Characterization of Homologous and Heterologous Rotavirus-Specific T-Cell Responses in Infant and Adult Mice

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Received 10 November 2004/Accepted 15 January 2005

During primary rotavirus (RV) infection, CD8+ T cells play an important role in viral clearance as well as providing partial protection against reinfection. CD4+ T cells are essential for maximal development of RV-specific intestinal immunoglobulin A. In this study, we took advantage of the cytokine flow cytometry technique to obtain a detailed map of H-2d- and H-2b-restricted CD8+ and CD4+ T-cell epitopes from the RV proteins VP6 and VP7. Three new CD8+ T-cell epitopes (H-2d and H-2b restricted) and one new CD4+ T-cell epitope (H-2d and H-2b restricted) were identified. Using these newly identified targets, we characterized the development and specificity of cellular immune responses in C57BL/6 and BALB/c mice during acute infection of infants and adults. We found that both the CD4+ and CD8+ responses peaked on days 5 to 7 after infection and then declined rapidly. Interestingly, both the response kinetics and tissue distributions were different when epitopes on VP6 and VP7 were compared. VP6 elicited a response which predominated in the intestine, while the response to VP7 was more systemic. Additionally, the T-cell responses elicited after homologous versus heterologous infection differed substantially. We found that during homologous infection, there was a greater response toward VP6 than that toward VP7, especially in the intestine, while after heterologous infection, this was not the case. Finally, in suckling mice, we found two peaks in the CD8+ response on days 7 and 14 postinfection, which differed from the single peak found in adults and likely mimics the biphasic pattern of rotavirus shedding in infant mice.

Rotavirus (RV) is the principal cause of severe diarrhea in young children worldwide, causing approximately 352,000 to 592,000 deaths a year (36). Further development and/or evaluation of effective RV vaccines depends upon a better understanding of the roles that various immune effectors play in protective immunity and identifying protective antigens that are recognized by these effector cells.

Although it has been shown in the murine model that antibodies are the principal mediators of protection against RV reinfection (11, 12, 29), T cells also play an important role in the RV-specific immune response. CD4+ T cells are essential for the development of more than 90% of the RV-specific intestinal immunoglobulin A (IgA) (11). Moreover, after intranasal immunization with a VP6 chimeric protein, CD4+ T cells are the only cells necessary to confer protection from reinfection (30). In addition, a VP6 T helper epitope has been identified in prior studies (1, 8).

Murine RV-specific CD8+ T cells have a direct antiviral effect, being involved in the timely resolution of primary RV infection and mediating partial short-term protection against reinfection (12, 15, 29). VP7, a glycoprotein that is the major constituent of the outer RV layer, has been shown to be the primary target for cross-reactive RV-specific cytotoxic T lymphocytes (CTLs) in C57BL/6 mice (33, 34). Likewise, VP6 and several other viral proteins, including NSP1 and VP3, can also be targets for CTLs (13, 18, 33). Previous epitope mapping studies using vaccinia virus recombinants expressing the VP6 and VP7 genes identified two Kb-restricted epitopes and one Kb-restricted epitope in VP7 and one Kb-restricted epitope in VP6 (4, 13, 14). However these previous epitope mapping studies did not provide quantitative data on the RV antiviral response, and little is known about the ontogeny, kinetics, and magnitude of the RV-specific CD4+ and CD8+ T-cell responses in humans or any other animal species.

Recently, in order to better identify and monitor specific T-cell responses, several new approaches have been developed which offer advantages compared to more traditional techniques like classic cytotoxicity and proliferation assays. These recently employed techniques include flow cytometry-based major histocompatibility complex (MHC) tetramer staining to directly enumerate virus-specific T cells, intracellular cytokine staining (ICS), and enzyme-linked immunospot (ELISPOT) assays, which detect cytokine secretion in response to specific antigen stimulation. Additionally, it has been shown that it is possible to map CD4+ and CD8+ T-cell epitopes by using pools of overlapping peptides representing the entire antigen sequence to stimulate gamma interferon (IFN-γ) production, as measured by intracellular staining or ELISPOT (23). This technique obviates the need for MHC-matched cell lines and the culturing of effector cells prior to assay and can be used in all samples regardless of HLA type.

Using both newly and previously described H-2d- and H-2b-restricted CD8+ and CD4+ T-cell epitopes from the RV proteins VP6 and VP7, we now report the tissue distribution and kinetics of the T-cell response after both homologous (murine RV in mice) and heterologous (non-murine RV in mice) infections in both adult and suckling mice.
Materials and Methods

Viruses. A stock of homologous wild-type murine RV (EC) was prepared as an intestinal homogenate, and the titer was determined as PFU per milliliter (19). Tissue-culture-adapted heterologous rhesus RV (RRV) was prepared as previously described, and the titer was determined as PFU per milliliter (19).

Mice and viral infection. C57BL/6 (H-2b) and BALB/c (H-2d) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) and bred in the Palo Alto Veterans Administration breeding facility. Suckling mice and 6- to 8-week-old female mice were inoculated by gastric gavage with (per mouse) 10^4 ID_50 of murine (EC) RV or 10^5 PFU of RRV. Prior to infection, adult mice received 100 μl of 1.33% sodium bicarbonate to neutralize stomach acid. Mice were sacrificed at indicated times after infection. All studies were approved by the Institutional Animal Care Committee.

Detection of anti-RV antibodies. For detection of RV-specific IgA and IgG in serum and stool samples, an enzyme-linked immunosorbent assay (ELISA) was carried out as previously described (10).

