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Evidence that Resolution of Rotavirus Infection in Mice Is due to Both CD4 and CD8 Cell-Dependent Activities

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The effector functions responsible for resolution of shedding in mice orally inoculated with the murine rotavirus strain EDIM were identified in B-cell-deficient and normal BALB/c mice after monoclonal antibody (MAb) depletion of CD4 and CD8 cells. When depleted of CD8 cells, B-cell-deficient μMt mice resolved their infections more slowly than nondepleted animals, but CD4 cell depletion caused chronic, high-level shedding. This finding indicated that CD4 cell-dependent immunological effectors other than, or in addition to, CD8 cells played roles in rotavirus resolution in μMt mice in the absence of antibody. The roles of CD4 and CD8 cells in resolution of rotavirus shedding were further characterized in immunologically normal BALB/c mice. Depletion of CD4 cells before EDIM inoculation resulted in rapid resolution of most shedding, but chronic, low-level shedding continued for weeks. When the CD4 cell-depleted BALB/c mice were subsequently depleted of CD8 cells, shedding levels increased significantly ($P < 0.001$), indicating that CD8 cells were responsible for the rapid but incomplete suppression of rotavirus shedding. Further experimentation revealed that little rotavirus antibody was made in CD4 cell-depleted BALB/c mice, and only after CD4 cells were repopulated did antibody production increase and virus shedding fully resolve. Thus, resolution of rotavirus shedding in both μMt and BALB/c mice was associated with CD4 and CD8 cell effector activities.

Rotaviruses cause severe gastroenteritis in young infants, which can lead to dehydration and, in the absence of appropriate rehydration therapy, can result in death. It is estimated that nearly 1 million infants die from rotavirus disease each year (8, 10). In immunocompetent children, the disease is normally self-limiting and symptoms resolve within days (10), resulting in at least partial protection against subsequent rotavirus illness (25). Resolution of acute rotavirus disease can, however, be prolonged in immunocompromised individuals, leading to chronic shedding of rotavirus and gastrointestinal symptoms (23, 28). Thus, immune mechanisms are involved in both the resolution of rotavirus disease and prevention of subsequent rotavirus illnesses.

Because of difficulties associated with identification of these immune mechanisms in humans, animal models have been developed. The most simple and easily manipulable model for studies on rotavirus infection and immunity is the mouse. Initial studies in mice demonstrated that rotavirus illness could be prevented in neonatal mice by passive immunization, either by suckling on immunized dams (17, 18) or by adoptive transfer of CD8 cells from immunized mice (19), thus suggesting that both antibody and cytotoxic T lymphocytes (CTLs) are capable of preventing rotavirus disease in this model. Furthermore, rotavirus shedding was resolved in mice with severe combined immunodeficiency following adoptive transfer of immune CD8 cells, indicating that CTLs are also able to resolve an active rotavirus infection in mice (4). The importance of antibody and CTLs in active immunity, in either resolution or prevention of rotavirus infection, was not determinable by these passive studies.

To define the effector functions involved in active immunity, we developed an adult mouse model (26). Because mice are susceptible to rotavirus disease for only the first 15 days of life, the endpoint of protection in this model was prevention of rotavirus infection. Using the model, it was determined that the best immunological correlates of protection were levels of serum and fecal rotavirus immunoglobulin A (IgA) (6, 14). Following oral immunization with a live murine rotavirus (strain EDIM), the titers of these antibodies remained elevated for at least 14 months, during which time the mice were totally resistant to reinfection (15).

Further studies conducted with a strain of genetically altered B-cell-deficient mice (i.e., J_HD), revealed that these mice could resolve virus shedding but were susceptible to reinfection within several weeks (7, 13). In addition, monoclonal antibody (MAb) depletion of CD8 cells prior to the initial infection prevented resolution of infection. These results suggested that CD8 cells played an important role in resolving rotavirus infection but that extended protection was dependent on antibody production in J_HD mice.

