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Role of B Cells and Cytotoxic T Lymphocytes in Clearance of and Immunity to Rotavirus Infection in Mice

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The immune mechanisms involved in clearance of and immunity to rotavirus infection are poorly understood. Although mice with severe combined immunodeficiency (SCID mice) become chronically infected, nude mice have been reported to clear rotavirus infection similarly to immunocompetent controls. To better characterize the role of cytotoxic T lymphocytes (CTLs) in clearance of and immunity to rotavirus infection, we infected naive or previously infected β_2-microglobulin (β_2m) knockout mice with murine rotavirus. Naive β_2m knockout mice shed rotavirus antigen 2 days longer than did normal control mice but completely resolved primary infection. β_2m knockout naive mice treated with depleting doses of an anti-CD8 monoclonal antibody before infection shed viral antigen for an additional day. Upon rechallenge, β_2m knockout mice, either treated with the anti-CD8 antibody or not treated, were completely resistant to reinfection. Clearance of rotavirus infection in naive β_2m knockout mice correlated with the development of intestinal rotavirus-specific immunoglobulin A. Before rechallenge, β_2m knockout mice had high levels of intestinal rotavirus-specific immunoglobulin A. These findings suggest that CTLs mediate rotavirus clearance but are not required for this function and that CTLs are not necessary for development of immunity to rotavirus reinfection. To further characterize the effector mechanisms involved in clearance and prevention of rotavirus infection, similar studies were performed with B-cell-deficient J_HD knockout mice. After primary infection, most naive J_HD mice had similar virus-shedding clearance curves as did control mice and completely resolved primary infection. However, 2 of 29 became chronically infected. All J_HD mice treated with anti-CD8 antibody became chronically infected with murine rotavirus. Upon rechallenge, J_HD mice which had cleared primary infection were all susceptible to reinfection. These findings suggest that B cells also play a role in clearance of primary infection but are absolutely necessary for development of immunity against rotavirus reinfection.

Rotaviruses are responsible for the death of 800,000 infants per year worldwide (15). The difficulties in developing a vaccine against rotaviruses are paralleled by our ignorance concerning the factors that determine both the clearance of and immunity to rotaviruses (4).

To understand these factors, we and others have used a mouse model of rotavirus infection (2, 10, 32). Previous studies with this model have produced conflicting results on the role of B cells and T cells in the resolution of primary infection and protection against rotavirus reinfection. It has been shown that mice with severe combined immunodeficiency (SCID mice) become chronically infected with murine rotaviruses (26). On the other hand, nude mice have been reported to clear rotavirus infection in the absence of a significant antibody response (8). The passive transfer of immune cytotoxic T lymphocytes (CTLs) has been shown both to protect against acute rotavirus-induced diarrhea in suckling mice (23) and to clear the chronic rotavirus infection from adult SCID mice (6). The rotavirus proteins recognized by the $CD8^+$ major histocompatibility complex-restricted T cells capable of mediating the clearance of chronic infection in SCID mice include both inner nonneutralizing and outer neutralizing proteins of the virus (5). Both clearance of primary infection and protection against reinfection have been correlated with the presence of rotavirus-specific intestinal immunoglobulin A (IgA) but not serum IgG (2, 10). Protection against reinfection in the mouse model has been shown to be relatively serotype independent and to correlate with the level of viral replication and serum and fecal rotavirus-specific IgA (10a, 21, 31).

To better clarify the role of B cells and CTLs in clearance of primary infection and the development of immunity against rotavirus reinfection, we have studied rotavirus infection in mice devoid of both T and B cells (Rag-2 gene knockouts [28]), CTLs (β_2 microglobulin [β_2m] knockout mice [20]), and B cells (J_HD knockout mice [3]). In our studies with adult mice, we have used a high-titer dose of the very infectious EC_w strain of rotavirus (2, 7), hoping to identify major determinants of both viral clearance and immunity.

MATERIALS AND METHODS

Cells. MA104 cells were grown as previously described (13). Plastic adherent MC57 (*H-2^b^*) cells were grown in Dulbecco's modified Eagle's medium (Biowhittaker, Walkersville, Mass.) containing 10% heat-inactivated fetal calf serum, 100 μg of streptomycin sulfate per ml, and 100 U of penicillin per ml.

Mice. All mice used in these studies were bred in our facilities except for the control adult C57BL/6 mice, which were obtained from Charles River, Portage, Mich., and 129/SvJ mice, which were obtained from the Jackson Laboratory, Bar Harbor, Maine. Because the 129/SvJ mice clear primary rotavirus infection and develop immunity to rotavirus reinfection similarly to C57BL/6 mice (data not shown), only C57BL/6 were used as controls in the experiments presented that involved animals derived from (129 × B6)F_2 founders. Breeding couples were obtained from the following sources: Rag-2 gene knockout mice [derived from (129 × B6)F_2 founders (H-2^b)] were from Genpharm International, Mountain View, Calif.; β_2m knockout mice [derived from (129 × B6)F_2 founders (H-2^b) and congenic with C57BL/6 (H-2^b)] were from the Jackson Laboratory; and J_HD mice [derived from (129 × B6)F_2 founder (H-2^b) mice] were obtained from D. Huszar (Genpharm International). As additional control mice for the Rag-2, J_HD, and β_2 knockout mice derived from (129 × B6)F_2, we used mice obtained

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by mating the latter mice with C57BL/6 mice (Charles River). Knockout and normal mice were weaned at 4 weeks of age and segregated according to sex into separate microisolator cages (five or fewer mice in each cage). All naive mice used did not have rotavirus-specific IgA in their stools before use. Sentinel animals housed in the cages previously used by the Rag-2 and J_HD knockout mice were periodically checked for serum anti-rotavirus antibodies and found negative.