Isolation of lymphocytes. At the selected times after infection (days 3, 5, 7, 10, and 15), adult mice were sacrificed by cervical dislocation and suckling mice were sacrificed by CO2 inhalation. Lymphocytes from spleen, mesenteric lymph nodes (MLNs), and Peyer’s patches (PPs) were isolated by mechanical disruption through a wire mesh. Erythrocytes (RBCs) in the spleen cell suspension were lysed with a commercial lysis buffer (Sigma-Aldrich, St. Louis, Mo.). Cells were washed twice with RPMI 1640 (Gibco BRL, Gaithersburg, Md.) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL) and 1 mM EDTA and stirred gently on a magnetic stirrer for 10 min at 37°C. The supernatant was then removed and centrifuged, and RBCs were lysed. An equal volume of 2% Dextran T500, and incubating the mixture at 37°C for 20 min. The supernatant was then removed and centrifuged, and RBCs were lysed.

For isolation of intestinal intraepithelial lymphocytes (IELs), small intestines from groups of two or three mice were pooled, cleared of PPs, longitudinally opened, and cut into 1-cm segments. Intestines were then incubated in RPMI 1640 containing 2% FCS, 100 μg of penicillin/ml, 100 μg of streptomycin per ml, and 1 mM EDTA and stirred gently on a magnetic stirrer for 10 min at 37°C. The supernatant, containing IELs and epithelial cell debris, was centrifuged at 400 × g for 5 min at 4°C, and the cells were then resuspended in RPMI 1640—2% FCS and passed through glass wool. The cell suspension was centrifuged and resuspended in 40% (vol/vol) Percoll (Amersham Pharmacia Biotech, Piscataway, N.J.). A discontinuous Percoll gradient made by layering 3 ml of the cell suspension above 2 ml of 75% Percoll (vol/vol) in a 15-ml tube was centrifuged at 600 × g for 30 min at room temperature. Cells were recovered from the 40 to 75% interface and washed twice with complete medium.

For isolation of fetal liver lymphocytes (PLLs), small intestines from groups of two or three mice were pooled, cleared of PPs, longitudinally opened, and cut into 1-cm segments. Intestines were then incubated in RPMI 1640 containing 2% FCS, 100 μg of penicillin/ml, 100 μg of streptomycin per ml, and 1 mM EDTA and stirred gently on a magnetic stirrer for 10 min at 37°C. The supernatant containing IELs and epithelial cell debris was centrifuged at 400 × g for 5 min at 4°C, and the cells were then resuspended in RPMI 1640—2% FCS and passed through glass wool. The cell suspension was centrifuged and resuspended in 400 × g for 5 min at 4°C, and the cells were then resuspended in 40% (vol/vol) Percoll (Amersham Pharmacia Biotech, Piscataway, N.J.). A discontinuous Percoll gradient made by layering 3 ml of the cell suspension above 2 ml of 75% Percoll (vol/vol) in a 15-ml tube was centrifuged at 600 × g for 30 min at room temperature. Cells were recovered from the 40 to 75% interface and washed twice with complete medium.

Isolation of peripheral blood mononuclear cells (PBMCs) was performed by diluting blood in 1 mM EGTA in phosphate-buffered saline (PBS), adding an equal volume of 2% Dextran T500, and incubating the mixture at 37°C for 20 min. The supernatant was then removed and centrifuged, and RBCs were lysed. All lymphocytes were allowed to recover in complete medium supplemented with 100 U of recombinant human interleukin-2 (IL-2) per ml (Pharmingen, La Jolla, Calif.) for 1 h at 37°C with 5% CO2 before stimulation with peptides.

Peptide and mixing of peptides. Fifteen-amino-acid-residue (15-mer) peptides overlapping by 9 amino acids were synthesized to span the entire amino acid sequence of the RV proteins VP6 and VP7. This required 97 peptides for VP6 and 79 peptides for VP7. The sequence of the homologous RVV was used as a template, and in the regions where little or no homology was found with the sequence of the murine strain EDIM, peptides were synthesized with the murine sequence (i.e., for VP6, the regions between amino acids 25 and 67, 160 and 183, 205 and 319, and 383 and 397 and for VP7 the regions between amino acids 1 and 51, 77 and 111, 133 and 163, and 201 and 239). Overall, the homology of the sequence of VP6 and VP7 used in this study was 94 and 91%, respectively, when compared to that of EDIM.

Peptides were reconstituted at 50 mg/ml in 100% dimethyl sulfoxide (DMSO). If necessary, 1 to 2 additional volumes of DMSO were added to fully dissolve those peptides that did not dissolve initially. Complete pools for VP6 and VP7 contained all reconstituted peptides of the respective set at equal concentrations (0.51 mg/ml for VP6 and 0.63 mg/ml for VP7). Smaller pools (subpools) containing 8 to 10 peptides (final concentration of each peptide, 5 to 6.25 mg/ml) were made in order to identify specific epitopes in both VP6 and VP7 by an intersecting checkerboard strategy as previously described (23, 26, 39). Briefly, row and column subpools (R-pool and C-pool, respectively) were arranged such that a checkerboard is formed. Subpools were made so that each 15-mer was contained in exactly one R-pool and one C-pool and ensuring that no overlapping peptides were contained in the same subpool. Therefore, if one C-pool and R-pool could stimulate a positive T-cell response, the common 15-mer at the intersection would be a possible T-cell epitope candidate. In order to test individual 15-mer peptides, reconstituted peptides were dissolved in 10% DMSO at a final concentration of 1 mg/ml. Finally, in order to do fine epitope mapping, 10-amino-acid-residue peptides (10-mers) overlapping by 9 amino acids were designed to span the 15-mer sequences identified as positive in the initial screen and were produced (Genspeed Synthesis, Inc., South San Francisco, Calif.). The 10-mers were solubilized in 100% DMSO at a concentration of 10 mg/ml and then diluted in PBS at a final concentration of 1 mg/ml. Pools, subpools, and individual peptides were aliquoted and stored at −80°C.