Studies conducted with a second B-cell-deficient mouse strain (μMt) indicated that CD8 cells may not always play a critical role in resolving rotavirus infection in the absence of antibody production (13). Following infection, these mice gradually resolved their infections, as determined by viral shedding, and the rate of resolution was slowed but not blocked by CD8 cell depletion in these mice. This finding led to the suggestion that other effector functions besides antibody or CD8 cells gradually resolve rotavirus infection in μMt mice.

A third immune component known to have effector activity in viral infections is CD4 cells. These cells act through several mechanisms to resolve and prevent viral infections. One mechanism is to provide help for B-cell expansion and maturation (3, 24). Cytokines that they produce can also stimulate expansion of CD8 cells and their activation into CTLs. Certain cytokines such as gamma interferon also have direct antiviral activities (11, 16, 22). Finally, CD4 cells can themselves acquire CTL activity (5). The purpose of this study was to determine the involvement of CD4 cells in resolution of rotavirus infection by using MAb depletion of these cells in normal and B-cell-deficient mice. Because of the previously established

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importance of CD8 cells in resolution of rotavirus infection in B-cell-deficient mice, the combined roles of CD4 and CD8 cells in virus resolution in normal mice were also investigated.

MATERIALS AND METHODS

Mouse strains. Two strains of mice were used in these studies. One was pathogen-free BALB/c which were purchased from Harlan-Sprague-Dawley when 6 weeks of age. No mouse had evidence of previous rotavirus infection as determined by serum rotavirus antibody titers. The other strain was genetically engineered and was unable to produce functional antibody. This strain was produced by Kitamura et al. (12), using targeted disruption of a membrane exon of the gene encoding the μ-chain constant region (μMT mutation) in mouse embryonic stem cells. The transfected stem cell clone D3 was injected into blastocysts from C57BL/6 mice, and the derived offspring were backcrossed multiple times to C57BL/6 mice. These mice, containing a μMT mutation on a C57BL/6 background, were purchased (females only) from Jackson Laboratories (Bar Harbor, Maine). They were included in this study with permission of K. Rajewsky. Experiments were conducted with adult mice (6 to 20 weeks of age). The μMT mice were found to produce no detectable antibody.

Rotaviruses. The murine EDIM strain of rotavirus was used throughout these studies. This virus was originally obtained from M. Collins (Microbiological Associates, Bethesda, Md.) in 1980. Both wild-type (wt) virus from mouse stool and cell culture-adapted (passage 9) EDIM preparations were used. Because adult C57BL/6 mice were totally resistant to infection when inoculated with passage 9 of EDIM (i.e., no rotavirus shedding or immune responses were detected) but were susceptible to infection with wt EDIM based on development of rotavirus antibody, the wt strain was used to inoculate μMt mice which have a C57BL/6 background. However, since the passage 9 preparation has been used routinely for all previous studies with BALB/c mice in our laboratory since the inception of the adult mouse model for rotavirus (26), it was used to inoculate BALB/c mice. Even though adult C57BL/6 mice are more susceptible to wt EDIM, the two virus preparations were nearly equally infective in adult BALB/c mice; i.e., the doses required to infect 50% of the mice were 240 and 560 focus-forming units (FFU) for the wt and passage 9 preparations, respectively. The large number of mice required to conduct an infectivity study precluded conducting this analysis in μMt mice. Thus, the results found with μMt and BALB/c mice may not be directly comparable because of differences in the EDIM preparations used for challenge. The wt EDIM preparation was obtained from stools of infected neonatal mice and purified as described previously (13). The final preparation contained 10^7 FFU per ml. The passage 9 preparation of EDIM was obtained as previously reported (26) and contained 2×10^6 FFU/ml.

Study plan. Mice were inoculated with the EDIM strain of rotavirus on day 0. On the day of inoculation and for specified days thereafter, two stool pellets per day were collected from each mouse and placed into 0.5 ml of Earle's balanced salt solution before being frozen. These were later thawed and homogenized before being tested for rotavirus antigen by an enzyme-linked immunosorbent assay (ELISA). To determine the effects of CD4 and CD8 cell depletion prior to virus inoculation, mice were injected intraperitoneally (i.p.) once per day with 1 mg of each ammonium sulfate-precipitated MAb preparation. Inoculations were initiated 5 days before virus administration (day −4) and were performed for 4 consecutive days and twice weekly thereafter for specified times. CD4 cells were depleted with the rat hybridoma cell line GK1.5, and CD8 cells were depleted with the rat hybridoma cell line 2.43, both purchased from the American Type Culture Collection. Depletion of CD4 and CD8 cells was monitored by using a fluorescence-activated cell sorter (FACS). These analyses were performed with different lymphoid tissues on the day of virus inoculation and at the times specified for each experiment. Following oral administration of EDIM, mice were monitored for shedding as described above to determine the effects of CD4 and CD8 cell depletion.

