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Immunity to Rotavirus in T Cell Deficient Mice

Manuel A. Franco[1] and Harry B. Greenberg

Department of Medicine, Department of Microbiology, and Department of Immunology, Stanford University School of Medicine, Stanford, California 94305; and Veterans' Affairs Palo Alto Health Care System, Palo Alto California 94304

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Rotavirus infection was studied in adult nude mice (BALB/c background), $\alpha\beta$ or $\gamma\delta$ and $\alpha\beta/\gamma\delta$ T cell receptor (TCR) knockout (−/−) mice (C57BL/6 and C57BL/6×129 backgrounds), and SCID mice (C57BL/6 background). The $\gamma\delta$ TCR −/− mice cleared infection just like control mice. All of the nude mice, $\alpha\beta$, and $\alpha\beta/\gamma\delta$ TCR −/− mice cleared primary rotavirus infection, with a short delay, compared to immunocompetent control mice and developed a rotavirus-specific intestinal IgA measured by ELISA. Elispot analysis with spleen and lamina propia cells showed that the virus-specific intestinal IgA response in immunocompetent C57BL/6 mice was similar to the $\gamma\delta$ TCR −/− mice and 7- to 60-fold higher than in the $\alpha\beta$ TCR −/− and $\alpha\beta/\gamma\delta$ TCR −/− mice. Likewise, the response of nude +/− mice was 20 times greater than that of nude −/− littermates. While the intestinal IgA antibodies of C57BL/6 mice, $\gamma\delta$ TCR −/− mice, and nude +/− mice recognized insect cells infected with recombinant baculovirus expressing rotavirus VP6 and VP4 proteins, those of the $\alpha\beta$ TCR −/−, $\alpha\beta/\gamma\delta$ TCR −/−, and nude −/− mice recognized only VP6. Immunocompetent C57BL/6 mice depleted of $CD4^+$ T cell developed similar levels of rotavirus-specific intestinal IgA as the $\alpha\beta$ TCR −/− mice, suggesting that this T cell-independent IgA response is present in normal mice. In contrast to previously published results with BALB/c SCID and RAG 2 −/− (C57BL/6×129 background) mice, all of which become chronically infected with murine rotavirus, 40% of the C57BL/6 SCID mice cleared primary rotavirus infection. These results suggest that both a T cell-independent antibody response and innate mechanisms can contribute to immunity to murine rotavirus and show that $\gamma\delta$ T cells are not necessary for efficient clearance of primary rotavirus infection in mice.

INTRODUCTION

Mice that lack both T and B cells (SCID mice on the BALB/c background (Riepenhoff-Talty *et al.,* 1987) and Rag 2 −/− mice on a mixed 129/C57BL/6 background (Franco *et al.,* 1997)) become chronically infected with murine rotavirus. Mice with gene targeted mutations that render them deficient in B cells have altered clearance of primary rotavirus infection: μMt −/− (C57BL/6 background) mice continue to shed reduced levels of rotavirus antigen up to 93 days after infection while a small fraction of B cell deficient J_HD mice (129/C57BL/6 background) also have altered clearance of primary rotavirus infection (Franco and Greenberg, 1995; McNeal *et al.,* 1995). If the B cell deficient mice are depleted of $CD8^+$ T cells by administration of an anti-CD8 monoclonal antibody they become chronically infected (J_HD mice) or shed more virus than undepleted mice (μMt −/−) (Franco and Greenberg, 1995; McNeal *et al.,* 1995). In contrast, if the J_HD mice are depleted of $\gamma\delta$ T cells they clear infection like undepleted mice (Franco and Greenberg, 1997). Immunocompetent mice and β_2 microglobulin −/− mice depleted of $CD8^+$ T cells have a short delay before completely clearing primary rotavirus infection (Franco and Greenberg, 1995; Franco *et al.,* 1997). In contrast with these recent studies that indicate that antibody and/or CD8 positive cells are necessary for clearance of primary rotavirus infection, it has also been shown that T cell deficient nude mice on the BALB/c background clear primary rotavirus infection like immunocompetent mice without the production of rotavirus specific antibodies (Eiden *et al.,* 1986).

Nude mice have been used in other viral systems to show that virus-specific IgM antibody production can be T cell-independent (Freer *et al.,* 1994). Although some systemic antiviral IgA can be detected in nude mice, most of the systemic IgA is T cell-dependent (Tyor *et al.,* 1989). Less is known about the intestinal antiviral IgA response of nude mice, but nude mice are unable to efficiently make intestinal IgA responses to cholera toxin, suggesting that this response is also T cell-dependent (Lycke *et al.,* 1987). The interpretation of these results is complicated by the fact that nude mice do have some T cells (Lin *et al.,* 1993; Speiser *et al.,* 1992). More recently T cell knockout mice with a targeted mutation in the $\alpha\beta$ and/or $\gamma\delta$ TCR have been produced (Itohara *et al.,* 1993; Mombaerts *et al.,* 1992). Like the nude mouse, $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice have been shown to have low levels of IgA antibodies, suggesting that T cells are im-

[1] To whom reprint requests should be addressed at Stanford University School of Medicine, Lab Surge, P304, Stanford CA 94305-5487. Fax: (415) 852-3259. E-mail: francoma@leland.stanford.edu.

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portant, but not essential, for the development of IgA antibodies (Mombaerts *et al.,* 1994). These T cell knockout mice have been recently used to demonstrate that IgM antibody responses can have an antiviral activity completely independent of T cells (Szomolanyi and Welsh, 1996). Like the nude mouse studies, results obtained with the T cell knockout mice should be viewed with the precaution that these mice can develop compensatory immune mechanisms that are present only at low levels or not at all in normal mice. An additional important factor that probably influences the interpretation of experiments with the $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice is that these mice develop autoimmune colitis (Mombaerts *et al.,* 1993).

We used several immunodeficient mice and immunodepleted normal mice to demonstrate that both a T cell-independent, virus-specific intestinal IgA response and an innate mechanism contribute to clearance of rotavirus infection in mice. We also found that $\gamma\delta$ T cells are not necessary for efficient clearance of primary rotavirus infection.

MATERIALS AND METHODS

Mice and virus inoculation

$\alpha\beta$ (C57BL/6 background *H-2*^{b}) and $\alpha\beta/\gamma\delta$ TCR $-/-$ (C57BL/6$\times$129 background *H-2*^{b}) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Nude mice (BALB/c background *H-2*^{d}), SCID mice (C57BL/6 background *H-2*^{b}), $\gamma\delta$ TCR $-/-$ mice (C57BL/6 background *H-2*^{b}), and C57BL/6J (*H-2*^{b}) mice were originally obtained from the Jackson Laboratory and bred in our facility (Franco and Greenberg, 1995). Nude mice were bred using nude $+/-$ dams mated to nude $-/-$ males. The nude $+/-$ offspring were used as control for the experimental nude $-/-$ litter mates. All naive mice studied were negative for rotavirus-specific IgA in their stools before use. Periodic health screens were performed on sentinel animals to ensure that animals were free of rotavirus and other mouse pathogens. Adult 6- to 8-week old mice were orally given 100 μl of sodium bicarbonate (1.33%) and then orally infected with intestinal homogenates derived from mouse pups infected with the wild type strain of rotavirus (ECw; G3, P16) (Burns *et al.,* 1995). After infection, viral antigen in stool samples and virus-specific intestinal and serum antibodies were detected using a previously described ELISA (Burns *et al.,* 1995; Franco *et al.,* 1997).

