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$CD8^+$ T Cells Can Mediate Almost Complete Short-Term and Partial Long-Term Immunity to Rotavirus in Mice

MANUEL A. FRANCO,* CRAIG TIN, AND HARRY B. GREENBERG

Veterans Administration Palo Alto Health Care System and Departments of Medicine, Microbiology, and Immunology, Stanford University School of Medicine, Stanford, California 94305

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We have recently shown that $CD8^+$ T cells mediate clearance of rotavirus infection in mice. B-cell-deficient J_HD knockout (−/−) mice depleted of $CD8^+$ T cells become chronically infected with murine rotavirus, and β_2 microglobulin −/− and other mice depleted of $CD8^+$ T cells have a 1- to 4-day delay in clearance of primary rotavirus infection. A role for $CD8^+$ T cells in protection from reinfection with rotavirus was suggested by these studies, because J_HD −/− mice rechallenged 6 to 8 weeks after primary infection shed smaller quantities of viral antigen and for fewer days than naive mice. Here we show that 8, 11, 13, and 18 days after primary infection the J_HD −/− mice are almost completely resistant to reinfection and that they are still partially protected from reinfection 6 weeks, 5 months, and 8 months after primary infection. Protection against reinfection was dependent on $CD8^+$ T cells, since J_HD −/− mice depleted of $CD8^+$ T cells by administration of an anti-CD8 monoclonal antibody became chronically infected with rotavirus upon rechallenge 13 days, 18 days, 6 weeks, and 5 months after primary infection. Thus, $CD8^+$ T cells can actively mediate almost complete short-term and partial long-term protection from reinfection.

Passive cell transfer experiments have shown that $CD8^+$ T cells can clear the chronic rotavirus infection of SCID (5) and Rag 2 knockout (−/−) mice (9) and prevent rotavirus-induced diarrhea in mouse pups (16). Recently, we and others have shown that $CD8^+$ T cells actively mediate clearance of primary rotavirus infection in J_HD −/− mice by demonstrating the inability of these mice to clear rotavirus infection if depleted of $CD8^+$ T cells by administration of an anti-CD8 monoclonal antibody (MAb) (6, 15). Further evidence of a role for $CD8^+$ T cells in clearing primary rotavirus infection is provided by experiments with β_2 microglobulin −/− mice (6) and immunocompetent mice depleted of $CD8^+$ T cells (9). Both types of mice completely clear primary rotavirus infection but with a short delay compared to the clearance time of immunocompetent nondepleted mice, and viral clearance correlates in these mice with the appearance of intestinal rotavirus-specific immunoglobulin A (IgA). Taken together, these experiments suggest that antibody also plays a role in clearance of primary rotavirus infection and that $CD8^+$ T cells mediate initial clearance of primary rotavirus infection. In the absence of $CD8^+$ T cells, clearance is delayed 1 to 4 days (6, 9). We have recently presented evidence that the antirotaviral mechanism used by the $CD8^+$ T cells is not dependent on killing of the hosts infected cells by a perforin- or fas-mediated mechanism or by the secretion of gamma interferon (9).

Although antibody seems to be the primary determinant of protection against rotavirus reinfection, a role for $CD8^+$ T cells in protection from viral reinfection was suggested by previous experiments (6, 15) in which J_HD −/− mice rechallenged 6 to 8 weeks after primary infection shed smaller quantities of viral antigen and for fewer days than naive J_HD −/− mice. Nevertheless, McNeal et al. showed that partial protection in B-cell-deficient mice was not abrogated if mice were depleted of $CD8^+$ T cells before being rechallenged with the EDIM strain of murine rotavirus (15). They suggested that this result may be due to resistance to depletion of memory $CD8^+$ T cells (15). Thus, it was not entirely clear if $CD8^+$ T cells actually mediated the partial protection seen in the J_HD −/− mice against rechallenge with the EDIM and ECw strains of rotavirus. In this paper, we address this issue by studying how protection against rechallenge with the ECw strain of rotavirus varies with time in J_HD −/− mice depleted or not depleted of $CD8^+$ T cells.