Stools were also collected from BALB/c mice to monitor rotavirus IgA titers. These were collected on the day of virus administration and at regular intervals thereafter, depending on the experiment. Blood specimens were collected (retro-orbital capillary plexus puncture) on the day of virus inoculation and at specified intervals thereafter to determine levels of serum rotavirus IgG and IgA.

Measurement of rotavirus shedding. Fecal specimens collected before and after EDIM inoculation were examined for the presence of rotavirus antigen, using a sensitive ELISA as previously described (14). The absorbancy values represent the average of two negative coated wells subtracted from the average of the two positive coated wells. Final absorbancies were considered positive if the values for positive wells were two times those for the negative wells and ≥0.15. The A_{490} values obtained were used not only to demonstrate the presence or absence of virus but also to quantify the amount of viral shedding. When the readings were ≥3.0 (maximum reading), the specimen was assigned a value of 4.0 for calculation purposes.

Measurement of rotavirus serum and stool antibody. Serum rotavirus IgG and IgA and stool rotavirus IgA titers were all analyzed by using methods reported in previous publications (14, 26, 27).

Isolation of lymphoid tissues and FACS analyses. Spleen, mesenteric lymph nodes, lamina propria, and intraepithelial lymphocytes (IELs) were all removed from mice, processed, and examined by FACS analysis, using methods reported previously (13). Two control and two depleted mice were included in each analysis.

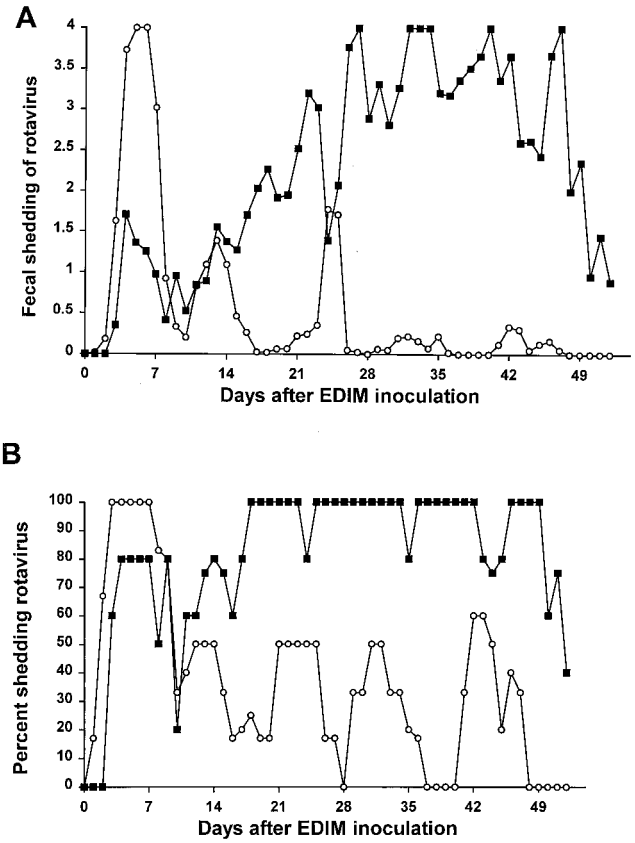

FIG. 1. Effect of CD4 cell depletion on fecal shedding of rotavirus following EDIM inoculation of μMt mice. Depletion by i.p. injection with 1 mg of MAb GK1.5 per day was performed on days −4, −3, −2, −1, 3, 7, 10, and 14. Mice were orally administered 5×10^5 FFU of unpassaged EDIM on day 0 and were monitored daily for rotavirus shedding following collection of two stool pellets. (A) Mean shedding (i.e., A_{490}) of six control (○) and five depleted (■) mice per group; (B) percentage of mice in either group that had detectable rotavirus shedding.