In the assay, peptides were used at a final concentration of 2 to 10 μg/ml and the DMSO concentration was kept below 0.5% (vol/vol) in all final assay mixtures.

Intracellular IFN-γ staining and flow cytometry analysis. To enumerate the number of IFN-γ-producing cells, 10^6 PBMCs was performed as previously described (32). Briefly, lymphocytes were incubated for 6 h in complete medium supplemented with 100 U of recombinant human IL-2 per ml and GolgiPlug (containing brefeldin A; BD Biosciences), which was added during the last 4 h. Cells were stimulated with either peptide pools, subpools, or individual peptides. Phorbol myristate acetate (PMA) (50 ng/ml; Sigma; ionomycin (500 ng/ml; Sigma) was used as a positive control, and DMSO was used as a negative control. After culture, the cells were harvested and washed once. Cells were then blocked with purified anti-FcγRIII monoclonal antibody (2.4G2; Pharmingen) and surface stained with anti-CD8α (53-6.7) conjugated with fluorescein isothiocyanate (FITC) and anti CD4 (RM4-5)-PerCP all from Pharmingen (San Diego, Calif.). In some experiments, cells were also stained with αβT (DAK 32) conjugated with allophycocyanin (APC); CD44 (IM7)-biotin, i-selectin (MEL-14)-APC; CD27 (LG.3A10)-APC; CD99 (H1.2F)-phycoerythrin (PE), T-cell receptor (TCR) β-chain (H57-597)-APC, and CD8 (53.5-8)-biotin. Cells were then permeabilized with the Cytofix/Cytoperm Kit (BD Biosciences) following the manufacturer’s instructions and stained with anti-IFN-γ (XMG1.2)-PE. After staining, the cells were washed once and fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Washington, Pa.). At least 500,000 cells were acquired, and four-color flow cytometry was performed with a FACSCalibur (BD Sciences) and analyzed with CellQuest software, version 3.1. Dead cells were excluded by forward and side scatter characteristics.

Statistical analysis. Statistical analysis was performed with StatView software (SAS Institute, Cary, N.C.). The background level (i.e., activity in the absence of peptides [DMSO] or using irrelevant peptides) of IFN-γ staining varied from tissue to tissue and animal to animal but was typically bellow 0.2% for both CD8+ and CD4+ lymphocytes. Only samples in which the IFN-γ staining was at least twice that of the background were considered positives. All values are reported after subtraction of the background-level staining. Differences between groups were evaluated with the nonparametric Mann-Whitney test. Significance was established if P was <0.05. Data are shown as the mean ± standard error.

Results

Mapping of H-2b-restricted CD8+ T-cell epitopes in RV VP6 and VP7. It has been shown previously that RV-specific CD8+ T cells can be detected following infection in C57BL/6 mice (35). In the present study, in order to identify CD8+ T-cell epitopes in RV proteins VP6 and VP7, C57BL/6 females were orally infected with murine RV (EC). These two proteins were chosen for detailed analysis because they appear to be highly immunogenic and VP7 has been shown to be a dominant target of the murine T-cell response against RV (34). Initially, splenocytes and MLN lymphocytes were harvested 7 days after infection.
infection, when antiviral CD8$^+$ responses frequently peak (7, 32) and restimulated in vitro for 6 h with complete pools of overlapping peptides corresponding to the entire sequence of VP6 and VP7, followed by intracellular IFN-γ staining. As shown (Fig. 1b), we were able to detect a CD8$^+$ T-cell response against both VP6 and VP7 pools but not a CD4$^+$ T-cell response. It appeared that most (>90%), if not all, of the responsive CD8$^+$ T cells were in the population of "large lymphocytes" as defined by forward scatter versus side scatter (Fig. 1a). For this reason, we have only presented results gating on this population of lymphocytes. Spleen and MLN cells from noninfected mice did not produce IFN-γ after stimulation with any of the peptides (data not shown), and spleen cells from RV-infected mice did not produce IFN-γ when stimulated with DMSO (Fig. 1b), confirming the specificity of the ICS.