Quantitation of virus-specific antibody secreting cells (ASCs) by Elispot assay

Multiscreen 96-well plates (MAIP N45 10) with Immobilon P membranes (Millipore, Molsheim, France) were coated with hyperimmune rabbit anti-rhesus rotavirus serum (1:1000 dilution) for 4 h at 37°. The plates were then washed with RPMI 1640 medium and incubated with RRV stock virus (a 1:5 dilution) overnight at 4°. After two washes with RPMI medium the plates were blocked with RPMI 1640 medium containing 10% fetal calf serum (FCS) for 1 h at 37°. Serial 10-fold dilutions of the cells to be analyzed were added to the plates subsequently and incubated at 37° with 5% CO_2 for 24 h. The plates were washed six times and incubated with either diluted peroxidase-conjugated anti-mouse IgA (0.25 μg/ml) or IgG (0.25 μg/ml) antisera (Kirkegaard and Perry Labs., Gaithersburg, MD). After 1 h of incubation at 37°, the plates were washed six times and developed with AEC (3-amino-9-ethyl-carbazole; Sigma) substrate. Spots formed by the precipitated substrate were enumerated using a stereo-microscope. As a negative control virus was omitted from the above protocol. The total number of antibody producing cells for each cell preparation was determined by coating plates with a diluted (4 μg/ml) antiserum against mouse immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD), blocking the plates, adding the cells, and then processing as for the virus-specific assay described above.

Isolation of lymphocyte populations

Isolation of spleen cells and IELs was done was previously described (Franco and Greenberg, 1995). Lamina propria cells were obtained as previously described (Mega *et al.,* 1991). After extraction of IELs, small pieces of small intestine (2 cm) were incubated twice for 20 min each at 37° in PBS containing 5 mM ethylenediamine tetracetic acid, with vigorous shaking between the changes of medium. The intestinal fragments were then washed three times in Joklik-modified medium and incubated in Joklik-modified medium containing 1.5 mg/ml Dispase II (Boehringer Mannheim, Mannheim, Germany) for 20 min each in three consecutive cycles, with agitation between the changes of medium. The cells present in the supernatant from the incubation with Dispase were pooled, washed with RPMI 1640 containing 10% heat-inactivated FCS, 100 μg/ml streptomycin sulfate, 100 U/ml penicillin, 2 mM L-glutamine, 2.5 mM sodium pyruvate, 20 mM HEPES, and 30 mM 2-mercaptoethanol (complete medium) and then placed on discontinuous gradients containing 75 and 40% Percoll (Pharmacia Biotech AM, Uppsala, Sweden) and centrifuged for 20 min (600 *g,* 25°). The cell population at the interface was collected, washed once in complete medium, and resuspended in fresh medium. The cells were then used for the different experiments, described below.

Quantitation of antibody levels to rotavirus proteins VP4, VP6, and VP7

The specificity of the intestinal antibody was determined using Sf-9 insect cells infected with recombinant

baculovirus expressing EW murine rotavirus proteins VP4, VP6, and VP7, as previously described, with minor modifications (Ishida *et al.,* 1996). Sf-9 cells were infected with baculovirus expressing individual rotavirus proteins and plated in 96-well tissue culture plates (Costar, Cambridge, MA) for 72 h incubation at 28°. The culture medium was removed and plates were dried in a vacuum oven at room temperature for 1 h. Cells were fixed with 10% formalin (Sigma, St. Louis, MO) for 30 min and permeabilized with 1% Triton X-100 (*t*-octylphenoxypolyethoxyethanol; Sigma) for 2 min. After washing twice, serial threefold dilutions of 10% fecal suspensions were added to the plates and incubated at 37° for 2 h. The plates were washed three times and incubated with diluted peroxidase-conjugated anti-mouse IgA (0.25 μg/ml) (Kirkegaard & Perry Laboratories). The plates were washed twice and AEC substrate was used to visualize stained cells. Diluted stool sample suspensions were also incubated with uninfected Sf-9 cells as a negative control. Monoclonal antibodies against VP4, VP6, and VP7 were used in all experiments to verify expression of the recombinant proteins in the infected Sf-9 cells (Ishida *et al.,* 1996). Negative samples were arbitrarily assigned a titer of 1:1 (threefold below 1:3) for statistical calculations.

Depletion of CD8$^+$, CD4$^+$, and $\gamma\delta$ T cells

Mice were depleted of CD8$^+$, CD4$^+$ and $\gamma\delta$ TCR$^+$ cells by administration of ascites fluid containing the rat anti-mouse CD8 (alpha chain) monoclonal antibody (mAb) 2.43 (Franco *et al.,* 1997), rat anti-mouse CD4 mAb GK 1.5, and anti-mouse $\gamma\delta$ TCR mAb UC7-13D5 (Franco and Greenberg, 1997), respectively. Hybridoma cells that produce all of the mAbs were obtained from the American Type Culture Collection (Rockville, MD) and ascites was produced in nude mice as previously described (Franco and Greenberg, 1995). Each mouse received 0.5 ml of ascites fluid on days 5, 4, and 3 before rotavirus infection, on the day of infection, and on days 3, 6, and 9 after infection. On the day of rotavirus infection, depleted and non-depleted control mice were killed to verify depletion in spleen and among intraepithelial lymphocytes (IEL) by FACS analysis (Franco and Greenberg, 1995, 1997). When mice were depleted of CD4$^+$ T cells, depletion was verified in lamina propia as well as spleen and IEL compartments. Depletion of CD4 cells was assessed by FACS analysis using biotinylated RM4-5 anti-CD4 mAb and streptavidin–phycoerythrin as a second stage (Pharmingen, San Diego, CA).

Statistical analysis

Statistical analysis was performed with Statview, a statistical package for Macintosh computers (Abacus Concepts, Inc., Berkeley, CA). Results are expressed as means plus and minus the SEM. Differences between means were compared with the Mann–Whitney *U* test.