Statistical analyses. Comparisons between groups to determine the significance (P values) of differences were all performed by a two-tailed, unpaired Student's t test. To measure differences in rotavirus antigen shedding between groups, the average quantity of virus shed (A_{490} values) for each group on each day was determined and used to compare differences. Comparisons were made on a week-to-week basis. Comparisons in antibody responses between groups were made by using the log of each titer for individual mice and determining differences between titers of groups on a day-to-day basis.

RESULTS

CD4 depletion in B-cell-deficient mouse strains. The initial studies to determine the role of CD4 cells in the resolution of rotavirus infection were with B-cell-deficient μMt mice. The purpose of these studies was to ascertain whether removal of CD4 cells by MAb depletion would alter the pattern of rotavirus shedding in mice in the absence of antibody. Following inoculation with 1 mg of MAb GK1.5 for 4 consecutive days, 93% of spleen and 86% of lamina propria CD4 cells were depleted as determined by FACS analysis. On the fifth day after CD4 depletion was initiated (day 0), the mice were orally inoculated with 5×10^5 FFU of wt EDIM. Nondepleted μMt mice shed large quantities of virus which gradually resolved to undetectable levels by 48 days after inoculation (Fig. 1). In contrast, CD4-depleted μMt mice shed significantly ($P <$

0.001) more rotavirus antigen from day 15 onward (based on a week-by-week average) which began to be resolved only approximately 50 days after virus inoculation, 5 weeks after the last injection with MAb GK1.5. By day 52, CD4 cells had begun to be repopulated, and their concentrations in spleen and lamina propria were reduced only 23 and 42%, respectively, relative to that found in the nondepleted mice. Thus, CD4 cell depletion of μMt mice caused chronic rotavirus shedding in high amounts which began to be resolved only when the CD4 cells were repopulated. Although CD4 cell-depleted mice shed significantly less ($P = 0.05$) rotavirus antigen during the first week following EDIM inoculation in this experiment, this difference was not significant in the subsequent experiment.

We previously reported (13) that when μMt mice were depleted of CD8 cells, rotavirus shedding following EDIM challenge was gradually resolved as occurred in nondepleted μMt mice, and shedding in the CD8-depleted animals was significantly greater only during week 3 of the 7-week observation period. Thus, EDIM shedding in CD4-depleted μMt mice observed in this present study appeared to be much more extensive than found previously in CD8-depleted animals. This result supports the suggestion that resolution of shedding in μMt mice may be through effectors other than antibody (which they do not produce) and in addition to CD8 cells.

To confirm the differences in shedding patterns between CD4- and CD8-depleted μMt mice following EDIM inoculation, the individual depletion studies were repeated in a combined experiment. Three groups of μMt mice (six mice/group) were included (nondepleted, CD4 depleted, and CD8 depleted), and the same depletion protocols were followed as used previously except that depletion of both CD4 and CD8 cells was continued by twice-weekly MAb inoculations until 42 days after EDIM administration. Results very similar to those found previously were obtained except that shedding during the first week after EDIM administration was not significantly different between any of the groups of mice ($P > 0.2$) and shedding after day 8 was fully resolved in this experiment in the nondepleted animals (Fig. 2). However, as found previously, shedding of rotavirus antigen was gradually resolved in the CD8-depleted mice but continued unabated for the 42-day observation period in the CD4-depleted animals. Thus, there was significantly ($P < 0.001$) more rotavirus shedding during any week after EDIM inoculation, except the first, in either CD4-depleted or CD8-depleted versus nondepleted mice and from the fourth week after inoculation onward in CD4-depleted versus CD8-depleted animals.