Viruses. Wild-type murine rotavirus strains EW_w (G3?, P17) and EC_w (G3, P17) were originally obtained from J. Wolf and T. Flewett, respectively (2, 7). Stocks of wild-type murine rotaviruses were prepared as intestinal homogenates, and their titers were determined in pups and adult mice as previously described (2). The titer of the EW_w stock used was 10^8 50% diarrhea doses (DD_{50}) per ml and 10^8 50% pup shedding doses (SD_{50}) per ml. The titer of the EC_w stock was 10^7 DD_{50} and 10^7 adult mouse SD_{50} per ml. The tissue culture-adapted RRV strain of simian rotavirus (G3, P3), was grown and subjected to titer determination in MA104 cells in the presence of trypsin as previously described (13). The titer of the stock used was 2.2×10^8 PFU/ml. The recombinant vaccinia virus (rVV) expressing RF bovine rotavirus VP7 (rVV7) and control nonrecombinant vaccinia virus (VVD4) were grown and subjected to titer determination as previously described (11).

Virus inoculation. Mouse pups (4 days old) were orally gavaged with 100 μl containing 10^6 EW_w DD_{50}. Each pup was then checked for diarrhea by gentle abdominal pressure (2). We chose the EW strain to infect mouse pups because it had been previously shown that SCID pups inoculated with EW became chronically infected (6). Previous studies had demonstrated that infection of suckling mice with EW_w fully protected mice from subsequent challenge with EC_w or any other murine strain for at least 1 year (2). Infected mice were kept in a laminar-flow hood in a separate room from naive mice. Naive mice (6 to 8 weeks old) or mice previously infected as pups were orally gavaged with 100 μl containing 10^5 EC_w SD_{50} following oral administration of 100 μl of 1.33% sodium bicarbonate to neutralize stomach acidity. Fecal samples were collected from each mouse on the day of challenge and for the 10 following days. Fecal samples were stored frozen at −70°C until used. For use in the enzyme-linked immunosorbent assays (ELISAs), 10% (wt/vol) stool suspensions were prepared with stool diluent (10 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$ [pH 7.4], 5% fetal bovine serum, 0.05% Tween 20, 10 mM sodium azide, protease inhibitor cocktail [Calbiochem, San Diego, Calif.] [containing 0.2 mM AEBSF [4-(2-aminoethyl) benzenesulfonylfluoride], 1 μg of aprotinin per ml, 1 mM benzamidine, 10 μg of leupeptin per ml, and 10 μg of pepstatin A per ml]).

Detection of viral antigen and virus specific IgA by ELISA. ELISAs were performed as previously described (2). For detection of viral antigen in fecal samples, 96-well polyvinyl chloride microtiter plates (Dynatech, McLean, Va.) were coated with a 1:2,000 dilution of hyperimmune rabbit anti-rhesus rotavirus serum diluted in TNC (10 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$ [pH 7.4]) and incubated at 37°C for 4 h. The plates were then blocked with 5% BLOTTO (5% [wt/vol] Carnation nonfat powdered milk in TNC [16]) at 37°C for 2 h. Suspended stool samples were diluted 1:1 in 1% BLOTTO, added to the plates, and incubated overnight at 4°C. The plates were washed three times with TNC–0.05% Tween 20 (TNC-T), and guinea pig anti-rhesus rotavirus hyperimmune serum (diluted 1:4,000 in 1% BLOTTO) was added to the plates for 1 h at 37°C. After two washes with TNC-T, horseradish peroxidase-conjugated goat anti-guinea pig IgG serum (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted 1:4,000 in 1% BLOTTO was added to the plates and incubated for 1 h at 37°C. ABTS (2,2′-azido-di[3-ethylbenzthiasoline sulfonate]) substrate (Kirkegaard and Perry Laboratories) was added after two washes in TNC-T, the plates were developed for 10 min at room temperature, and the reaction was stopped by the addition of 10% sodium dodecyl sulfate. The A_{405} was read with a plate reader (BIO-TEK instruments, Burlington, Vt.). Our negative controls for the viral antigen ELISA were the stool samples from naive mice. The mean optical density of the values of day 0 from naive mice ($n = 17$) in the experiments shown was -0.008 ± 0.003 (standard error of the mean [SEM]). A stool sample was considered to be positive for antigen if the optical density (OD) value was above 0.15.