Naive 6- to 8-week-old J_HD −/− mice (obtained from Genpharm International (Mountain View, Calif.) and bred in our facility as described previously (6) were orally infected with an intestinal homogenate containing 10^5 shedding doses 50 (SD_{50}) of the ECw strain of murine rotavirus as previously described (6). Stool samples were then collected from the mice and analyzed for viral antigen by the previously described enzyme-linked immunosorbent assay (ELISA) (2). Briefly, microtiter plates (Dynatech, McLean, Va.) were coated with diluted hyperimmune rabbit anti-rhesus rotavirus serum and blocked, and then 5% stool sample suspensions were added to individual wells and incubated overnight at 4°C. Plates were washed, and diluted guinea pig anti-rhesus rotavirus hyperimmune serum was added to the plates for 1 h at 37°C. After two washes, diluted horseradish peroxidase-conjugated goat anti-guinea pig IgG serum (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added to the plates and incubated for 1 h at 37°C. After two washes, ABTS (2,2′-azido-di-3-ethylbenzthiazoline sulfonate) substrate (Kirkegaard and Perry Laboratories) was added, the plates were developed for 10 min at room temperature, and the reaction was stopped by the addition of 10% sodium dodecyl sulfate. The absorbance at 405 nm was read with a plate reader (BIO-TEK Instruments, Burlington, Vt.). A sample was considered positive for viral antigen if its optical density (OD) was at least 0.1 unit greater than the OD of samples from naive mice on the day of infection. Mice were rechallenged with the same dose of rotavirus at different times after infection, and stool samples were analyzed similarly. The total amount of viral antigen shed by each mouse was obtained by calculating the area under the antigen shed-

* Corresponding author. Mailing address: Stanford University, School of Medicine, Lab Surge, P304, Stanford, CA 94305. Phone: (415) 493-5000, ext. 63124. Fax: (415) 852-3259. E-mail: francoma@leland.stanford.edu.

ding curve. Due to minor variations in the sensitivity of the ELISA between experiments, this last value was obtained after dividing all the values from an experiment by the mean of the peak amounts of antigen shedding of naive mice in the experiment. Immunocompetent sentinel mice kept during the period of the experiments described did not develop rotavirus infection or infection with other common mouse pathogens. As previously described (6), none of the J_HD −/− mice infected with rotavirus developed rotavirus-specific stool IgA (data not shown).

Mice were depleted of $CD8^+$ T cells by administration of ascites fluid containing the rat anti-mouse CD8 MAb 2.43 as previously described (6). Each mouse received 0.5 ml of ascites fluid intraperitoneally 5, 4, and 3 days before rotavirus infection or rechallenge, on the day of rotavirus infection or rechallenge, and on days 3, 6, and 9 after infection or rechallenge. On the day of rotavirus infection or rechallenge, depleted and nondepleted control mice were killed to verify depletion of $CD8^+$ T cells in spleens and among intraepithelial lymphocytes (IELs) by fluorescence-activated cell sorter (FACS) analysis. Spleen cells and IELs were stained with fluorescein isothiocyanate-labeled anti-CD8 MAb 53 6.7 and either anti-γδ T-cell receptor (TCR) (MAb GL3) or anti-αβ TCR (MAb H57-597) MAb, both of which were labeled with phycoerythrin (PE) (all labeled MAbs were obtained from Pharmingen, San Diego, Calif.).