RESULTS

BALB/c nude −/−, $\alpha\beta$ TCR −/−, and $\alpha\beta/\gamma\delta$ TCR −/− mice have delayed clearance of primary rotavirus infection but are protected from rotavirus reinfection

To study if T cells are necessary for clearance of primary rotavirus infection in mice, we infected athymic adult nude −/− and control nude +/− mouse littermates with 10^5 shedding dose 50 (SD_{50}) of the EC strain of murine rotavirus. As we previously reported with β_2 microglobulin −/− mice, nude −/− mice cleared infection with a small delay (1–3 days) compared to control nude +/− mice (Fig. 1a). The mean number of antigen shedding days of nude −/− mice was significantly greater than that of nude +/− mice ($P = 0.01$). Since nude −/− mice have CD8$^+$ T cells among the IEL (Lin *et al.,* 1993), we investigated whether these CD8$^+$ cells were participating in clearance of rotavirus infection by treating the nude −/− mice with an anti-CD8 monoclonal antibody (Franco *et al.,* 1997). More than 95% of the CD8 cells were depleted in the spleen and IELs of the treated mice. CD8-depleted nude −/− mice cleared infection like untreated mice, while CD8-depleted nude +/− mice cleared infection with a small delay compared to untreated nude +/− mice and in a similar manner to treated and untreated nude −/− mice (Fig. 1a). This result suggests that the delay in viral clearance in nude −/− is due to their lack of CD8$^+$ thymus derived T cells.

To confirm and extend the results with nude mice, we infected adult $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice with 10^5 SD_{50} of the EC strain of murine rotavirus. Like the nude mice, both types of knockout mice cleared infection with a short delay (2–4 days and 1–3 days for the $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice, respectively), compared to immunocompetent C57BL/6 mice (Fig. 2a). The mean number days that antigen was shed by $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice was significantly greater than that of C57BL/6 mice ($P < 0.01$). When the $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice were rechallenged 8 weeks after primary infection they were resistant to rechallenge (Fig. 2b). In separate rechallenge experiments from that shown in Fig. 2b, one of five $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice shed very low levels of viral antigen for 1 to 4 days (data not shown). Among nude mice, three were resistant to rechallenge and two were reinfected for 1 to 3 days (data not shown).

$\gamma\delta$ TCR −/− mice efficiently clear primary rotavirus infection, and $\alpha\beta$ TCR −/− depleted of $\gamma\delta$ T cells clear infection like undepleted mice

To determine if $\gamma\delta$ T cells were necessary for clearance of primary rotavirus infection, we infected $\gamma\delta$ TCR

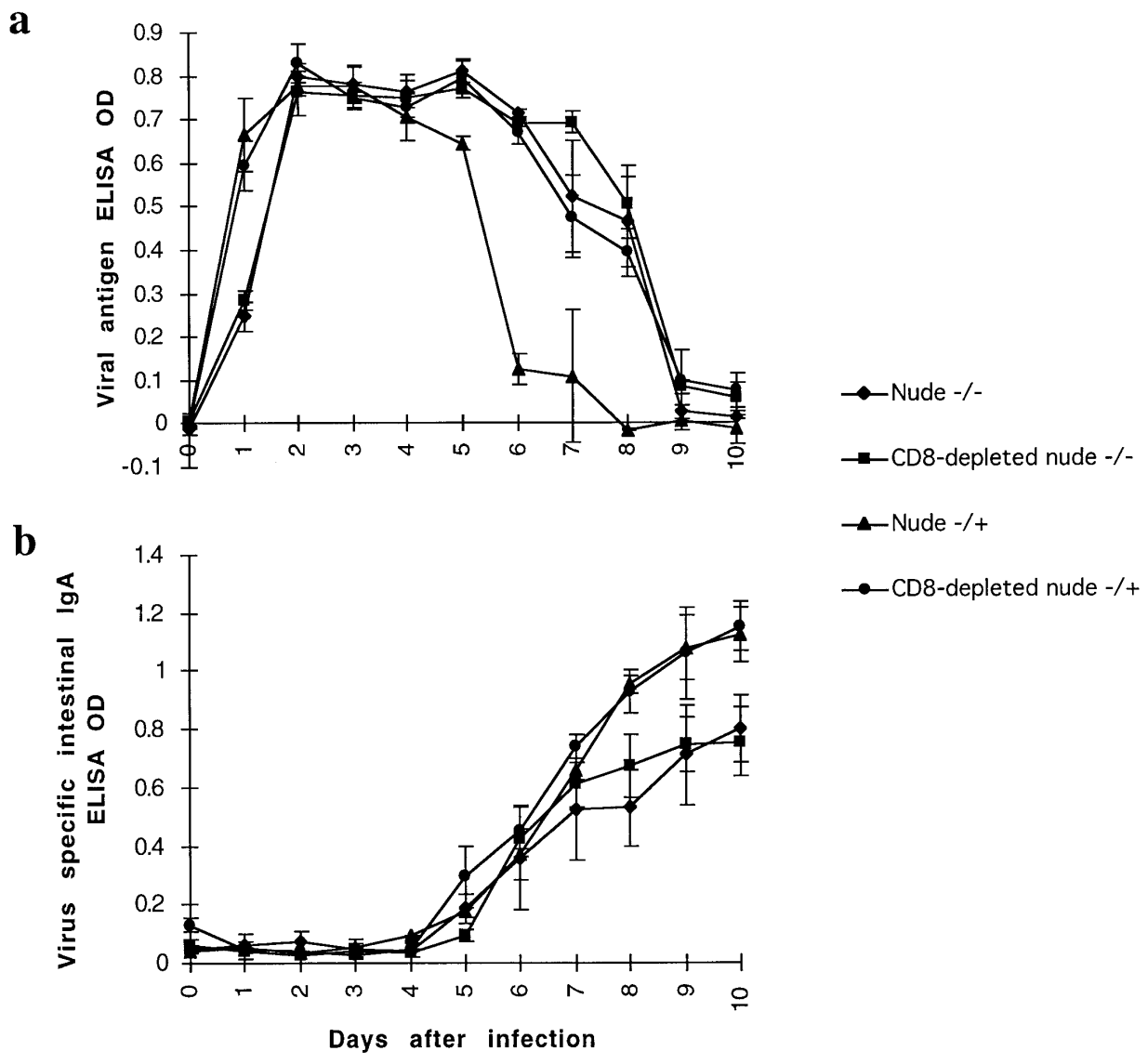

FIG. 1. Fecal viral antigen (a) and virus-specific intestinal IgA (b) shedding curves of nude +/− and nude −/− mice littermates depleted or not depleted of $CD8^+$ cells by administration of 2.43 anti-CD8 mAb. Mice were infected at 6 to 8 weeks of age with 10^5 SD_{50} of the ECw strain of murine rotavirus. Fecal rotavirus was measured by ELISA and results are expressed as OD readings. Each data point represents the mean of two untreated nude +/− and three mice in the other groups, plus and minus the corresponding SEM. This experiment has been repeated three times with the undepleted nude −/− mice and twice with the CD8-depleted −/− mice with similar results.