For screening of VP7 epitopes, 18 C- and R-pools, as detailed in Materials and Methods, were tested. Four subpools (C4, C6, R7, and R9) elicited high IFN-γ responses in CD8$^+$ T cells (3.88% ± 0.38%, 3.59% ± 0.36%, 4.5% ± 0.84%, and 3.75% ± 1.12%, respectively) followed by three subpools (C3, C8, and R1), which elicited lower responses (1.02% ± 0.3%, 1.22% ± 0.21%, and 1.34% ± 0.04%, respectively) at day 7 postinfection (Fig. 2). The remaining subpools, as exemplified by subpool C5 in Fig. 2, were negative. Specific antigenic target regions were identified by finding the common individual 15-mer in the positive intersecting subpool checkboard. All 15-mers common to two positive subpools were tested, and four were able to stimulate an IFN-γ response. Of these, two were the overlapping 15-mers VP7$^{281-295}$ and VP7$^{285-299}$ and the other two were also overlapping peptides (VP7$^{29-43}$ and VP7$^{33-47}$). This indicated that CD8$^+$ T-cell target epitopes were present in these two overlapping regions. To fine map the epitopes within these 15-mers, 10-mers spanning the entire sequences from VP7$^{281-295}$ and VP7$^{29-47}$ and overlapping by 9 amino acids, were tested. The 10-mers VP7$^{286-295}$, VP7$^{287-296}$, and VP7$^{288-297}$ elicited clear IFN-γ responses, the highest being to the 10-mer VP7$^{286-295}$ (3.43% ± 0.14%). Also, the 10-mers VP7$^{31-40}$, VP7$^{32-41}$, and VP7$^{33-42}$, induced clear responses, the highest being with VP7$^{33-42}$. Of note, the percentages of CD8$^+$ T cells stimulated by the entire VP7 pool, the more positive subpools, the common 15-mers VP7$^{281-295}$ and VP7$^{285-299}$ and the 10-mer VP7$^{286-295}$ were similar or identical (Fig. 2). Interestingly, the 10-mer VP7$^{286-295}$ did not contain a peptide carrying the canonical motif for H2-D$^b$-restricted epitopes (N in position 5 and M or L at the C terminus) (9), but computational analysis using the SYFPEITHI and BIMAS databases identified within the VP7$^{286-295}$ sequence the nonamer VNWKKWWQV (residues 287 to 295) as a potential D$^b$ binder. The sequence of this epitope is highly conserved among different group A RV strains (Table 1), and it is located in a region that has been reported as a conserved domain between RV groups A, B, and C (22). We also confirmed the presence of the K$^b$-restricted epitope IVYRFLFV, residues 33 to 40, which was previously identified (14). Interestingly, VP7$^{286-295}$ gave a substantially stronger response than the previous identified VP7$^{33-42}$ with 3.4% ± 0.14% versus 1.1% ± 0.34% of CD8$^+$ T cells responding on day 7 postinfection (Fig. 2).

![Image](http://jvi.asm.org/)

**FIG. 1.** VP6- and VP7-specific CD8$^+$ and CD4$^+$ T-cell responses in adult C57BL/6 mice after oral EC infection. Splenocytes from EC-infected C57BL/6 mice were harvested 7 days after primary infection. Cells were restimulated with the complete overlapping peptide pools representing the entire VP6 or VP7 sequence, and responses were visualized by intracellular IFN-γ staining. Positive responses were exclusively detected in the large lymphocyte gate (forward scatter [FSC] versus side scatter [SSC]) (a). For analysis, cells were gated on large CD4$^+$ or CD8$^+$ lymphocytes. PMA and DMSO were used to restimulate cells as positive and negative controls, respectively. The percentage of CD8$^+$ IFN-γ$^+$ or CD4$^+$ IFN-γ$^+$ T cells is indicated in the top right corner of each plot (b). These data are derived from at least three independent experiments with two to three mice per group.
Using the same strategy, two H-2 b-restricted epitopes were mapped in VP6 as well. Fine epitope mapping, using 10-mers identified from the overlapping subpools, restricted the VP6 epitopes to VP6 53-62 and VP6 357-366. Using the SYFPEITI and BIMAS databases, a D b-restricted epitope was predicted within the VP6 53-62 sequence (NLPLRNWNFD) and a K b-restricted epitope was predicted in the VP6 357-366 sequence (VGPVFPPGM). The latter epitope corresponds to a previously identified H-2 b-restricted epitope in VP6 (13). The IFN-γ responses elicited by these two epitopes were very similar at day 7 postinfection in the spleen (0.68% ± 0.12% versus 0.73% ± 0.23%; data not shown).

Tissue distribution and kinetics of CD8+ T-cell response after RV homologous infection in adult mice. Once class I-restricted epitopes in both VP6 and VP7 were identified, we sought to determine the distribution of RV CD8+ T cells following acute homologous murine RV infection of the gastrointestinal tract in adult mice. We detected significant numbers of CD8+ T cells in each tissue we examined on day 7 postinfection (Fig. 3). The highest frequencies of RV CD8+ T cells were found in the liver, followed by lung, spleen, and intestinal lamina propria. This result correlates well with recent findings using VP6 and VP7 tetramers to monitor infection with the murine strain EDIM in adult mice (28). After RV infection, we detected an overall increase in the level of total CD8+ T cells in the liver (data not shown) on days 5 and 7 postinfection, but by day 10, the percentage returned to normal levels. No significant changes were observed in any other tissue (data not shown). Non-antigen-specific increases in CD8+ T cells have also been observed following LCMV infection (6). In addition, in our RV-infected mice, hematoxylin and eosin staining of liver sections at days 5 and 7 postinfection revealed mononuclear inflammatory infiltrates in the liver parenchyma remote from portal tracts.