Since, in some viral models, the capacity of $CD8^+$ T cells to mediate protection against viral reinfection has been reported to be short-lived (11), we first determined if J_HD −/− mice rechallenged within days after primary infection were better protected than mice rechallenged 6 to 8 weeks after primary infection. A prior study indicated that 4-day-old J_HD −/− pups infected with rotavirus and then rechallenged with the ECw strain of murine rotavirus 6 to 8 weeks after primary infection were all reinfectable (6). In our study, 0, 9, 33, and 50% of J_HD −/− mice rechallenged 8, 11, 13, and 18 days, respectively, after primary infection as adults were reinfectable (Fig. 1a and 2a and Table 1). The mice infected on days 11, 13, and 18 shed virus for only 1 to 3 days compared to the 5 to 6 days of viral shedding following primary infection (Table 1). To determine if this early protection against reinfection was mediated by $CD8^+$ T cells, we administered anti-CD8 MAb to a group of J_HD −/− mice starting on day 8 after primary infection and rechallenged these mice on day 13 after primary infection (Fig. 1b). More than 95% of the $CD8^+$ T cells in the spleens and the IELs of control mice that received the anti-CD8 MAb were depleted (Fig. 3). All the mice depleted of $CD8^+$ T cells on day 8 were reinfected by day 10 postinfection and shed virus to levels comparable to those shed after the primary infection (Fig. 1b). The early reshedding in CD8-depleted mice (day 10) was probably due to reinfection with residual virus present in the mouse cages or possibly to virus that the mice had not completely cleared (although they had stopped shedding detectable quantities of viral antigen in the stool by day 8 [Fig. 1b]). Two of the mice continued shedding rotavirus until day 19 after infection, when stool sample collection was stopped, and two mice cleared infection by day 17 after primary infection (Fig. 1b). Because in the previous experiment it was unclear whether the reinfection in the CD8-depleted mice was due to incomplete viral clearance or actual reinfection, we performed an experiment in which the CD8 depletion was started 13 days after primary infection and the challenge was done 5 days later (Fig. 2). In contrast to the CD8-undepleted mice, 50% of which shed virus for 1 to 3 days, four CD8-depleted mice that were not shedding detectable viral antigen at the time of rechallenge started shedding viral antigen at high levels upon

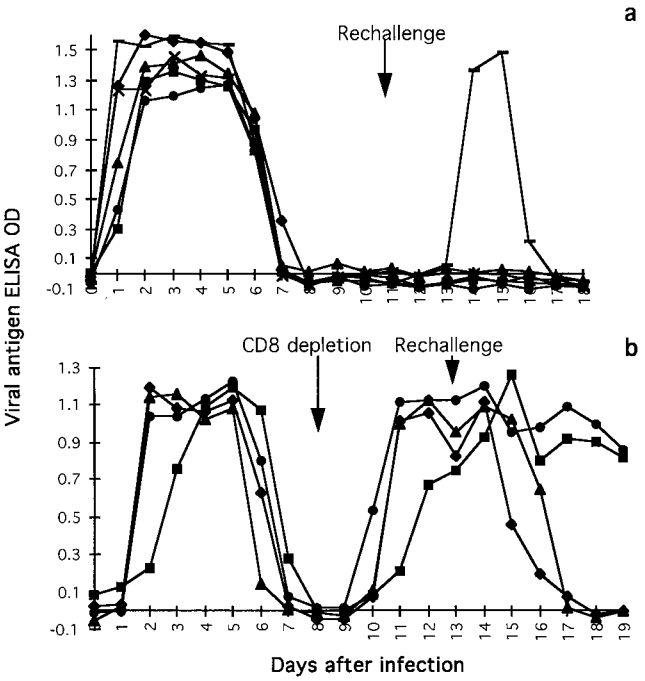

FIG. 1. Fecal viral antigen shedding curves of 6- to 8-week-old J_HD −/− mice orally infected with 10^5 SD_{50} of the ECw strain of murine rotavirus and then rechallenged with the same virus dose. (a) Nondepleted mice rechallenged on day 11; (b) mice depleted of $CD8^+$ T cells by administration of anti-CD8 MAb 2.43 starting on day 8 after the primary infection (indicated by the arrow) and rechallenged on day 13. Fecal rotavirus antigen was measured by ELISA, and results are expressed as OD units. Shedding curves of individual mice are shown.

rechallenge and continued to do so until the experiment was finished. This result confirms that the early protection against reinfection was due to $CD8^+$ T cells. The effect of the anti-CD8 MAb was not due to a nonspecific effect of the ascites treatment, because comparable administrations of ascites containing a rat anti-interleukin-2 MAb or a hamster anti-γδ TCR (clone S4B6-1 and clone UC7-13D5, respectively, both obtained from the American Type Culture Collection) did not alter normal clearance of primary rotavirus infection of J_HD −/− mice or prevent the partial protection from reinfection when the mice were rechallenged 6 weeks after the second challenge (Table 1 and data not shown). We interpret these results as indicating that $CD8^+$ T cells are capable of mediating complete or almost complete protection against rotaviral reinfection within the first 18 days after primary infection. This timing makes it likely that the protection was mediated primarily by effector lymphocytes.