−/− mice with 10^5 SD_{50} of the EC strain of murine rotavirus. These mice (Fig. 2c) cleared rotavirus infection similarly to C57BL/5 control mice (Fig. 2a). This result suggests that, in adult mice, $\gamma\delta$ T cells either play no role in immunity to rotavirus, or if $\gamma\delta$ T cells do play a role, other cells must be able to compensate for their absence. If $\gamma\delta$ T cells play a role in clearance of rotavirus infection, their effect may be seen in $\alpha\beta$ TCR −/− mice that would be expected to have diminished compensatory mechanisms. To determine if this was the case, we infected $\alpha\beta$ TCR −/− mice depleted of $\gamma\delta$ T cells with rotavirus. Depletion of $\gamma\delta$ T cells was more than 82% among spleen cells and IELs. These depleted mice (Fig. 2c) cleared primary rotavirus infection like undepleted $\alpha\beta$ TCR −/− mice (Fig. 2a). We could not find evidence of a role for $\gamma\delta$ T cells in rotavirus immunity of adult mice.

BALB/c nude −/−, $\alpha\beta$ TCR −/−, and $\alpha\beta/\gamma\delta$ TCR −/− mice develop rotavirus-specific intestinal IgA

To determine if nude mice and $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice that lack $\alpha\beta$ T helper cells developed a T cell-independent antibody response, we measured rotavirus-specific antibodies in stool and serum samples from rotavirus infected mice. All three types of mice developed rotavirus-specific IgA responses in their stool samples that

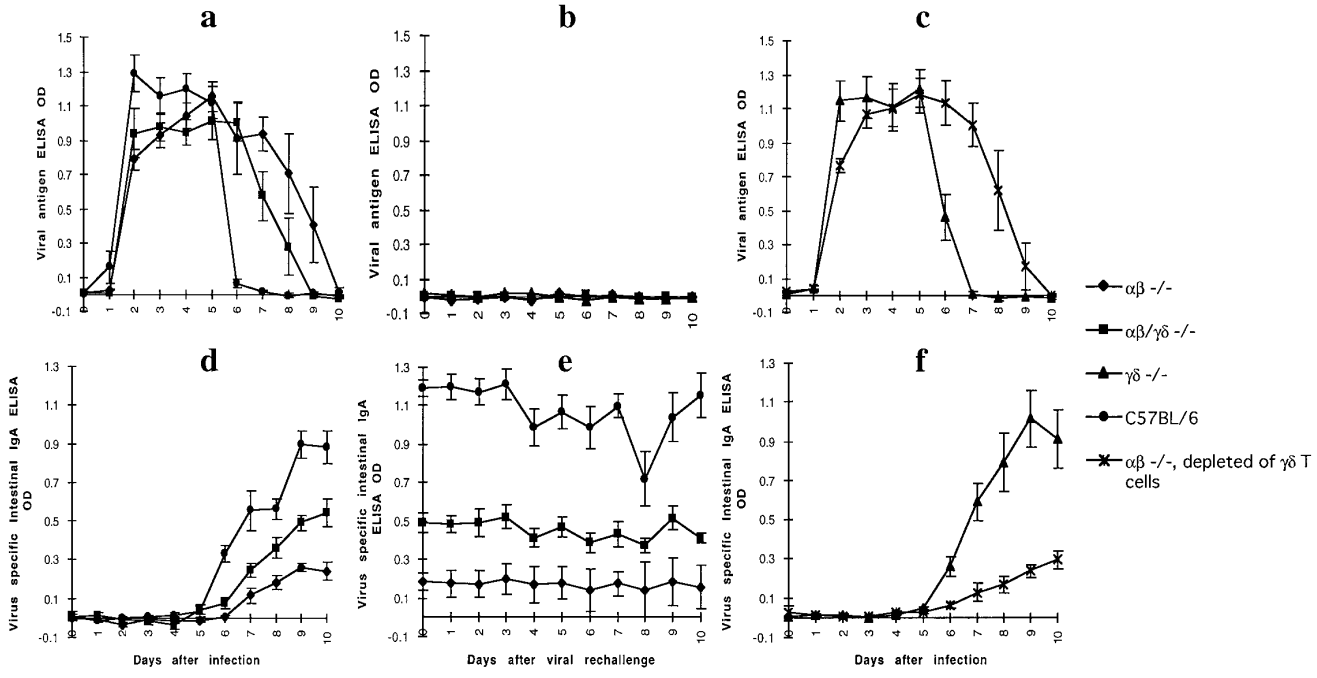

FIG. 2. Fecal viral antigen (top row) and virus-specific intestinal IgA (bottom row) shedding curves of TCR $-/-$ mice infected or rechallenged with 10^5 SD_{50} of the ECw strain of murine rotavirus. Primary infection of $\alpha\beta/\gamma\delta$ TCR $-/-$, $\alpha\beta$ TCR $-/-$, and control immunocompetent C57BL/6 mice are shown in (a) and (d). The same mice rechallenged 8 weeks after primary infection are shown in (b) and (e). Primary infection of $\gamma\delta$ TCR $-/-$ and $\alpha\beta$ TCR $-/-$ mice depleted of $\gamma\delta$ T cells by administration of UC7-13D5 anti-$\gamma\delta$ TCR mAb are shown in (c) and (f). Fecal rotavirus antigen and virus-specific intestinal IgA were measured by ELISA and results are expressed as OD readings. Each data point represents the mean of three $\alpha\beta$ TCR $-/-$ mice depleted of $\gamma\delta$ T cells, and five mice in each of the other groups plus and minus the corresponding SEM. Experiments have been repeated at least twice with similar results.

correlated temporally with viral clearance (Figs. 1b and 2d). The intestinal response of the T cell deficient mice was 4 to 40 times lower than that of immunocompetent control mice (Table 1). In serum, $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice developed a similar IgM response to C57BL/6 mice (Table 2). The $\alpha\beta/\gamma\delta$ TCR $-/-$ mice developed very low levels of virus-specific serum IgG and IgA, while the $\alpha\beta$ TCR $-/-$ mice did not have detectable levels of these serum antibodies. The $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice had low levels of serum and stool antibodies immediately after primary infection and before rechallenge 8 weeks after primary infection (Table 1 and 2). In accord with their lack of reinfec-

TABLE 1

$\alpha\beta$, $\alpha\beta/\gamma\delta$ TCR $-/-$, and CD4-Depleted C57BL/6 Mice Develop Low Levels of Rotavirus-Specific Intestinal IgA

Mouse strain	Geometric mean anti-rotavirus stool IgA titer as a function of time (titer range)		
	Ten days after primary infection	Eight weeks after primary infection (day of rechallenge)	Ten days after rechallenge
C57BL/6	48.5 (9–243)	243 (27–729)	156 (81–729)
$\gamma\delta$ TCR$-/-$	65 (9–243)*	ND	ND
CD4-depleted C57BL/6	7.2 (3–27)**	ND	ND
$\alpha\beta$ TCR $-/-$	1.25 (3–9)**	1.37 (3–9)**	0.87 (<3–9)**
$\gamma\delta$-depleted $\alpha\beta$ TCR $-/-$	1.66 (3–9)**,***	ND	ND
$\alpha\beta/\gamma\delta$ TCR $-/-$	13.97 (9–27)**	12.51 (3–27)**	11.21 (3–27)**

Note. Serial threefold dilutions of 10% stool suspensions from mice infected with the ECw strain of murine rotavirus were assayed for rotavirus-specific antibodies by ELISA. Results shown are the geometric mean (range) from 3$\gamma\delta$-depleted $\alpha\beta$ TCR $-/-$ mice and five or more animals in each of the other groups. ND, not done.