<table>
<thead>
<tr>
<th>Strain</th>
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<tr>
<td>Wa</td>
<td>Human</td>
<td>1</td>
<td>VNWKKWQWQV</td>
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<tr>
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<td>Human</td>
<td>2</td>
<td>VNWKKWQWQV</td>
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<tr>
<td>SA11</td>
<td>Simian</td>
<td>3</td>
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<tr>
<td>RRV</td>
<td>Simian</td>
<td>3</td>
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<tr>
<td>EDIM</td>
<td>Mouse</td>
<td>3</td>
<td>INWKKWQWQV</td>
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<td>St Thomas 3</td>
<td>Human</td>
<td>4</td>
<td>VNWKKWQWQV</td>
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<td>OSU</td>
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<td>F45, WI-61</td>
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a Discrepant residues are in boldface.
We next examined the kinetics and tissue distribution of the RV CD8+ T-cell response following oral infection. Based on the data in Fig. 3 and the fact that RV primarily causes morbidity due to effects on the small intestine, we chose to monitor the T-cell response in the IEL intestinal compartment, PPs, MLNs, spleen, and liver. Since no significant differences were observed between LPL and IEL responses and numbers of CD8+ T cells are higher in the IELs, we elected to focus our study on the enteric effector response in the IEL compartment. The CD8+ T-cell response peaked within days 5 and 7 of infection in all tissues (at which time viral shedding had substantially decreased) and dropped quickly thereafter (Fig. 4). The newly identified VP6357-366 epitope had a very similar kinetic and quantitative response to VP6357-366 (data not shown), so we chose to only present the results obtained with the VP6357-366 epitope. The kinetics of VP6357-366 and VP733-42 specific CD8+ T cells were very similar in all of the tissues studied. Of interest, these peak responses consistently occurred earlier than the one to VP7266-295, 5 versus 7 days postinfection, respectively.

The maximal response to individual peptide epitopes also varied, depending on the tissue examined. For instance, specific CD8+ T cells to VP6357-366 and VP733-42 were detected in higher percentages in PPs, IELs, and the liver than in the MLNs and spleen, at day 5 postinfection. On the other hand, a low response to VP7266-295 was detected in the IEL compartment, at both days 5 (P = 0.003 when compared to VP6357-66 and VP733-42) and 7 postinfection, and in contrast, a much higher response appeared in the spleen at day 7, significantly different from the response to the other two epitopes (P = 0.04). Hence, the CD8+ T-cell response to various RV epitopes varied temporally and by organ distribution.

The phenotype of RV-specific T cells detected during acute infection corresponded to that of effector memory T cells (CD45hi, CD27int, Lsel-) (Fig. 5). Since RV-specific T cells are thought to be predominantly primed in the small intestine, we examined expression of the intestinal homing receptor α4β7. We found that regardless of the tissue to which they migrated, almost all RV-specific CD8+ T cells were α4β7+ by days 5 to 7 postinfection (Fig. 5) (data not shown).

Finally, since the IEL compartment contains both CD8αα as well as CD8αβ cells, we examined the TCR phenotype of the responsive cells in this compartment. We found that all IFN-γ-positive cells were present in the CD8αβ subset (data not shown).

Comparison of the T-cell responses after homologous versus heterologous RV infection in adult mice. One of the major current approaches to produce RV vaccines relies on using heterologous (host range restricted) viruses as a vaccine candidate. Therefore, it was of interest to compare the CD8+ T-cell response following homologous versus heterologous infection in the mouse model. A heterologous, host-range-restricted vaccine using simian virus (RRV) reassortants was the first vaccine licensed for use in the United States and showed efficacy against moderate to severe diarrhea in the 70 to 90% range (37). This vaccine was subsequently withdrawn from the market because of its association with intussusception. We used RRV as a model for heterologous virus infection in this study. We chose to use VP6357-366 and VP7266-295 peptides to compare the responses to two different epitopes after homologous and heterologous infections, since responses to these two epitopes were distinct from each other (Fig. 4). As shown in Fig. 6 the CD8+ T-cell response was both quantitatively and qualitatively distinct, depending on whether a homologous or heterologous infection took place. For instance, when mice were immunized with RRV, a very low VP6 response was observed in all of the tissues (PPs, IELs, MLNs, spleen, and liver). When the response against VP6 in RRV-infected mice was compared to the response after EC infection, the most striking difference was found in the IEL compartment, where the peak response at day 5 postinfection showed 5.4% ± 1.06% of CD8+ T cells producing IFN-γ after murine EC infection versus 1.25% ± 0.22% after simian RRV infection (P = 0.04) (Fig. 6). Significantly higher peak responses against VP6 after EC infection were also detected in the PPs and in the liver at day 5 postinfection (P = 0.04 and 0.03, respectively). In order to ensure that sequence variation in the target peptides was not responsible for these differences, we resequenced our
two viral strains and found no differences between RRV and EC in the indicated peptide epitope of VP6 (results not shown).

In marked contrast to the VP6 response, higher responses toward VP7 were elicited after heterologous RRV infection in most of the tissues examined (Fig. 6). When comparing the peak VP7 responses (day 7 postinfection) between homologous and heterologous infections, a somewhat higher response after homologous infection was observed in the PPs (5.2% ± 0.7% after EC infection versus 3.1% ± 0.7% after RRV infection; *P = 0.05). A substantially greater response following RRV infection was observed in the spleen (8.8% ± 1.1% versus 3.7% ± 0.4%; *P = 0.0002). A higher response, although not statistically significant, was also detected in the MLNs (3.5% ± 0.7% versus 1.6% ± 0.3%; *P = 0.08) after heterologous infection. Similar responses were detected in the liver (15.0% ± 3.2% after RRV infection versus 14.7% ± 2.7% after EC infection; *P = 0.4) and in the IEL compartment (0.7% ± 0.2% and 1.3% ± 0.4% following RRV and EC infections, respectively; *P = 0.2).