To determine if $CD8^+$ T cells could mediate protection from viral reinfection at later time points after primary infection, we rechallenged CD8-depleted and -nondepleted J_HD −/− mice 5 months after primary infection (Fig. 4 and 5 and Table 1). Some of the mice (described above) that had been rechallenged 8 to 13 days after primary infection were also rechallenged 6 weeks or 8 months after the second challenge (Fig. 4 and 5 and Table 1). All CD8-nondepleted J_HD −/− mice rechallenged 5 months after primary infection or 8 months after the second challenge were reinfected (Table 1). Sixty percent of nondepleted J_HD −/− mice rechallenged for a second time after 6 weeks were also reinfected (Table 1 and Fig. 4a). However, the mean number of antigen shedding days and the mean total amount of viral antigen shed from all rechallenged mice were significantly less than the mean num-

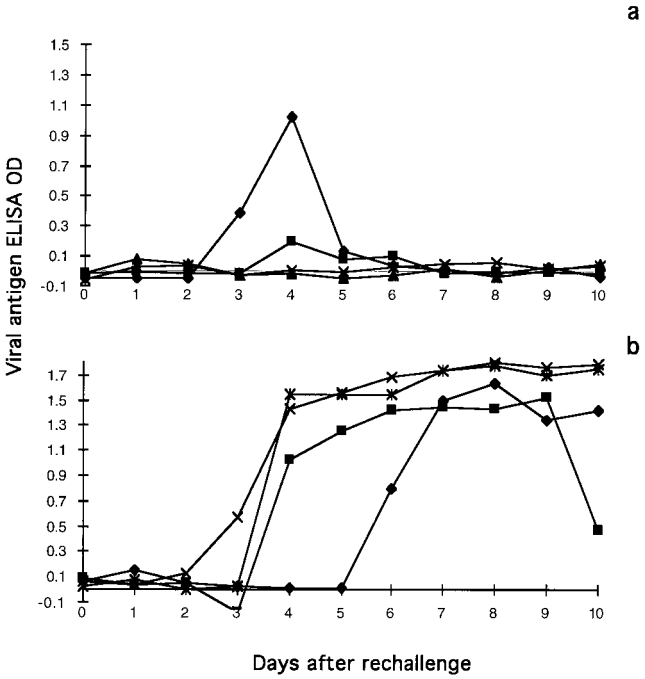

FIG. 2. Fecal viral antigen shedding curves of J_HD −/− mice rechallenged with 10^5 SD_{50} of the ECw strain of murine rotavirus 18 days after primary infection. (a) Nondepleted mice; (b) mice depleted of $CD8^+$ T cells by administration of anti-CD8 MAb 2.43 starting 5 days before rechallenge. Fecal rotavirus antigen was measured by ELISA, and results are expressed as OD units. Shedding curves of individual mice are shown.

ber of antigen shedding days or mean total amount of viral antigen shed ($P < 0.002$ according to the Mann-Whitney U test) of naive mice (Table 1). This partial protection from reinfection was mediated by $CD8^+$ T cells, because most of the mice that were depleted of $CD8^+$ T cells before rechallenge at 6 weeks or 5 months after primary infection shed viral antigen for at least 10 days after rechallenge (Fig. 4 and 5 and Table 1). These results indicate that $CD8^+$ T cells can mediate partial long-term protection (up to 5 months) from viral reinfection. Mice rechallenged for a second time 8 months following primary infection (the first rechallenge was on day 13) shed virus for only 1 day less than naive mice (Table 1) and on day 3 after the second rechallenge shed virus to levels comparable to those of mice with a primary infection (Fig. 5b), suggesting that the CD8 effect may eventually disappear.