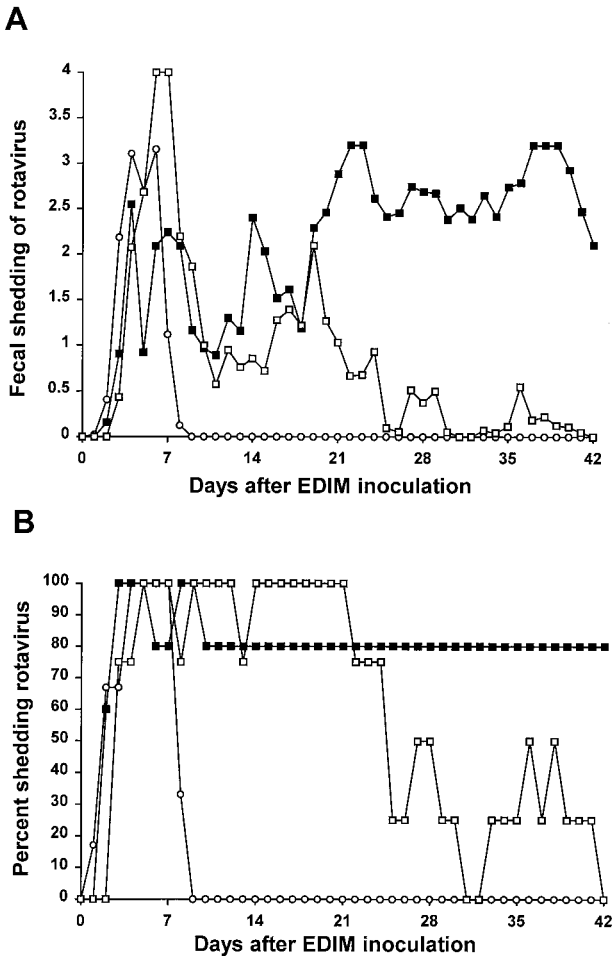

FIG. 2. Effect of CD4 and CD8 cell depletion on fecal shedding of rotavirus following EDIM inoculation of μMt mice as determined in a simultaneous experiment. Depletion by i.p. injection with 1 mg of either MAb GK1.5 (CD4 cells) or MAb 2.43 (CD8 cells) per day was performed on days −4, −3, −2, and −1 and twice weekly thereafter until day 42. Mice were orally administered 5×10^5 FFU of unpassaged EDIM on day 0 and were monitored daily for rotavirus shedding. (A) Mean shedding of rotavirus antigen (A_{490}); (B) percentage of mice with detectable rotavirus shedding. ○, Nondepleted; ■, CD4 depleted; □, CD8 depleted.

CD4 and CD8 depletion of normal mice. Resolution of rotavirus shedding in genetically altered, B-cell-deficient μMt mice was delayed in CD8-depleted mice but appeared to have an absolute dependence on the presence of CD4 cells in these mice. Because genetically altered mice have a propensity to utilize compensatory effector mechanisms (3, 21), the relative importance of CD4 and CD8 cells was further examined in a normal strain of inbred mice (i.e., BALB/c).

Depletion of CD4 cells in adult BALB/c mice was initiated 5 days before EDIM inoculation on day 0, by which time 83% of splenic CD4 cells had been eliminated as determined by FACS analysis. When these mice were orally inoculated with 4×10^4 FFU of EDIM (passage 9), shedding was observed 2 days after infection, peaked between days 3 and 6, and was resolved by day 9 (Fig. 3). A similar rapid decrease in shedding was observed in mice depleted of CD4 cells. In contrast to nondepleted mice, however, shedding was not fully resolved and continued sporadically up to at least 48 days after EDIM inoculation. The immunological effectors responsible for the suppression of virus shedding and the effectors missing in the CD4 cell-depleted BALB/c mice responsible for complete resolution were further investigated.

To determine whether CD8 cells were responsible for suppression of virus shedding in CD4 cell-depleted mice, half of these mice were also depleted of CD8 cells beginning at 18 days after infection. After 4 days of CD8 depletion, 99% of splenic CD8 cells were eliminated. By day 28, these doubly depleted mice were shedding significantly ($P < 0.001$) more virus during any weekly period than the group depleted only of CD4 cells (Fig. 3). This increased level of shedding continued until the experiment was stopped 49 days after infection. Thus, the observed suppression of shedding in CD4-depleted mice appeared to be due to CD8 cells.