Finally, we were able to detect a positive CD4+ T-cell response after heterologous (RRV) infection when cells were stimulated with the VP6 pool. In contrast, and as noted above, no CD4+ T-cell response was detected after homologous infection. The epitope mapping for CD4+ T-cell epitopes was carried out as previously described, and LILs were used since the response was more apparent in the liver than in other tissues. The highest positive response was obtained with the 15-mer VP6245-259 (3.1% ± 0.6%, sequence ATWYFNPVIL RPNNV), which elicited a similar response to the one obtained with the complete VP6 pool or the positive subpools. The CD4+ T-cell response was only detectable at day 5 postinfection.

**Kinetics of CD8+ T-cell responses after RV homologous infection in suckling mice.** RV infection of suckling mice is a more complete model of natural infection in infants, since pups, but not adult mice, develop diarrhea along with viral
infection and fecal shedding during rotavirus infection. Hence we examined the T-cell response to homologous infection in suckling mice and compared this to infection in adults. Five-day-old C57BL/6 suckling mice were infected with EC, and at day 7 postinfection, splenocytes were restimulated with the peptide VP7286-295 for 6 h. Cells were stained with different combinations of CD8, CD44, CD62L (L-selectin [Lsel]), CD27, α4β7, CCR7, and IFN-γ MAbs and analyzed by flow cytometry. For analysis, cells were gated on large CD8+ lymphocytes.

Mapping of H-2d-restricted CD8+ T-cell epitopes and CD4+ T-cell epitopes in RV VP6 and VP7. We wished to extend the epitope mapping to BALB/c mice (H-2b) since this strain has been commonly used in a variety of studies of protection in the mouse model (5, 10). However when splenocytes and lymphocytes from the MLNs were harvested 5 and 7 days post-homologous or -heterologous infection in BALB/c mice, no CD4+ or CD8+ T-cell responses were detectable. Based on our results in C57BL/6 mice, we decided to examine LILs for CD8+ T-cell response after both homologous and heterologous infection. At day 5 postinfection with RRV, we were able to detect IFN-γ production from both CD4+ and CD8+ T cells stimulated with pooled VP6 peptides (Fig. 8a). A very low CD8+ T-cell response (0.4% ± 0.2%) was inconsistently detected after stimulation with the VP7 pool during both homologous and heterologous infections, and only a CD8+ T-cell response but no CD4+ T-cell response was detected after EC infection with the VP6 pool. Both the CD4+ and the CD8+ responses against VP6 in BALB/c peaked on day 5 and then declined rapidly, similar to the kinetics of these responses in C57BL/6 mice.

Epitope mapping for CD8+ and CD4+ epitopes in BALB/c mice was carried out as previously described. When analyzing the CD4+ T-cell response, the highest positive response was obtained with the same 15-mer mapped in the H-2b background, VP6245-259 (2.19% ± 0.76%). Interestingly, none of the common peptides in the positive subpools corresponded to the recently described CD4 protective VP6 epitope, VP6289-302 (8). However, the 15-mer containing this sequence was present in one of the positive subpools, and when individually tested, the 15-mer VP6289-303 was able to stimulate an IFN-γ response, but this was significantly lower than that stimulated by the new CD4+ H-2b epitope VP6245-259 (0.44% versus 2.19%) (Fig. 8b; P = 0.02).

Concerning the CD8+ T-cell response, only VP6157-171 elicited a positive response (2.4% ± 0.7%). Computational analysis with the SYFPEITHI and BIMAS databases identified within the VP6157-171 sequence the decamer IFPYSASFTL (residues 160 to 169) as a potential Kd binder. Of note, no response was detected toward the Kd-restricted epitope described by Buesa et al. (4), VP75-13.

DISCUSSION

To our knowledge, this is the first study to characterize in detail the time course, organ distribution, and quantitative T-cell response to two of the most immunogenic RV proteins, VP6 and VP7, during an acute infection.

Interestingly, in the two comparisons we examined, namely homologous versus heterologous infection and infection of adults versus that of sucking mice, we observed both qualitative and quantitative differences in the responses to the different VP6 and VP7 epitopes. For instance, we showed that there was a significantly higher response to VP653-62, VP6357-366, and VP733-42 versus VP7286-295 in the following scenarios: homologous infection, sucking mice, and intestinal tissues. The common factor in these three situations may be a higher viral replication rate in the intestine with synthesis of greater amounts of viral protein. On the other hand, the response against the newly identified H-2b VP7 epitope (VP7286-295) was weaker in the intestinal compartment but higher in the spleen.
and MLNs than that in the other epitopes, higher during het-

erologous RRV infection, higher in adults than in infant mice,

and finally peaked later (day 7 versus day 5 for the VP6

epitopes and VP7286-295). It has been previously shown that

during RRV infection there is extraintestinal spread (31), and

these findings might suggest that the VP7286-295 epitope may be

better presented systemically, as opposed to locally in the gut.