$CD8^+$ T cells have been operationally classified as naive, effector, or memory cells (19). The efficient short-term protection against viral reinfection seen in our model is most probably mediated by $CD8^+$ effector T cells activated by the primary infection (Fig. 1 and 2 and Table 1). This result is consistent with the previously reported capacity of effector $CD8^+$ cells transferred into mouse pups to afford complete protection from rotavirus-induced diarrhea (16). In some models, memory $CD8^+$ T cells have been shown to retain effector characteristics like the lysis of target cells in vitro without the need of restimulation (19). Nevertheless, memory cells, probably because they are lower in number or not fully activated, generally do not prevent viral reinfection but limit the extent of the second infection by accelerating resolution (1). Memory $CD8^+$ T cells that are involved in the accelerated response are most probably responsible for limiting the number of days and quantity of viral antigen shedding after reinfection in the J_HD −/− mice rechallenged more than 18 days after a previous infection. We have recently shown that memory $CD8^+$ T cells home to the gastrointestinal tract in rotavirus infection on the basis of $\alpha4\beta7$ integrin expression (17).

Whether memory $CD8^+$ T cells can mediate significant protection against rotavirus-induced disease (as opposed to shedding) is difficult to evaluate in this model, as mice become maturationally resistant to rotavirus diarrhea after 15 days of age (18). Because protection from viral reinfection is a more stringent parameter than protection from disease, it is probable that $CD8^+$ T cells can also mediate some degree of protection from rotavirus-induced diarrhea. However, human studies with another mucosal pathogen, influenza virus, suggest that although $CD8^+$ T cells can mediate antiviral immu-

TABLE 1. Summary of rotavirus rechallenge experiments performed with J_HD −/− mice

Time interval between last two viral challenges	Treatment prior to last viral challenge	No. of mice	% of mice shedding antigen	Mean no. of days of viral antigen shedding (range)		Standardized mean total antigen shedding (SEM)	
				All mice	Reinfected mice only	All mice	Reinfected mice only
0 days (naive mice)	None	28	100	5.8 (5–7)		5.09 (0.11)	
0 days (naive mice)	Depletion of γδ T cells	4	100	5.75 (5–6)		5.60 (0.38)	
8 days	None	3	0	0		0	
11 days	None	11	9	0.27 (0–3)[c]	3 (3)[e]	0.1 (0.21)[c]	2.13[e]
13 days	None	9	33	1 (0–3)[c]	3 (3)[c]	0.71 (0.42)[c]	1.92 (0.37)[c]
13 days	Depletion of $CD8^+$ cells	4	100	(7–10 or more)[b]		6.66 (0.96)[d]	
18 days	None	4	50	1.25 (0–3)[c]	2.5 (2–3)[e]	0.33 (0.22)[c]	0.59 (0.27)[e]
18 days	Depletion of $CD8^+$ cells	4	100	(5–8 or more)[b]		5.98 (0.7)[d]	
6 wk[a]	None	5	60	1.8 (0–3)[c]	3 (3)[c]	0.80 (0.45)[c]	1.5 (0.2)[c]
6 wk[a]	Depletion of γδ T cells	6	83	1.66 (0–3)[c]	2 (1–3)[c]	0.85 (0.29)[c]	0.98 (0.31)[c]
6 wk[a]	Depletion of $CD8^+$ cells	6	100	(4–10 or more)[b]		6.96 (0.75)[d]	
5 mo	None	6	100	3.6 (2–6)[c]		1.87 (0.51)[c]	
5 mo	Depletion of $CD8^+$ cells	6	100	(10 or more)[b]		7.51 (0.70)[d]	
8 mo[a]	None	6	100	4.5 (3–5)[c]		2.57 (0.24)[c]	

[a] These mice had been initially rechallenged 8, 11, or 13 days after primary infection; all other mice were rechallenged only once.
[b] Two of four, four of four, four of six, and six of six of the mice had not cleared infection when the respective experiments were finished.
[c] Significantly different ($P < 0.002$) from the value for naive mice.
[d] Significantly different ($P < 0.004$) from the value in the preceding row.
[e] Statistical analysis was not performed with these values because of the low number of mice.

