and parasitic pathogens that are known to cause human illness as well (3, 6, 16, 22, 35, 37, 38). Because of this, combined with the findings that very little rectal shedding of rotavirus has been observed in conventionally reared captive adult rhesus macaques but that these animals invariably have high levels of serum antibodies, it seems likely that rotavirus infections in adult macaques are primarily if not solely subclinical (35). Consistent with this premise, we investigated cell-mediated immunological correlates of rotavirus infection of <1-year-old juvenile rhesus macaques rather than adult animals.

In order to study recall immune responses to rotavirus in vitro, we used either DL or TL virus particles derived from our new simian rotavirus isolate (TUCH) for restimulation of T lymphocytes. Differentiated cultures of monocyte-derived DCs were used for rotavirus antigen presentation (26). T lymphocytes were derived from peripheral blood of convalescent juvenile macaques with a recent history of rotavirus infection. In rotavirus-infected humans and rodents, both CD4⁺ and CD8⁺ T lymphocytes were found to contribute to virus clearance (12, 18, 24, 32). In addition, there is some evidence to suggest that rotavirus VP6 T-helper cell epitopes may be able to form ligands with CD4⁺ cells derived from different host species (2). Moreover, we have reported that CD4⁺ T lymphocytes are the only effector cells needed to elicit protection after mucosal immunization with VP6 and adjuvant in the mouse challenge model (24). It follows, therefore, that rotavirus vaccine design may shift from primarily targeting the traditional humoral immune responses toward more actively targeting cell-mediated responses in future studies.

In our study, several potential candidates for rotavirus-associated cell-mediated immune responses were tested. Cytokines measured in cell culture supernatants or through intracellular

staining of CD4⁺ and CD8⁺ T lymphocytes were selected based on their relevance in controlling viral infections (IFN- γ), mucosal IgA responses (IL-6), and cell-mediated responses (IL-12) (34, 39, 40). Previously, elevated levels of both IL-6 and IFN- γ were reported in studies with rotavirus-infected human patients (12, 13, 15, 31). In a study with IL-6-deficient mice, it was found that both IL-6⁻ and IL-6⁺ mice were able to clear rotavirus infection associated with the production of virus-specific IgA (39). This finding, however, does not negate the role that IL-6 plays during the promotion of IgA production. One interpretation is that in addition to IL-6, there are other alternative cytokine pathways that lead to the development of rotavirus-specific IgA-secreting B cells. More recent evidence generated in studies with rotavirus-infected children suggests that IL-6 is one of the cytokines positively correlated with recovery time (13, 31). Interestingly, it has been suggested that in patients with possible rotavirus-induced encephalopathy, IL-6 may positively correlate with levels of nitric oxide in serum and cerebrospinal fluid and with convulsive seizures (15). In our study, IL-6 secretion was detected from both CD4⁺ and CD8⁺ T lymphocyte subsets obtained from the peripheral blood of juvenile rhesus macaques with a history of rotavirus infection. While stimulation of these cells with negative control (mock) antigen resulted in no or very little IL-6 production, stimulation with a positive (mitogen) control and/or rotavirus resulted in significantly increased levels of IL-6 as measured by both ELISA and intracellular flow cytometry. These findings are consistent with the aforementioned studies that indicate the importance of IL-6 during rotavirus infection.

Although rotavirus has been defined as a poor inducer of IFN- γ secretion from memory T cells in vitro (12), in naturally exposed children with rotavirus diarrhea, significantly higher levels of IFN- γ have recently been reported (1, 13). Experiments with a mouse model demonstrated that mice deficient in IFN- γ are still capable of generating an immune response to rotavirus and resolving an infection (40). In our study with naturally infected juvenile macaques, moderate but consistent levels of IFN- γ ⁺ cells were found in both CD4⁺ and CD8⁺ T lymphocytes upon in vitro stimulation with TUCH rotavirus. Since juvenile macaques are exposed to a much broader spectrum of pathogens than their human counterparts, it is possible that persistence of rotavirus memory T cells is more robust in juvenile macaques than in children. We were able to detect these T-cell recall responses at 2 months after detection of rectal viral shedding, which is approximately two times longer than in an analogous study with human CD4⁺ and CD8⁺ cells (12). In our study, however, it was not possible to determine whether the rotavirus that was shed in the feces of naturally infected animals was the result of primary or secondary exposure. Furthermore, the different means of antigen presentation utilized by the immunological assays in our study compared to those used in the human study (12) may also have contributed to the observed differences.

In addition to IL-6 and IFN- γ secretion, we evaluated lymphocyte proliferation in the two animals with the highest numbers of available PBMCs. The intensity of lymphocyte proliferation was directly related to the number of antigen-presenting cells that were loaded with rotavirus. The novelty of the present study was that differentiated cultures of DCs were

utilized for rotavirus antigen presentation. Increased levels of IL-12 in lymphocyte cultures likely reflected T-cell interactions with antigen-presenting DCs, as these have previously been reported to correlate with IL-12 levels (34).

Here we report for the first time that there are similarities between rhesus and human immune responses to rotavirus at the cellular level. Our findings are consistent with studies conducted with human patients (12, 18) and those generated with an adult mouse model of rotavirus infection (24, 32) that identified CD4⁺ and/or CD8⁺ T lymphocytes as the effectors of rotavirus-induced immune responses. Furthermore, trials to evaluate the efficacy of novel rotavirus vaccine candidates in experimentally inoculated juvenile macaques are currently being conducted.

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