For the detection of virus-specific intestinal and serum IgA, plates were coated and blocked as for the antigen detection ELISA and then incubated with a dilution of RRV stock virus (1:8 in 1% BLOTTO) overnight at 4°C. After two washes with TNC-T, 5% stool samples in 1% BLOTTO or dilutions of serum in 1% BLOTTO were added to the plates. After 2 h of incubation at 37°C, the plates were washed three times in TNC-T and peroxidase-conjugated anti-mouse IgA or IgG antiserum (Kirkegaard and Perry Laboratories) (diluted 1:2,000 and 1:4,000, respectively, in 1% BLOTTO) was added. After 1 h of incubation at 37°C, the plates were washed and developed as described above for the antigen ELISA. In pilot experiments, the detection of rotavirus-specific IgA and IgG was compared with RRV or intestinal homogenate of EC_w murine rotavirus as antigen and found to be similar. The former antigen was chosen for ease of manipulation. Our negative controls for the IgA ELISA were the stool samples from naive mice. The mean OD of the values of day 0 from naive mice ($n = 12$) in the experiments shown was 0.027 ± 0.011 (SEM). A stool sample was considered to be positive for virus-specific IgA if the OD value was above 0.3. We determined only virus-specific stool IgA because, in our experience, virus-specific IgM and IgG levels are much lower or undetectable after natural infection.

Cytotoxicity assay. The cytotoxicity assay used is similar to the one previously described (11), with slight modifications. For preparation of effector cells, mice were sacrificed 10 days after primary infection and spleen single-cell suspensions were prepared with RPMI 1640 containing 10% heat-inactivated fetal calf serum, 100 μg of streptomycin sulfate per ml, 100 U of penicillin per ml, 2 mM L-glutamine, 2.5 mM sodium pyruvate, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), and 30 μM 2-mercaptoethanol (complete medium) and mixed in a 2:1 ratio with stimulator cells. The latter, obtained from spleens of nonimmune animals, had been previously incubated (10^7 cells per ml in complete medium) for 20 min at 37°C with mitomycin (25 μg/ml; Sigma Chemical Co., St. Louis, Mo.), washed three times, and then incubated with RRV at a PFU/cell ratio of 1 for 30 min. The cell mixture was adjusted to 5×10^6 cells per ml, distributed to 24-well plates, and cultured with 5% CO_2 at 37°C for 5 days. For some of the experiments, effector cells were prepared by addition of RRV (PFU/cell ratio of 0.3) to spleen cells of immune animals, and 5×10^6 cells per ml in CM was distributed to 24-well plates and cultured with 5% CO_2 at 37°C for 5 days. Similar responses were obtained with effectors prepared by either method.

MC57 target cells were infected in suspension (Dulbecco's modified Eagle's medium with 20 mM HEPES, without fetal calf serum) at a multiplicity of infection of 100 for RRV or of 2 to 5 for the VV strains. After 2 h at 37°C, cells (1×10^6 to 3×10^6) were centrifuged, labeled with 50 μCi of ^{51}Cr (Amersham, Arlington Heights, Ill.), and incubated for 1 h at 37°C. They were then washed three times and distributed in 96-well plates (10^4 cells per well). Effector cells were added at the appropriate concentration to obtain the desired effector-to-target cell ratios in a total volume of 200 μl. After 5 h of incubation at 37°C, 50-μl samples were taken from each well and counted in a gamma counter. For calculating the spontaneous and maximum release of ^{51}Cr, target cells were incubated with medium only and 10% Triton X-100, respectively. Spontaneous release was generally below 20%. The percentage of specific ^{51}Cr release was calculated by the following formula: (experimental release − spontaneous release)/(maximum release − spontaneous release). More than 90% of target cells were infected by RRV and rVV7 as shown by immunofluorescence with an antirotavirus-specific antiserum. The assay was done in duplicate wells. The SEM for duplicate wells was below 10% of the mean. A response was considered positive if specific lysis was above 10% and at least twice the value for the negative control (targets treated with medium or infected with the nonrecombinant VVD4 strain [11]).

In vivo depletion of $CD8^+$ cells and flow cytometry. Mice were depleted of $CD8^+$ cells by administration of anti-CD8 monoclonal antibody (MAb) produced by 2.43 rat hybridoma cells obtained from the American Type Culture Collection, Rockville, Md. The MAb was prepared as ascites fluid by inoculating 5×10^6 2.43 hybridoma cells into pristane (2,6,10,14-tetramethylpentadecane [Sigma Chemical Co.])-primed outbred nude mice (Simonsen Inc., Gilroy, Calif.). Ascites fluid was pooled, aliquoted, and stored at −70°C until use. A 1:10,000 dilution of the ascites fluid pool was used to stain $CD8^+$ spleen cells (from normal mice) for fluorescence-activated cell sorter (FACS) analysis with fluorescein isothiocyanate-labeled anti-rat serum as a second stage. Each mouse received 0.5 ml of ascites fluid intraperitoneally 6, 5, and 4 days before rotavirus infection, on the day of rotavirus infection, and on days 3, 6, and 9 after infection. On the day of rotavirus infection, depleted and nondepleted control mice were killed to verify depletion of $CD8^+$ cells in the spleen and among intraepithelial lymphocytes (IELs) by FACS analysis.