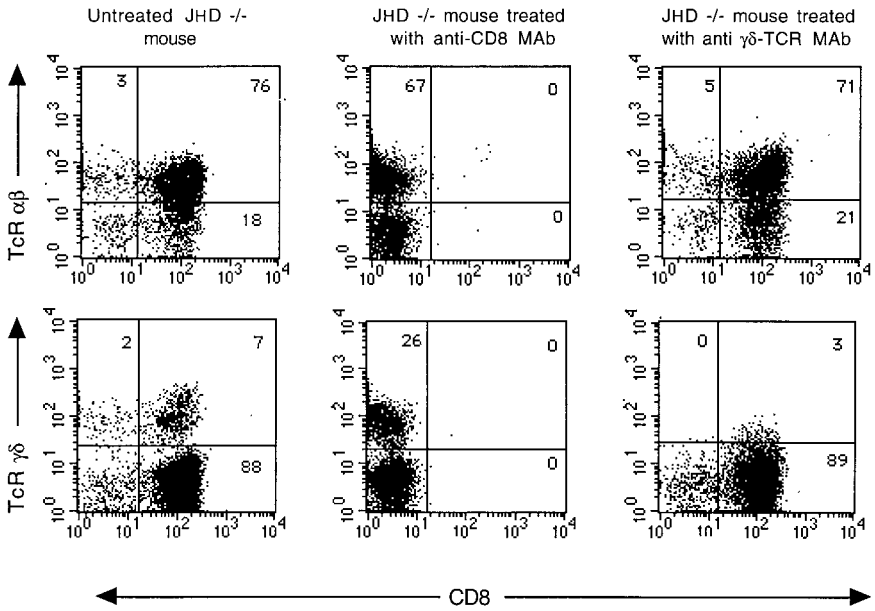

FIG. 3. FACS analysis of the IELs from J_HD −/− mice not depleted of $CD8^+$ T cells (first column), depleted of $CD8^+$ T cells by administration of anti-CD8 MAb 2.43 (second column), and inoculated with anti-γδ TCR MAb UC7-13D5 (third column). IELs were reacted with MAb 53 6.7-fluorescein isothiocyanate (anti-CD8) and either MAb GL3-PE (anti-γδ TCR) or MAb H57-597–PE (anti-αβ TCR). Numbers in the quadrants indicate percentages of stained cells in the respective compartments.

nity, they probably do not protect against disease after natural infection (13, 14).

One should be cautious before directly extrapolating the results seen in the B-cell-deficient J_HD −/− mice to normal mice. $CD8^+$ T cells are probably not needed for developing complete protection from reinfection in immunocompetent mice, since β_2 microglobulin −/− mice depleted of $CD8^+$ T cells are completely protected from rotavirus rechallenge (6). In addition, the protective effect of the $CD8^+$ T cells we observed in the J_HD −/− mice may be mediated by a mechanism that develops in these mice only as a compensatory response for their lack of B cells and may be absent in normal mice. However, it does seems likely that rotavirus-specific memory $CD8^+$ T cells persist in normal mice. A recent study of $CD8^+$ T-cell memory in μMT B-cell-deficient mice showed that the antiviral response of $CD8^+$ T cells in these mice was very similar in specificity and duration to that of immunocompetent mice (1). Determining directly whether $CD8^+$ T cells can actively mediate protection against rotavirus infection in immunocompetent mice could be done by vaccinating such mice with peptides containing previously described class 1-restricted rotavirus T-cell epitopes and then rechallenging these mice (7, 8). The relevance of the experiments presented here to rotavirus immunity in other species and in humans in particular remains to be established.