* Value is not significantly different from the value of C57BL/6 mice ($P > 0.25$).

** Values are significantly different from the values of C57BL/6 mice ($P < 0.01$).

*** Value is not significantly different from the value of nondepleted $\alpha\beta$ TCR $-/-$ mice ($P = 0.37$).

TABLE 2

$\alpha\beta$, $\alpha\beta/\gamma\delta$ TCR $-/-$, and CD4-Depleted C57BL/6 Mice Develop Very Low Levels of Rotavirus-Specific Serum Antibody

	Geometric mean anti-rotavirus serum titer of different antibody isotypes as a function of time (titer range)								
	Two weeks after primary infection			Eight weeks after primary infection (before rechallenge)			Two weeks after rechallenge		
Mouse strain	IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
C57BL/6	277 (16.7–4,050)	13,548 (4,050–109,350)	1,425 (1,350–36,450)	187 (450–4,050)	70,397 (36,450–109,350)	5,040 (4,050–12,150)	289 (150–450)	87,695 (36,450–109,350)	6,279 (4,050–12,150)
$\gamma\delta$ TCR $-/-$	450 (450)	18,837 (12,150–36,450)	3,248 (1,350–4,050)	ND	ND	ND	ND	ND	ND
CD4-depleted C57BL/6	191 (16.7–4,050)	16 (16)	27.13 (16–50)	ND	ND	ND	ND	ND	ND
$\alpha\beta$ TCR $-/-$	62 (50–150)	16 (16)	16 (16)	96 (50–450)	25 (16–50)	20 (16–50)	120 (50–450)	16 (16)	16 (16)
$\alpha\beta/\gamma\beta$ TCR $-/-$	560 (450–1350)	40 (16–150)	25 (16–50)	62 (50–150)	49 (16–150)	49 (16–150)	120 (50–150)	49 (16–150)	96 (50–150)

Note. Serum titers of rotavirus-specific antibodies in mice orally infected and rechallenged with EC rotavirus were determined by ELISA. Results are given as the geometric mean (range) from five or more mice per group. All values from the $\gamma\delta$ TCR $-/-$ were not significantly different from equivalent values of C57BL/6 mice ($P > 0.1$). The IgM value of $\alpha\beta$ TCR $-/-$ mice, 2 weeks after infection, is significantly different from the equivalent value of C57BL/6 ($P = 0.007$). All other IgM values from $\alpha\beta$ TCR $-/-$, CD4-depleted C57BL/6, and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice were not significantly different from the values of C57BL/6 mice ($P > 0.07$). All the IgG and IgA values from CD4-depleted C57BL/6, $\alpha\beta$ TCR $-/-$, and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice were significantly different from the values of C57BL/6 ($P < 0.0001$). ND, not done.

tion after rechallenge, the intestinal IgA levels of $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice were unchanged (Fig. 2e and Table 1). Serum antibody levels were also not changed by viral rechallenge (Table 2). These results demonstrate that a low but clearly detectable virus-specific IgA response, principally localized to the intestine, can develop and persist for up to 8 weeks in completely T cell deficient animals. This response is associated with clearance of primary infection and resistance to reinfection.

$\gamma\delta$ T cells do not influence the development of rotavirus-specific intestinal IgA

Fujihashi *et al.* have recently shown that $\gamma\delta$ TCR $-/-$ mice develop lower intestinal IgA in response to tetanus toxoid and cholera toxin than $\gamma\delta$ TCR $+/+$ mice. We used these mice to study if the rotavirus-specific intestinal IgA was also altered. $\gamma\delta$ TCR $-/-$ mice developed an intestinal IgA response of similar kinetics and magnitude to control C57BL/6 mice (Fig. 2f and Table 2). The serum antiviral response in $\gamma\delta$ TCR $-/-$ mice was also comparable to control mice. The $\alpha\beta/\gamma\delta$ TCR $-/-$ mice developed an intestinal IgA response that was slightly higher than that of $\alpha\beta$ $-/-$ mice (Table 1), indicating that, if anything, the $\gamma\delta$ T cells could be downregulating the IgA response. However, $\gamma\delta$ T cells do not seem to be up or downregulating the intestinal IgA response in $\alpha\beta$ TCR $-/-$ mice, because these mice, depleted of $\gamma\delta$ T cells, developed an IgA response of similar kinetics and magnitude to undepleted mice (Fig. 2f and Table 1). The small difference in levels of intestinal IgA response could be explained by the different genetic background of the $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$. Taken together, these results suggest that $\gamma\delta$ T cells do not significantly modulate the antirotavirus intestinal IgA response in mice.

Elispot quantitation of virus-specific ASC in the spleen and lamina propria of T cell deficient mice

We performed Elispot analysis with lamina propria and spleen cells from nude and TCR $-/-$ mice 2 weeks after primary infection to quantify better the magnitude of the IgA response they developed (Table 3). Results consistent with the ELISA findings were obtained. $\gamma\delta$ TCR $-/-$ mice developed similar numbers of rotavirus-specific IgA and IgG ASC to C57BL/6 mice in their spleens and lamina propria. The $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice developed roughly 60 and 7 times lower numbers of rotavirus-specific IgA ASC and no virus-specific IgG ASCs in the lamina propria. The $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice did not develop any virus-specific ASC in the spleen, confirming that the antibody response was highly localized to the intestinal mucosal compartment. Results with the nude $-/-$ mice were similar to the $\alpha\beta$ TCR $-/-$ mice, showing no ASC in the spleen and an exclusive IgA response in the lamina propia of a magnitude 20 times less than their nude $+/-$ littermates (Table 3).