Spleen cells were isolated for FACS analysis by standard procedures. IELs were isolated as previously described (30) with small modifications. Briefly, Peyer's patches were removed from the small intestine of individual mice. Each small intestine was then opened longitudinally, cut into 2-cm segments, and washed in RPMI 1640 medium. The tissues were then transferred to 20 ml of RPMI 1640 medium–2% heat-inactivated fetal calf serum–100 μg of streptomycin sulfate per ml–100 U of penicillin per ml and incubated for 30 min at 37°C with gentle stirring. The tissues were then transferred to 50-ml tubes and shaken vigorously, and the medium containing the IELs was saved at 4°C. This process was repeated twice. Medium containing IELs was centrifuged, and the pelleted cells were resuspended in the same medium and passed over a glass wool column. Eluent, containing IELs, was centrifuged, and the pellet was resuspended in 3 ml of 40% Percoll (Pharmacia, Uppsala, Sweden), layered onto 2 ml of 75% Percoll, and centrifuged at 25°C for 20 min at $600 \times g$. IELs obtained at the interface were washed and stained with MAbs for FACS analysis.

Spleen cells and IELs were stained with anti-CD8 MAb 53 6.7 labeled with fluorescein isothiocyanate and either anti-γδ T-cell receptor (clone GL3) or anti-αβ T-cell receptor (clone H57-597) MAbs both labeled with phycoerythrin (all labeled MAbs were obtained from Pharmingen, San Diego, Calif.). To avoid false-negative results due to competition, the anti-CD8 MAb used for staining was chosen because it recognizes a different epitope from that recognized by the 2.43 MAb used for the depletion. Two-color FACS analysis was performed with a FACScan (Becton Dickinson, San Jose, Calif.). Analysis of IELs was done after gating cells for forward and side scatter properties of IELs. Demarcation bars for analysis of cells positive for a given marker were set by using unstained cells of the corresponding origin.

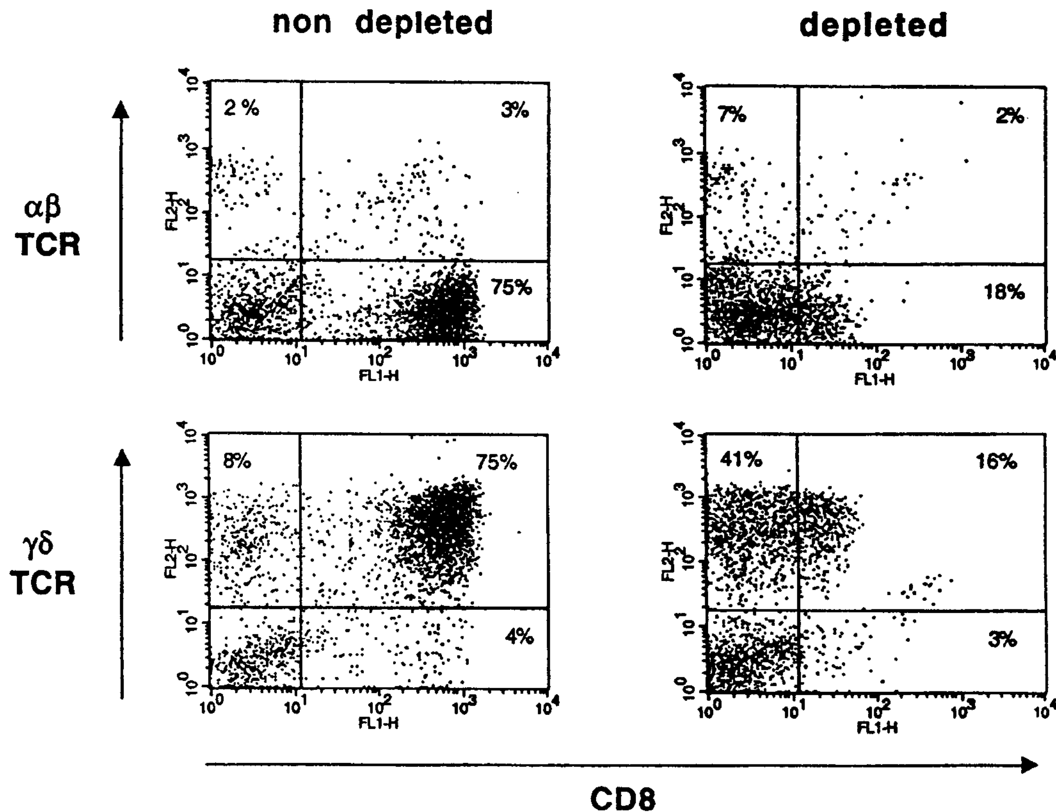

FIG. 1. Two-color FACS analysis of the IELs of β_2m mice depleted of CD8$^+$ cells by administration of 2.43 anti-CD8 MAb or not depleted. IELs were reacted with MAb 53 6.7-FITC (anti-CD8) and either MAb GL3-PE (anti-γδ TCR) or MAb H57-597-PE (anti αβ TCR). The percentages shown indicate percentages of stained cells in the indicated compartments.