The capacity of rotavirus-specific memory $CD8^+$ T cells to protect against viral reinfection in mice appears to be less efficient than that of memory $CD8^+$ T cells specific for lymphocytic choriomeningitis virus and mouse cytomegalovirus, in which complete protection mediated by $CD8^+$ T cells can be evidenced months after primary infection with the challenge virus (1, 3). In contrast, antirotavirus memory $CD8^+$ T cells seem to be more efficient at mediating protection than the $CD8^+$ T cells induced by recombinant vaccinia viruses (rVV) expressing respiratory syncytial virus (RSV) or influenza virus glycoproteins (11, 12). $CD8^+$ T cells induced by rVV protect from reinfection only if the RSV challenge occurs within 10

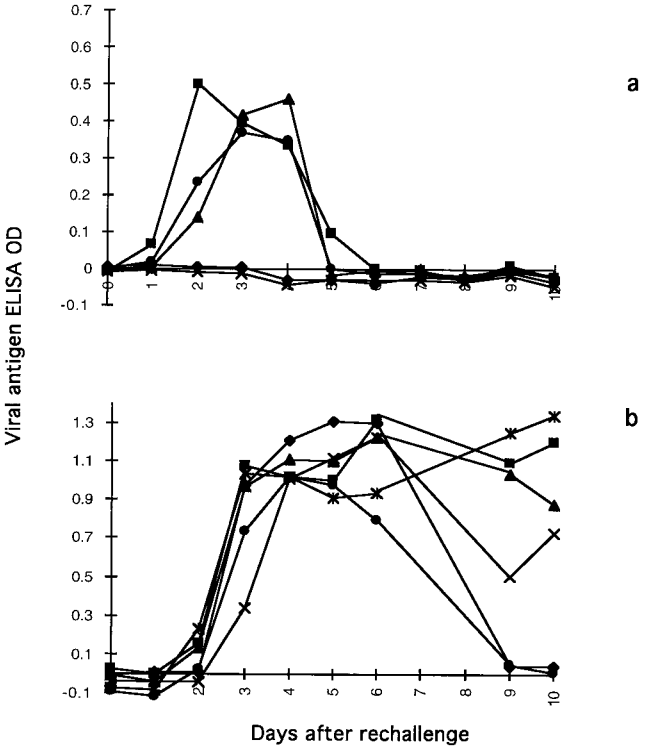

FIG. 4. Fecal viral antigen shedding curves of J_HD −/− mice rechallenged with 10^5 SD_{50} of the ECw strain of murine rotavirus 6 weeks after a second rechallenge 11 to 13 days after primary infection. (a) Nondepleted mice; (b) mice depleted of $CD8^+$ T cells by administration of anti-CD8 MAb 2.43 starting 5 days before rechallenge. Fecal rotavirus antigen was measured by ELISA, and results are expressed as OD units. Shedding curves of individual mice are shown. Stool samples were not collected from mice on days 7 and 8 for the experiment reflected in panel b.

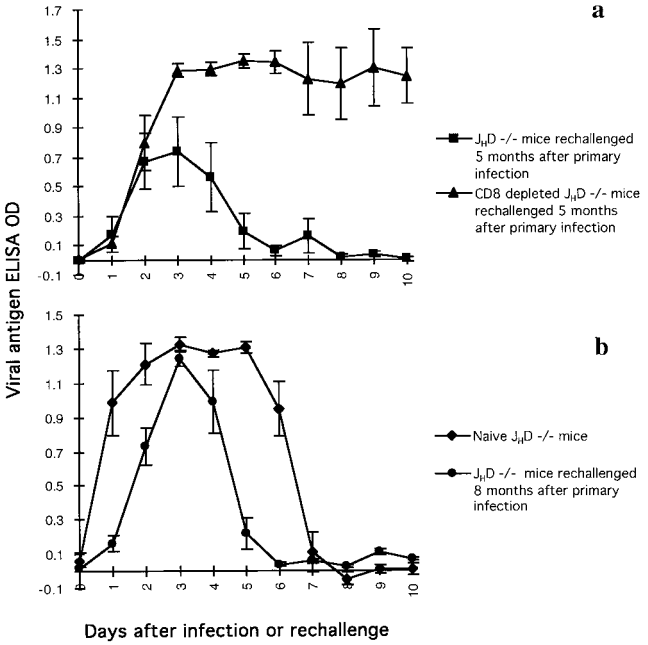

FIG. 5. (a) Fecal viral antigen shedding curves of J_HD −/− mice rechallenged 5 months after primary infection and either nondepleted or depleted of $CD8^+$ T cells by administration of anti-CD8 MAb 2.43; (b) fecal viral antigen shedding curves of naive J_HD −/− mice and J_HD −/− mice rechallenged 8 months after primary infection. The mice rechallenged 8 months after primary infection had been rechallenged a second time 11 to 13 days after primary infection. Mice were initially infected and rechallenged with 10^5 SD_{50} of the ECw strain of murine rotavirus. Fecal rotavirus antigen was measured by ELISA, and results are expressed as OD units. Each time point represents the mean of results with six mice ± the corresponding standard error of the mean.