Intestinal IgA from nude $-/-$, $\alpha\beta$ TCR $-/-$, and $\alpha\beta/\gamma\delta$ TCR $-/-$ recognizes the rotavirus VP6 protein expressed by a recombinant baculovirus

Work from our laboratory has recently shown that IgA monoclonal antibodies specific for VP6, a nonneutralizing rotavirus protein, can mediate immunity to murine rotavirus (Burns *et al.,* 1996). In light of this finding, it was

TABLE 3

Elispot Quantitation of Rotavirus-Specific ASC in Immunocompetent and TCR Deficient Mice

	No. of ASC in the lamina propria per 10^5 cells (SEM)				No. of ASC in the spleen per 10^5 cells (SEM)			
	Total		Rotavirus-specific		Total		Rotavirus-specific	
Mouse strain	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
Nude +/− (n = 3)	4,533 (2,820)	453.3 (374)	303.3 (248.8)	1.6 (1.6)	146.7 (78.6)	223.3 (101.1)	1.3 (1.3)	1 (0.5)
Nude −/− (n = 4)	1,635 (514)	460 (282)	14.25 (6.5)*	1.7 (1.7)**	34 (15.3)	575 (159.9)	0**	0**
C57BL/6 (n = 5)	8,013 (3,467)	1560 (801.3)	893.2 (172.8)	6 (3.6)	64 (20.4)	216 (97)	8 (3.1)	1.7 (0.6)
TCR $\gamma\delta$ −/− (n = 5)	17,980 (7,078)	2155 (1340)	809 (284.7)***	2.3 (2.1)***	60 (12.6)	140 (26.8)	3.08 (0.5)***	2.6 (1.3)***
TCR $\alpha\beta$ −/− (n = 5)	3,041 (1,741)	558.1 (258.6)	14.3 (9.7)†	0†	11.2 (2)	36 (12)	0†	0†
TCR $\alpha\beta/\gamma\delta$ −/− (n = 5)	5,607 (608.8)	642.2 (514.9)	131 (18.1)†	0†	26 (5.8)	118.8 (71)	0†	0†

Note. Two weeks after primary infection with the EC strain of murine rotavirus, spleen and lamina propria cells from the different strains of mice were assayed by Elispot analysis.

* Value is significantly different from the value of nude +/− mice (P = 0.05).

** Values are not significantly different from the values of nude +/− mice ($P > 0.15$).

*** Values are not significantly different from the corresponding values of C57BL/6 mice ($P > 0.11$).

† Values are significantly different from the corresponding values of C57BL/6 mice ($P < 0.03$).

of interest to determine the specificity of the rotavirus-specific intestinal IgA seen in the T cell deficient mice. We used a recently described immunohistochemical assay with baculovirus-expressed neutralizing rotavirus proteins VP4 and VP7 and the nonneutralizing VP6 protein (Ishida *et al.,* 1996). C57BL/6 mice developed a predominant intestinal IgA response against VP6, a smaller response against VP4, and no response against VP7, as was demonstrated with BALB/c mice (Ishida *et al.,* 1996). The response of $\gamma\delta$ TCR −/− mice was similar to that of C57BL/6 mice, while the $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR −/− mice developed a response of lower magnitude and only against VP6 (Table 4). This result is consistent with the view that IgA antibodies against VP6 can mediate an antiviral effect.

C57BL/6 mice depleted of $CD4^+$ T cells efficiently clear primary rotavirus infection and develop low levels of virus-specific intestinal IgA

The rotavirus-specific intestinal IgA response described above in the T cell knockout mice could, in theory, be a response that compensates for their lack of T cells and T cell dependent antibody. To determine if immunocompetent mice also develop a T cell-independent rotavirus-specific intestinal IgA, we infected C57BL/6 mice depleted of $CD4^+$ T helper cells with murine rotavirus. More than 85% of the CD4 population was depleted in lamina propria, IELs and spleen of treated mice. As can be seen in Fig. 3 these mice cleared rotavirus infection like undepleted mice and produced levels of rotavirus-specific intestinal IgA similar to the $\alpha\beta$ TCR −/− mice. The levels of virus-specific antibody in stool and serum were also very similar to those of the $\alpha\beta$ TCR −/− mice (Tables 1 and 2). For comparison we studied C57BL/6 mice that were depleted of $CD8^+$ T cells (Fig. 3). More than 95% of the CD8 cells was depleted in the spleen and IELs of treated mice. As previously reported with mice of C57BL/6 and 129 mixed genetic background (Franco *et al.,* 1997), the CD8-depleted C57BL/6 mice

TABLE 4

Intestinal IgA from T Cell Deficient Mice Recognizes Recombinant VP6 Rotavirus Protein

	Geometric mean stool IgA titer against recombinant rotavirus proteins 10 days after primary infection (titer range)		
Mouse strain	VP4	VP6	VP7
Nude +/−	1.7 (<3–3)	67 (27–243)	<3
Nude −/−	<3	6.2 (3–27)*	<3
C57BL/6	2.4 (<3–9)	43.9 (9–243)	<3
$\gamma\delta$ TCR −/−	3 (<3–9)**	27 (9–81)**	<3
$\alpha\beta$ TCR −/−	<3	6.8 (3–27)***	<3
$\gamma\delta$-depleted $\alpha\beta$ TCR −/−	<3	6 (3–9)***†	<3
$\alpha\beta/\gamma\delta$ TCR −/−	<3	14.4 (3–27)***	<3

Note. Serial threefold dilutions of 10% stool suspensions from mice 10 days after primary infection were assayed for reactivity against Sf-9 insect cells infected with recombinant baculoviruses expressing VP4, VP6, and VP7 rotavirus proteins from the EW strain of murine rotavirus. Results represent the geometric mean of five or more mice in each group.

* Value is significantly different from the value of nude +/− mice (P = 0.001).

** Values are not significantly different from the values of C57BL/6 mice ($P > 0.18$).

*** Values are significantly different from the corresponding value of C57BL/6 mice ($P < 0.001$).

† Value is not significantly different from the value of nondepleted $\alpha\beta$ TCR −/− mice (P = 0.55).