RESULTS

Rag-2 gene knockout mice become chronically infected with murine rotavirus. Since the genetic defect of SCID mice has been shown to affect the sensitivity of their enterocytes to ionizing radiation (1), it seemed possible that these mice had abnormal enterocytes and that this defect could influence the capacity of the mice to become chronically infected with murine rotavirus. To confirm the absolute requirement of B and/or T cells for clearing a primary rotavirus infection, we infected Rag-2 gene knockout pups with the EW_w strain of murine rotavirus and Rag-2 gene knockout adult mice with the EC_w strain of murine rotavirus. Both pups and adults became chronically infected and continuously shed rotavirus antigen in their stools for at least 10 months (results not shown). We interpret these findings as confirming a requirement of specific immune T- and/or B-cell functions for the successful clearance of primary rotavirus infection in mice. As was reported with SCID mice (26), Rag-2 gene knockout pups and pups of all strains of mice studied (see below) developed diarrhea of similar intensity and duration to that of control mice after infection with murine rotavirus (results not shown).

β_2m knockout mice have delayed clearance of primary rotavirus infection but are fully immune to reinfection. To determine if CTLs are important in clearance of primary rotavirus infection in normal mice, we infected adult β_2m knockout mice with the EC_w strain of murine rotavirus. As β_2m knockout mice have been reported to have residual class 1-restricted T cells (19), we included an experimental group which was further depleted of CD8$^+$ T cells by administration of an anti-CD8$^+$ MAb. The protocol used to deplete CD8$^+$ T cells completely eliminated these cells from the spleen of a normal C57BL/6 mouse (results not shown) and depleted 75% of the population of γδ CD8$^+$ intestinal IELs present in β_2m knockout mice (Fig. 1). This result supports the conclusion that the residual αβ CD8$^+$, class 1-restricted T cells present in the β_2m knockout mice were completely or almost completely eliminated by treatment with the anti-CD8 MAb. Following primary inoculation with murine rotavirus, β_2m knockout mice shed viral antigen for two more days than did control C57BL/6 mice (Fig. 2a). β_2m knockout mice treated with anti-CD8 MAb shed viral antigen for an additional day. The difference between the mean number of viral antigen-shedding days in C57BL/6 and β_2m mice was statistically significant as judged by a two-tailed Student t test ($P = 0.00001$). The difference between the mean number of viral antigen-shedding days of β_2m and the CD8-depleted β_2m mice was also statistically significant ($P = 0.04$) according to the same test.

We next investigated whether CTLs are necessary for the development of immunity to rotavirus reinfection. Both the β_2m knockout mice treated with the anti-CD8 MAb and the untreated β_2m knockout mice were completely resistant to reinfection with a challenge dose containing 10^5 SD_{50}. Figure 2b shows that β_2m knockout mice had high levels of rotavirus-specific IgA present in their stools prior to rechallenge and that they developed this rotavirus-specific IgA coincident with the clearance of a primary infection. Taken together, these results indicate that class 1-restricted CD8$^+$ T cells play a role in clearance of primary rotavirus infection but are not necessary for this function, since clearance occurred even in their absence. CD8$^+$ T cells do not appear to be necessary for the

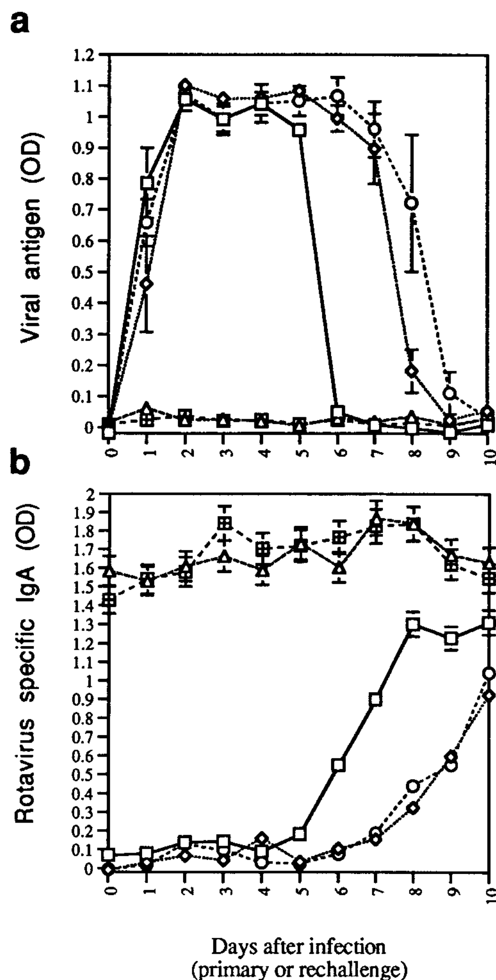

FIG. 2. Fecal viral antigen (a) and rotavirus-specific IgA (b) shedding curves of control naive C57BL/6 mice (□) and naive and previously infected β_2m knockout mice depleted of $CD8^+$ cells by administration of 2.43 anti-CD8 MAb (○) or not depleted (◇). CD8-depleted (⊞) and nondepleted (△) mice were rechallenged or infected at 6 to 8 weeks of age with 10^5 SD_{50} of the EC_w strain of murine rotavirus. The β_2m mice which were rechallenged had been previously infected with 10^6 DD_{50} of the EW_w strain of rotavirus when they were 4 days old. Fecal rotavirus antigen and rotavirus-specific IgA were measured by ELISA, and results are expressed as OD readings. Each time point represents the mean for three to six mice ± SEM (points without SEM bars had SEM below 0.05 OD unit).