days of immunization, and accelerated clearance of RSV infection is not evidenced if the challenge is performed 45 days after primary infection with the rVV (11). It has been proposed that the capacity of a virus to spread into lymphopoietic cells and replicate efficiently in the host (for example, lymphocytic choriomeningitis virus) determines the development of a strong $CD8^+$ T-cell response (20). Although rotavirus antigen has been detected in lymphoid organs, particularly in cells expressing major histocompatibility complex class II molecules after primary infection (4), it is not known if the virus can replicate in cells of lymphopoietic origin. For future studies of $CD8^+$ T-cell memory in mice, in addition to determining if rotavirus can replicate in cells of lymphopoietic origin, it will be important to ascertain whether rotavirus can persist for long periods of time in the host without evidence of antigen shedding. The persistence of viral antigen capable of constantly restimulating the $CD8^+$ T cells has been proposed as a factor that would induce an efficient long-term antiviral memory response (19). At present, there is no evidence available to support the proposal that rotavirus persists for long periods in the normal host.

As a control for detecting nonspecific effects following the anti-CD8 MAb administration, we also administered an anti-γδ TCR MAb to the J_HD −/− mice. The anti-γδ TCR MAb had no effect on clearance of primary rotavirus infection or on the partial protection from reinfection seen when the J_HD −/− mice were rechallenged for a second time 6 weeks after the first rechallenge (Table 1). Interestingly, when FACS analysis of nondepleted J_HD −/− mice was performed, we detected low percentages of γδ T cells compared to those of immunocompetent mice of the same genetic background (C57BL/6x129Sv) (Fig. 3 and data not shown). At present we do not have a clear explanation for this finding. The administration of the anti-γδ MAb depleted approximately half of the γδ IELs present in the J_HD −/− mice, and the remaining γδ T cells showed a reduction in the intensity of staining, suggesting modulation of TCR expression, similar to what has been reported by others (10). Although our depletion of γδ T cells was not complete, the result suggests that, in this model, γδ T cells are not necessary for immunity to rotavirus.

Our results differ somewhat from those of McNeal et al., who found that J_HD −/− mice depleted of $CD8^+$ T cells prior to rechallenge 6 weeks after primary infection shed reduced amounts of rotavirus antigen just like nondepleted mice (15). The ECw strain of rotavirus used in our study is more infectious for adult mice than the wild-type EDIM EWw strain of rotavirus (2), and the EDIM strain used by McNeal et al. is a tissue culture-adapted strain derived from the same original stock as the wild-type EWw strain. Thus, the ECw strain is probably more infectious for adult mice than the EDIM strain of rotavirus used by McNeal and colleagues. One could imagine that an immune mechanism distinct from $CD8^+$ T cells, or as suggested by McNeal et al., a very small number of memory $CD8^+$ T cells remaining after depletion treatment, could mediate protection against a less infectious virus. In support of this hypothesis is our finding that all CD8-depleted naive mice (6) and mice depleted of $CD8^+$ T cells 5 months after primary infection (Fig. 5a) continued to shed virus for at least 10 days after primary infection or rechallenge. On the other hand, among mice depleted of $CD8^+$ T cells 8 days after primary infection (Fig. 1b) or 6 weeks after the second rechallenge (Fig. 4b), two of four mice in the first case and two of six mice in the second case stopped shedding viral antigen despite continued anti-CD8 MAb treatment. The virus clearance seen in these subsets of mice appears to occur in the absence of $CD8^+$ T cells. In this circumstance, clearance could be explained by either memory $CD8^+$ cells that are resistant to depletion or another antiviral mechanism, as was hypothesized by McNeal et al.

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