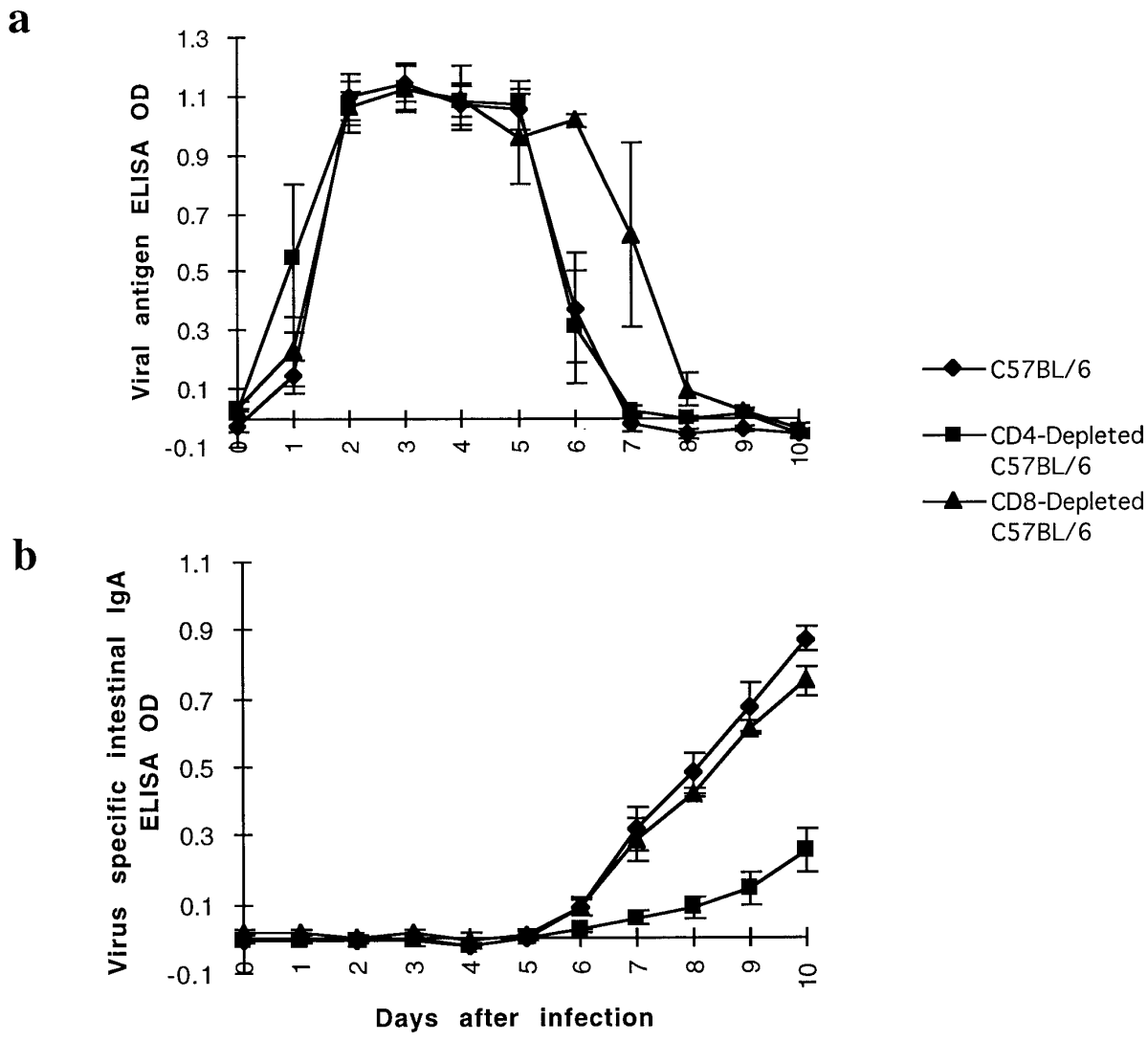

FIG. 3. Fecal viral antigen (a) and rotavirus specific intestinal IgA (b) shedding curves of C57BL/6 mice depleted or not depleted of $CD4^+$ or $CD8^+$ cells by administration of GK1.5 anti-CD4 or 2.43 mAb, respectively. Mice were challenged at 6 to 8 weeks of age with 10^5 SD_{50} of the ECw strain of murine rotavirus. Fecal rotavirus was measured by ELISA and results are expressed as OD readings. Each time point represent the mean of five C57BL/6 mice and three depleted mice plus and minus the corresponding SEM. Experiments with the CD4- and CD8-depleted mice have been repeated three and two times, respectively, with similar results.

had a 1- to 2-day delay in clearance of primary rotavirus infection but developed similar levels of virus specific intestinal IgA (Fig. 3). The mean number of antigen shedding days of CD8-depleted C57BL/6 mice was greater than that of nondepleted C57BL/6 mice ($P = 0.0027$).

SCID mice on the C57BL/6 background can clear primary rotavirus infection

The experiments with T cell knockout mice reported above were performed with mice on a C57BL/6 background. We infected T and B cell deficient SCID mice on a C57BL/6 background to determine if other factors, in addition to the antibody response identified in the T cell knockout mice, could also be contributing to immunity to rotavirus infection. In contrast to prior findings in T and B cell deficient Rag 2 −/− mice (129×C57BL/6 background) and BALB/c SCID mice (Franco and Greenberg, 1995; Riepenhoff-Talty *et al.,* 1987), 40% of the C57BL/6 SCID mice cleared primary rotavirus infection and mice that chronically shed virus for more than 10 days shed variable levels of viral antigen. The experiment of Fig. 4 is representative of a total of three experiments performed with SCID mice. In this experiment mouse S4 was able to completely clear primary rotavirus infection while its other four littermates became chronically infected (Fig. 4). In total, approximately 40% of SCID mice of the C57BL/6 background cleared infection. SCID mice that cleared infection did not develop rotavirus-specific IgM, IgG, or IgA antibodies in serum or intestine (data not

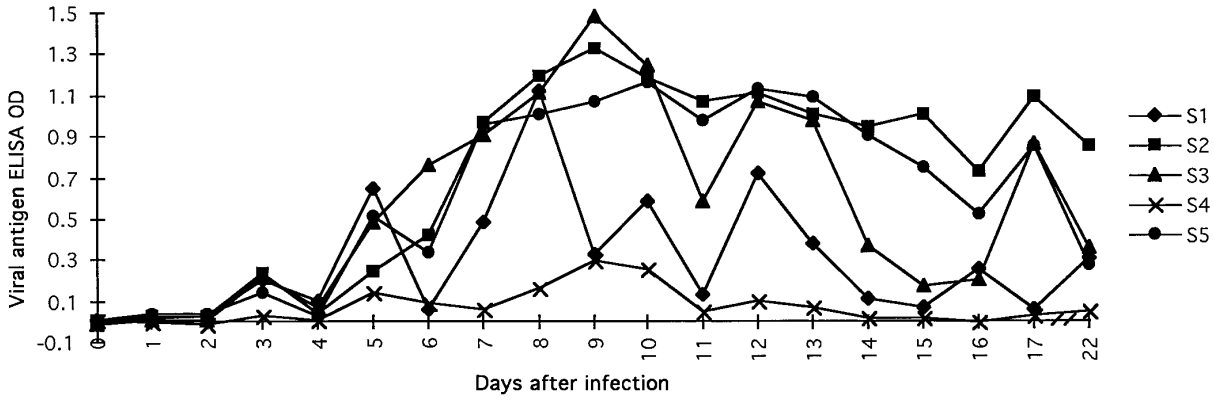

FIG. 4. Fecal viral antigen shedding curves of C57BL/6 SCID mice. Mice were challenged at 6 to 8 weeks of age with 10^5 SD_{50} of the ECw strain of murine rotavirus. Fecal rotavirus was measured by ELISA and results are expressed as OD readings. Shedding curves of individual mice are shown. This experiment has been repeated three times.

shown). This result suggests that in mice with a C57BL/6 background, innate or nonimmunological mechanisms can also mediate partial immunity to rotavirus.