development of immunity to rotavirus reinfection. Results of initial studies performed with β_2m mice derived from (129 × B6)F_2 founders were identical to those reported above for the congenic β_2m mice. Mice obtained from the cross between C57BL/6 mice and β_2m knockout mice derived from (129 × B6)F_2 founders cleared and developed immunity against murine rotavirus infection in an identical manner to C57BL/6 mice (results not shown).

Some B-cell-deficient J_HD knockout mice have altered clearance of primary rotavirus infection and are susceptible to reinfection. To determine if B cells play a role in clearance of primary rotavirus infection in normal mice, we infected adult B-cell-deficient J_HD knockout mice with murine rotavirus. Most J_HD knockout mice cleared primary rotavirus infection in an identical manner to control C57BL/6 mice (Fig. 3). The difference between the mean number of viral antigen-shedding days for C57BL/6 and J_HD knockout mice was not statistically significant by a two-tailed Student *t* test ($P = 0.14$). However, in separate experiments, 3 of 29 mice studied (six different litters) shed small quantities of viral antigen (ELISA OD values below 0.2) for 1 day on either day 7 or day 9 postinfection (results not shown) and 2 mice chronically shed viral antigen up to 27 days postinfection (data not shown). A stool suspension (day 27 postinfection) from one of these J_HD mice was used to infect three naive J_HD knockout mice. All three mice were infected and shed viral antigen for only 5 days (results not shown). None of the J_HD knockout mice that cleared primary infection developed detectable antibody responses in the serum or feces (data not shown).

To determine if B cells play a role in the development of immunity against rotavirus reinfection, we rechallenged J_HD knockout mice that had been infected as pups with the EW_w strain of murine rotavirus and had resolved primary infection. As can be seen in Fig. 3, all rechallenged mice were reinfected with rotavirus. The reinfected mice shed slightly lower levels of viral antigen in their stools and for fewer days than did the naive controls (Fig. 3). The difference between the mean numbers of viral antigen-shedding days of naive and rechallenged J_HD knockout mice was statistically significant by a two-tailed Student *t* test ($P = 0.0004$). We interpret these results as indicating that B cells were absolutely necessary for the development of complete resistance to rotavirus reinfection in mice.

β_2m and J_HD knockout mice do not have detectable CTLs against murine rotavirus. To confirm that rotavirus-infected β_2m knockout mice did not have rotavirus-specific CTLs and that the J_HD knockout mice did have them, we took advantage of a protocol used to detect cross-reactive CTLs specific for heterologous rotaviruses (11). We used this protocol because it has been difficult for us to establish a CTL assay with cell culture-adapted murine rotavirus, presumably because of its low titer (2). As expected, the β_2m mice did not have detectable rotavirus-specific CTLs (Table 1). Unexpectedly, we could not detect rotavirus-specific CTLs in the spleen of the J_HD

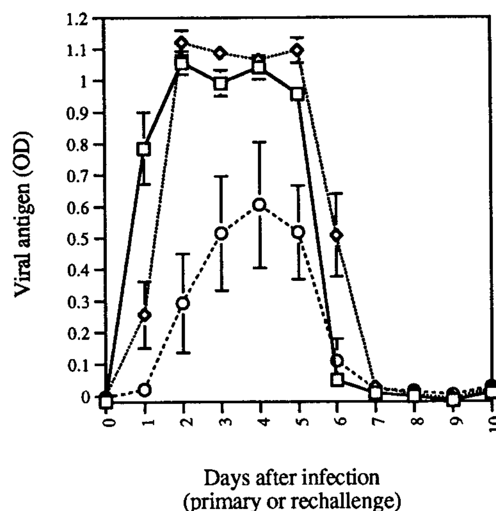

FIG. 3. Fecal viral antigen-shedding curves of control naive C57BL/6 (□) and naive (◇) and previously infected (○) J_HD knockout mice. Mice were rechallenged or infected at 6 to 8 weeks of age with 10^5 SD_{50} of the EC_w strain of murine rotavirus. J_HD mice which were rechallenged had been previously infected with 10^6 DD_{50} of the EW_W strain of rotavirus when they were 4 days old. Fecal rotavirus antigen was measured by ELISA, and results are expressed as OD readings. Each time point represents the mean of five to six mice ± SEM (points without SEM bars had SEM below 0.05 OD unit). This experiment has been repeated twice with similar results.