It will be interesting in future studies to use CD8

T-cell populations isolated by tetramer-based sorting to test the abil-

ity of the various epitope-specific CD8

T cells to kill target cells and speed the resolution of infection. Since heter-

ologous viruses form the basis of one of the major new vaccine

candidates, it is highly relevant to know if the differences ob-

served here are generic for differences between homologous

and heterologous infection and whether they actually have

functional sequelae. It has been reported that there can be

discrepancies between the lytic activity of CD8

T cells for a
given determinant and the number of activated cells identified

by ICS (7), and these differences may affect protective efficacy

of heterologous versus homologous immunogens. It will also

be interesting to determine if one of these CD8

T-cell pop-

ulations is predominant in the memory subset and if, after

challenge of previously immunized mice, one of these popula-

tions is preferentially boosted compared to the others. All of

FIG. 6. Kinetics of VP6357-366- and VP7286-295-specific CD8

T cells in different organs after homologous murine EC or heterologous RRV

infection of adult mice. C57BL/6 mice were infected orally with 104 ID50 of EC or 107 PFU of RRV. At the indicated days, cells from different

tissues were restimulated with the peptides VP6357-366 and VP7286-295 for 6 h. Cells were stained with anti-CD8-FITC and PE-conjugated IFN-γ and

analyzed by flow cytometry. Graphs indicate percent values of IFN-γ

CD8

T cells from different tissues and represent the mean ± standard
deviation from three independent experiments (two to three mice pooled in each experiment). Asterisks indicate significant differences between

homologous and heterologous infection. (P < 0.05, Mann-Whitney test).
suckling mice (independent experiments (two to three mice pooled in each experi-
ence) infected orally with 10⁴ ID₅₀ of EC. At the indicated days, cells from the
livers of adults versus sucking mice following oral murine EC infection. Five-day-old and 6- to 8-week-old C57BL/6 mice were in-
shown).

tified the two class I epitopes mapped by Buesa et al. (4), VP75-13 and VP78-16, in the C57BL/6 and BALB/c backgrounds, re-
these studies would be particularly relevant to the design of live attenuated RV vaccines. We do not believe these differences
can be explained by differences in the sequences of the two RV strains, since the sequences of the VP6 and VP7 epitopes identified in this study are conserved (data not shown).

During acute RV infection in adult mice, we identified a typical primary response time course of CD8⁺ T cells, well
characterized in the lymphocytic choriomeningitis virus (LCMV) model, that involves an extensive proliferation phase (activation and expansion, 1 to 8 days postinfection), a con-
traction or death phase (days 8 to 30), during which 95% of the antigen-specific cells die, and finally a long-term memory phase (32). However this typical progression was not what we ob-
served in suckling mice (Fig. 7), where the proliferation phase appeared to be longer and there were two separate peaks of response (days 7 and 14). This finding mimics an old observa-
tion that infection in mouse pups frequently resulted in a biphasic pattern of antigen shedding distinct from that in adults, where a monophasic RV antigen-shedding profile is commonly observed (5). In addition, in pups the length of the shedding is longer than that in adults: in adults, antigen shedding ends 7 days postinfection, but in pups shedding is detected until day 9 or 10 postinfection, which is about 14 days of age. Since CD8⁺ T cells are responsible for the timely clearing of virus, one can hypothesize that in pups the CD8⁺ T cells generated during the first peak of the response are not suffi-
cient to fully control the infection. It remains to be determined if this is due to the actual quantity of CD8⁺ T cells, since the numbers detected in the first peak are significantly lower than those in either adults or the second peak of response. Alter-
natively the quality of the T-cell response (lytic activity and cytokine release) may change between the first and second peaks. It seems reasonable to speculate that the second peak constitutes a better response.

Three new CD8⁺ T-cell epitopes (one H-2d and two H-2b restricted) and one new CD4⁺ T-cell epitope (H-2d and H-2b restricted) were identified in this study by the ICS strategy and overlapping peptide libraries (24, 39). In addition, the presence of previously characterized H-2b CD8⁺ epitopes VP7₁₄₋₁₆ and VP6₃₅₇₋₆₆ and the H-2d CD4⁺ epitope VP6₂₉₉₋₃₀₂ (8, 13, 14) was confirmed. Interestingly, our mapping strategy didn’t iden-
tify the two class I epitopes mapped by Buesa et al. (4), VP7₅₋₁₃ and VP7₆₋₁₆, in the C57BL/6 and BALB/c backgrounds, re-
RV proteins are responsible for a more robust CD8+ T-cell response. The individual 15-mers containing these epitopes failed to elicit a positive response in our analysis. One possible explanation for these differences might be that Buesa immunized mice with RV strains different from those used in our experiments (SA11, EDIM, and WA versus EC and RRV), although there is a high level of homology in the VP7 sequences between these strains. In any case, the identification of these new epitopes and confirmation of several previous identified targets should be useful for future studies of the T-cell response to RV infection and immunization.

In this study, we were able to quantify, for the first time, the RV-specific T-cell response in two different mouse strains, C57BL/6 and BALB/c. As previously shown by Buesa et al. (4), we detected a significantly lower CD8+ T-cell response in BALB/c versus C57BL/6 mice. In the BALB/c background, there was an extremely low response detected after stimulation with the VP7 peptide library, the VP6 response could only be detected in the liver, and the peak response after stimulation with the respective VP6 epitopes was 2.4% ± 0.7%, versus 8.06% ± 1.58% in C57BL/6. The possibility exists that other RV proteins are responsible for a more robust CD8+ T-cell response in the H-2d background. For instance, it has been shown that there is a CTL response in C57BL/6 mice toward VP3 (13) and toward the nonstructural protein NSP1 (18), but no studies to our knowledge have explored the CD8+ response toward these or other RV proteins in BALB/c mice. Of note, besides the differences in the CD8+ T-cell response between the two strains, there were also significant differences in the antibody responses after homologous RV infection (data not shown). Higher titers of RV-specific serum IgA were detected in BALB/c mice than in C57BL/6 mice during the first 15 days postinfection. Since the antibody response in the C57BL/6 background did not rise as rapidly as in BALB/c mice during the first 2 weeks of infection, one could hypothesize that viral replication might be enhanced in these mice during this early phase, leading to greater CTL priming.