DISCUSSION

The detection of virus-specific intestinal IgA in T cell deficient mice was surprising since the switch to this class of immunoglobulin is generally felt to be dependent on the interaction between T helper cells (classically using an $\alpha\beta$ TCR) and B cells through the interaction mediated by CD40 and its ligand (Oxenius *et al.,* 1996). The finding that $\alpha\beta$ and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice cleared primary rotavirus infection much more efficiently than C57BL/6 SCID mice suggests that the T cell independent local antibody response in the former mice is responsible for clearance of rotavirus infection and in protecting these mice from reinfection.

Our results with the nude mice differ somewhat from those reported previously (Eiden *et al.,* 1986). Eiden *et al.* observed that rotavirus-specific antibody was not detected in rotavirus infected BALB/c nude $-/-$ mice. We do not think the difference in these experiments is due to the strain of rotavirus employed since we have obtained results similar to those found in Fig. 1 using 10^2 SD_{50} of the EW strain of murine rotavirus (data not shown). This strain of virus is derived from the same original virus stock as that used by Eiden *et al.* The competitive ELISA to measure local antibody responses used in the prior studies may have been unable to detect the rotavirus-specific IgA antibody reported here. The fact that 100% of BALB/c SCID mice become chronically infected with murine rotavirus (Riepenhoff-Talty *et al.,* 1987 and MF unpublished data) suggests that the virus-specific T cell independent antibody in BALB/c nude $-/-$ mice is contributing to viral clearance. Nonetheless, we cannot exclude the possibility that a mechanism similar to that of C57BL/6 SCID mice or thymus-independent T cells present in nude $-/-$ mice (Lin *et al.,* 1993) could also be contributing to viral clearance.

We did not find any difference in the rate of clearance of primary rotavirus infection or in the development of the virus-specific IgA response in $\gamma\delta$ TCR $-/-$ mice compared to normal C57BL/6 mice (Fig. 2c). We also did not see any alteration in viral clearance or in the production of low levels of virus-specific intestinal IgA in the $\alpha\beta$ TCR $-/-$ mice depleted of $\gamma\delta$ T cells (Fig. 2f). These results, taken together with our previous studies in J_HD $-/-$ B cell deficient mice depleted of $\gamma\delta$ T cells (Franco and Greenberg, 1997), suggest that $\gamma\delta$ T cells are not necessary for clearance of rotavirus infection. These results do not formally exclude the possibility that $\gamma\delta$ T cells may play a role during rotavirus infection, but its role is compensated by another mechanism or is too small to be detected in our experiments. Our finding that $\gamma\delta$ TCR $-/-$ mice do not have altered intestinal antiviral IgA responses differs somewhat from the findings of Fujihashi *et al.* that showed that $\gamma\delta$ TCR $-/-$ mice had reduced intestinal IgA responses to cholera toxin and tetanus toxoid (Fujihashi *et al.,* 1996). The fact that our mice were responding to rotavirus, a replicating antigen could account for the difference between the two studies.

Interestingly the intestinal IgA response of nude, $\alpha\beta$, and $\alpha\beta/\gamma\delta$ TCR $-/-$ mice appeared to be directed exclusively to VP6. VP6 is a structural viral protein against which most of the host antibody response following infection is directed (Ishida *et al.,* 1996). It has been shown that the repetitive structure of the virus-associated form of the glycoprotein of vesicular stomatitis virus is key to its immunogenicity for B cells (Bachmann *et al.,* 1993). In particular this oligomeric structure affects its ability to stimulate T cell-independent B cell responses (Bachmann *et al.,* 1995) without necessarily inducing a polyclonal B cell response (Fehr *et al.,* 1996). VP6, assembled in the middle layer of the rotavirus particle, forms a repeti-

tive structure. Recombinant VP6 is also known to self-assemble into complex oligomers (Kapikian and Chanock, 1996; Tosser *et al.,* 1992). It is likely that VP6, like the glycoprotein of vesicular stomatitis virus, is presented to the immune system as a highly repetitive antigen capable of inducing strong T cell-independent as well as T cell-dependent B cell responses. In the immunocompetent mice, the intestinal IgA response to VP4 is more than 10-fold lower than to VP6 (Table 4). Therefore the lack of a detectable antibody response against VP4 in the T cell deficient mice could be due to a general decrease in IgA responses or to other unknown factors in these mice.

The presence of intestinal rotavirus-specific IgA in CD4-depleted C57BL/6 mice suggests that the T cell-independent IgA response observed in the T cell knockout mice exists or can exist in normal mice under some circumstances. Due to the low levels of the T cell-independent antibody produced by the CD4 depleted mice, it is improbable that these antibodies play an essential role in resolving infection or preventing reinfection in normal mice. It is tempting to speculate that the T cell-independent IgA response participates in regulating the equilibrium between the host and the commensal intestinal microorganisms, as has been recently suggested for intestinal IgA antibodies produced by B1a cells (Bos *et al.,* 1996). In contrast to the small delay in viral clearance observed in CD8-depleted C57BL/6 mice, CD4-depleted C57BL/6 mice cleared primary rotavirus infection just as rapidly as nondepleted mice (Fig. 3). This result indicates that in C57BL/6 mice, $CD8^+$ T cells, but not $CD4^+$ T cells, are necessary for efficient clearance of primary rotavirus infection.

Complete clearance of primary rotavirus infection by a subset of C57BL/6 SCID mice clearly implicates an innate mechanism in clearance of rotavirus infection (Fig. 4). This mechanism seems to be particularly operative in immunodeficient mice, and varies in effectiveness depending on the strain of mice. In one experiment with T cell deficient mice, 4 $\alpha\beta$ TCR $-/-$ and 3 $\alpha\beta/\gamma\delta$ TCR $-/-$ mice shed lower levels of viral antigen than naive C57BL/6 mice in a manner similar to the SCID mouse S4 in Fig. 4, suggesting that this mechanism can also exist to a certain degree in the T cell deficient mice. In addition, we have observed that outbred and some C57BL/6 nude $-/-$ mice behave similarly to SCID mouse S4. SCID mice have been shown to have NK cells with enhanced antiviral function compared to those from normal mice (Taterka *et al.,* 1995). From our data it is not possible to determine whether the anti-rotavirus effect we observed in the SCID mice is due to these enhanced NK cells or to other immunological or no immunological mechanisms (e.g., due to changes in the intestinal epithelial cells). Of note, the present studies further document the redundant nature of the protective immune response to rotavirus and the multiple immunologic and possible nonimmunologic pathways available to the host to respond to this highly important and ubiquitous pathogen.

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