TABLE 1. J_HD but not β_2m knockout mice have rotavirus-specific CTLs

Mouse strain	In vivo priming[a]	% Specific lysis against target[b]:			
		Medium	RRV	VVD4	rVV7
C57BL/6	RRV	7.2	**40.9**	5.9	**39.7**
J_HD	RRV	9.2	**33**	4.5	**27.3**
C57BL/6	EC	7.2	**27.2**	8.7	**24.7**
β_2m	EC	26.2	20.7	20	19.7
J_HD	EC	15.9	19.5	13.3	23
J_HD	Not primed	12.1	11.1	10.6	12.1

[a] Effector cells obtained from mice orally infected with EC, intraperitoneally with RRV, or nonimmunized were restimulated in vitro with RRV.

[b] The percent specific lysis against the different target cells is shown. Shown is an effector-to-target-cell ratio of 30. Ratios of 10 and 5 showed a clear dose effect. Values in boldface type (above 10% and at least double the value of the negative control) are considered positive.

mice orally infected with murine rotavirus (Table 1). Nonetheless, we could detect rotavirus-specific CTLs in the spleen cells of a J_HD mouse inoculated intraperitoneally with 10^7 PFU of RRV (Table 1).

J_HD knockout mice become chronically infected with rotavirus after administration of depleting doses of anti-CD8 MAb. Since we could not clearly detect rotavirus-specific CTLs in the spleens of J_HD mice infected with the murine rotavirus, we treated a group of J_HD mice with the 2.43 anti-CD8 MAb to directly determine if $CD8^+$ T cells were indeed mediating virus clearance in vivo in these animals. As can be seen in Fig. 4, the J_HD mice treated with the anti-CD8 MAb became chronically infected with rotavirus. This result indicates that $CD8^+$ T cells are the mediators of viral clearance in J_HD knockout mice following primary infection, despite our inability to detect them in an in vitro cytotoxicity assay.

DISCUSSION

Despite previous work showing the importance of T and/or B cells in the immune response against rotavirus, the precise effectors of immunity to and resolution of infection have not been fully identified. In the present work, we have taken advantage of several genetically altered strains of mice to address this issue with a murine model of rotavirus infection. We have confirmed the importance of both T and B cells in clearing primary rotavirus infection. In the absence of class 1-restricted $CD8^+$ T cells (CTLs, β_2m knockout mice) but not of B cells (J_HD knockout mice), clearance of a rotavirus infection is delayed. Thus, resolution of primary rotavirus infection is probably a function of CTLs. $CD8^+$ T cells are absolutely necessary for clearance of a rotavirus infection in J_HD knockout mice but not in β_2m knockout mice that are able to produce intestinal rotavirus-specific antibody. These findings suggest that CTLs are not absolutely necessary for clearance and that B cells can probably mediate clearance in the absence of $CD8^+$ T cells. This latter fact is further supported by the altered clearance of rotavirus infection seen in some of the J_HD knockout mice studied (see below).

Protection from reinfection by rotavirus seems to be, on the other hand, a primary B-cell function: β_2m knockout mice devoid of CTLs are completely protected from reinfection, but all B-cell-deficient J_HD knockout mice tested have been susceptible to reinfection.

Our results with the Rag-2 gene knockout mice confirm the previous observation made with SCID mice (26) that T and/or B cells are necessary for clearance of a primary rotavirus infection. This observation and the fact that β_2m knockout mice have delayed clearance of a primary rotavirus infection are inconsistent with the report that nude mice clear rotavirus infection similarly to normal mice and without the development of detectable antirotavirus antibodies (8). One factor that could explain this apparent difference is the fact that the previous study of nude mice used a virus that was less infectious for adult mice than for pups (8), while we have used the EC strain of murine rotavirus, which is equally infectious for both adults and pups (2). We are currently investigating the capacity of nude mice to clear a rotavirus infection with our strains of highly infectious murine rotavirus.

Our results are in agreement with most but not all previous known facts about clearance and immunity to rotavirus in mice and other animals (2, 10). As seen with our β_2m knockout mice, delayed rotavirus clearance was observed in calves treated with an anti-CD8 MAb (24). Our inability to show complete protection against reinfection in the B-cell-deficient mice (which have $CD8^+$ cells capable of clearing a rotavirus infection) contrasts with the report that CTLs can afford, upon passive transfer, complete protection against rotavirus-induced diarrhea in pups (23). Considering that we did obtain some degree of decreased viral shedding in the rechallenged J_HD mice, it is possible that we were not looking at an optimal time point to demonstrate the protective activity mediated by CTLs. In other murine models of viral infection (18), the protective activity of CTLs can be very short-lived. Also, the dose we used to challenge mice was much higher than the dose used in the passive-transfer study (23). An alternative explanation is that CTLs have the capacity to protect against disease but not against viral replication as measured in our study.