Our results also show that, independent of the mouse strain studied, the overall RV-specific CD4+ response against VP6 and VP7 is very low compared to the RV CD8+ response or CD4+ responses to other viruses (40). This low response could only be detected following RRV infection, despite the fact that the sequence of the epitope VP6245–259 only differs in the first amino acid between the homologous and heterologous strains used. This finding supports our previous study in humans (20), where very low frequencies of RV-specific CD4+ T cells were detected in children and adults with RV diarrhea. One possible explanation for missing the CD4+ T-cell response could be the length of the peptides (15-mers) we used. In a previous study, Tobery et al. (38) successfully detected CD4+ T-cell responses to both the L1 and E1 proteins of HPV16, by using 20-mer peptides overlapping by 10 amino acids and ELISPOT assays, and it’s possible that longer peptides with even longer overlaps than the one used in this study are more suitable for the detection of a CD4+ response. However, we do not think this is the likely explanation since, in other studies, even shorter peptides (12-mers, 2-amino-acid overlap) have been used successfully to detect CD4+ responses in both C57BL/6 and BALB/c mice (17). In addition, when directly compared, CD4+ responses to 15-mer and 20-mer mixes were generally indistinguishable (26). The possibility remains, as for the CD8+ response, that other RV proteins are responsible for the CD4+ T-cell response. It also remains to be determined if these two strains differ in their Th1/Th2 cytokine profiles after RV infection and if, by only measuring IFN-γ responses, we are underestimating the total anti-RV CD4+ T-cell response. Lymphoproliferation studies and limiting dilution analysis might help address this issue. Finally, it is possible, as we suggested in our previous study (20), that an important number of RV-specific T cells are unpolarized and that direct detection of RV-reactive T cells with tetramers could be a more sensitive approach to quantify the RV-specific T-cell response. Further studies in this area are indicated.

Interestingly the H-2d-restricted CD4+ epitope that has been described as highly protective following intranasal immunization (VP6289–303) was not an immunodominant epitope in our study but rather elicited a significantly lower response than the dominant epitope, VP6245–259 (Fig. 8). One possible explanation for this discrepancy might be the different routes of immunization and different antigen formulation used in these studies: intranasal immunization of protein or peptide with LT adjuvant in the study by Choi et al. versus oral infection with live virus in our study. It has been recently shown (16) that the route of virus infection can affect the selection and expansion of subpopulations of virus-specific CTLs, and considering our results, this might also be the case for CD4+ T-cell populations. We are currently testing this hypothesis by comparing the RV-specific CD8+ and CD4+ T-cell populations induced after oral, intranasal, and intramuscular immunization with RRV. Hence, it appears from these studies that the CD4+ protective response observed by Choi et al. is not elicited or is elicited at very low levels following homologous or heterologous infection of the gastrointestinal tract and is unlikely to be an important contributor to protection under these immunization conditions or after natural infection.

Our results are in general agreement with the recent finding by Letfrancois et al. (27, 28) that showed that activated RV-specific CD8+ T cells migrate preferentially to tertiary organs, since the highest percentages of RV-specific T cells were found in the liver and lungs (Fig. 3). However, these results need to be interpreted very carefully since recent findings indicate that systemic antigenemia or viremia is present during RV infection (2) and some RV strains, like RRV, can spread to and replicate in the liver (31)—hence the transient inflammatory process seen on liver histology after homologous RV infection may not be completely nonspecific. In addition, inflammation in the liver, associated with accumulation of high numbers of CD8+ T cells, has been described in other models (3). The critical question during RV infection remains, then, if the presence of CD8+ in all of these tissues actually reflects ongoing extraintestinal infection or simply nonspecific migration of lymphocytes to tertiary organs. Interestingly, during the peak response (day 5 or 7 postinfection, depending on the epitope), almost all of the RV-activated CD8+ T cells expressed the intestinal homing receptor α4β7, regardless of the tissue where they were detected (data not shown). In contrast to the findings by Masopust et al. (27), we do not see a down-regulation of α4β7 on day 7 postinfection, when most of the cells still express high levels of this integrin. One interpretation of this finding would be that, since RV replicates preferentially
in the small intestine, all the RV-specific CD8+ T cells were initially primed in the intestine, and therefore express αβ7, and then migrate to different tissues. It would be of great interest to characterize the coexpression of other integrins and chemokine receptors (CCR9, CXCRL6, and CCR5 for example) involved in homing to lymphoid and nonlymphoid organs in order to better understand the homing potential of these cells, especially since it has been shown that expression of αβ7 is not essential for CD8+ T cells to migrate to the intestine or provide immunity to RV (25).

Until now, no RV-specific T-cell epitopes had been described in humans, and it is not simple to evaluate the efficacy of different vaccine strategies in generating protective CD8+ or CD4+ responses in people. Epitope mapping in humans will likely be challenging, since our results indicate that these responses are very short lived and that the frequencies of cells localized in the small intestine, all the RV-specific CD8+/H11001 T cells to cytotoxic T lymphocytes secreting gamma interferon after acute natural rotavirus infection in children and adults. J. Virol. 76:4741–4749.


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