We have shown that $CD8^+$ cells are the effectors of viral clearance in J_HD mice by using in vivo depletion assays. Nonetheless, we were not able to detect CTLs in these mice in an assay in which they are readily detectable in C57BL/6 mice (Table 1) and mice derived from the F_2 of a cross between 129 and C57BL/6 mice (10b). Considering that the spleens of the J_HD mice contain 1/5 to 1/10 the number of cells of normal

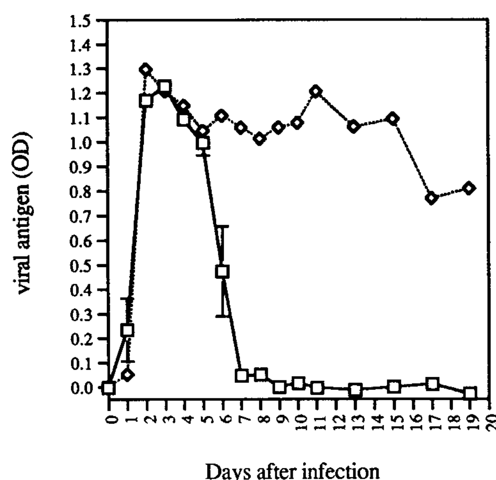

FIG. 4. Fecal viral antigen shedding curves of J_HD knockout mice depleted of $CD8^+$ cells by administration of 2.43 anti-CD8 MAb (◇) or not depleted (□) as described in Materials and Methods. Mice were challenged at 6 to 8 weeks of age with the EC_w strain of murine rotavirus. Fecal rotavirus antigen was measured by ELISA, and results are expressed as OD readings. Each time point represents the mean of three depleted or five nondepleted J_HD mice ± SEM (points without SEM bars had SEM below 0.05 OD unit). This experiment has been repeated twice with similar results.

C57BL/6 mice or of the mice derived from the cross between C57BL/6 mice and 129 mice (results not shown), it is possible that the conditions used to detect the CTLs were not optimal for the J_HD mice. Our ability to detect rotavirus-specific CTLs in the spleens of J_HD mice immunized with the heterologous RRV (a better antigen to detect CTLs with the assay used) supports this conclusion. An alternative explanation for our findings is that the $CD8^+$ cells that mediate clearance in J_HD mice are not necessarily cytotoxic. The $CD8^+$ cells could, in theory, be mediating their effect by the release of cytokines (25). We are currently testing this hypothesis by studying rotavirus infection in perforin knockout mice that lack most T-cell-mediated cytotoxic functions.

Viral shedding was prolonged one more day in β_2m knockout mice by using a protocol to deplete $CD8^+$ cells. Since $\gamma\delta$ $CD8^+$ IELs are present in the β_2m knockout mice and the CD8 cell depletion protocol did not completely eliminate these cells, we favor the hypothesis that the prolongation of shedding observed after 2.43 MAb treatment is due to the depletion of potentially residual class 1-restricted $CD8^+$ cells present in these mice (19) and not to the partial depletion of the $\gamma\delta$ $CD8^+$ IELs. This hypothesis is in accord with the finding that the anti-rotavirus CTL activity of IELs from mice inoculated with heterologous rotaviruses is due to the $\alpha\beta$ and not $\gamma\delta$ $CD8^+$ IELs (22).

Of 29 J_HD mice tested, 2 shed viral antigen for up to 27 days after viral infection and 15% of the mice shed viral antigen for one additional day after cessation of shedding. The noncongenic nature of the J_HD mice could be determining the variability in viral clearance of infection seen in these mice. It is not likely that a virulent variant of the EC virus was selected in the J_HD mouse that was chronically infected, since three naive J_HD mice infected with a stool sample from the chronically infected mouse cleared the virus infection in a normal period. It seems probable that for an unknown reason, the chronically infected J_HD mice could not mount an anti-rotavirus CTL response or that the virus gained access to a site in the gut where it was protected from the effect of CTLs. A B-cell-deficient child has also been reported to have become chronically infected with rotavirus (27).

Our data demonstrating delayed viral clearance of primary infection and development of complete immunity against rotavirus reinfection in β_2m knockout mice are very similar to the data describing the way these mice behave after infection with influenza virus (9). In both models, antibody seems to play the major antiviral role. β_2m knockout mice seem to develop a local rotavirus-specific IgA response more slowly than control mice do (Fig. 2b). Ten days after infection, β_2m knockout mice had significantly lower rotavirus-specific IgG (but not IgA) end point antibody titers in serum than did control mice (data not shown). β_2m knockout mice infected with influenza virus develop lower levels of virus-specific antibody at day 10 after infection than do control mice (9). In contrast, β_2m knockout mice infected with Sendai virus develop an increased antiviral IgA response as measured 18 days after infection (14). As suggested previously, these results indicate that a class 1-restricted cell probably influences antibody responses (29). As opposed to our model, lymphocytic choriomeningitis virus, a virus against which antibodies play a relatively minor role, produces a chronic infection of β_2m knockout mice (20). To our knowledge, there is no other study of viral infection of B-cell-deficient knockout mice comparable to ours. Previous work with mice depleted of B cells by treatment with anti-μ antibodies identified a primary role for antibodies in protection from reinfection and a minor role for antibodies in clearance of primary infection due to respiratory syncytial virus and influenza virus (12, 17). These results are comparable to our findings with the J_HD knockout mice and reinforce the notion that humoral immune mechanisms play the primary role in immunity to viruses at mucosal surfaces.

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