When the CD4 cell depletion experiment with BALB/c mice was repeated with additional groups of BALB/c mice, EDIM shedding was again fully resolved within 10 days in the nondepleted animals but continued sporadically for up to 23 days in the CD4 cell-depleted group (results not shown). To establish that this sporadic shedding was not due merely to reexposure to virus shed by other animals within the cage, these two

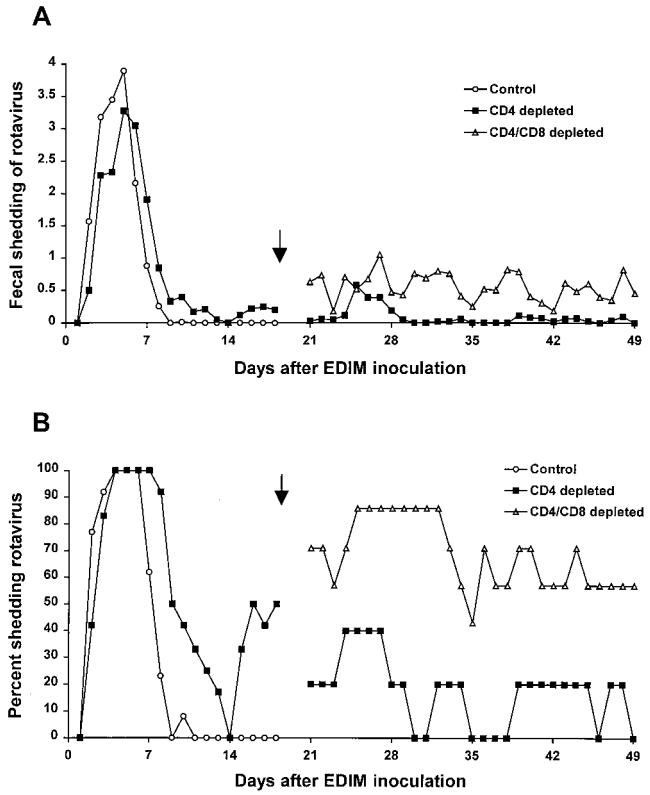

FIG. 3. Effect of CD4 cell depletion on fecal rotavirus shedding following administration of EDIM to BALB/c mice and the combined effect of CD4-CD8 cell depletion on this shedding pattern. BALB/c mice either were not depleted or were depleted of CD4 cells by i.p. injection of MAb GK1.5 on days −4, −3, −2, −1, 1, 4, 8, 11, 14, 18, 21, and 25 and inoculated with 4×10^4 FFU of EDIM (passage 9) on day 0. Two stool pellets were collected each day and monitored for rotavirus up to day 18. The values represent the mean rotavirus shedding (A_{490}) of 6 control and 12 CD4 cell-depleted mice. Beginning on day 18 (arrow), seven of the CD4 cell-depleted mice were also depleted of CD8 cells by i.p. injections with MAb 2.43 on days 18, 19, 20, 21, and 25. These and the five other CD4 cell-depleted mice continued to be monitored for rotavirus shedding until day 49. (A) Mean shedding of rotavirus antigen (A_{490}); (B) percentage of mice with detectable rotavirus shedding.

groups of mice were rechallenged on day 28 with 4×10^4 FFU of EDIM passage 9. None of the mice shed detectable quantities of rotavirus antigen even though CD4 cell depletion was maintained by twice-weekly anti-CD4 MAb inoculation throughout the entire period. Thus, sporadic shedding was not due merely to reexposure to EDIM because the immune responses responsible for resolution of shedding in these animals protected against infection.

Because CD4 depletion delayed complete resolution of EDIM shedding in BALB/c mice and the low level of shedding in these mice was magnified when they were additionally depleted of CD8 cells on day 18 after EDIM inoculation (Fig. 3), it appeared that CD8 cells were responsible for suppression of rotavirus shedding. However, complete resolution of shedding was greatly delayed in CD4-depleted mice even in the presence of CD8 cell activity. Possibly, the full complement of rotavirus-specific CD8 cells was not generated in the absence of CD4 cell helper function. It is also possible, however, that another CD4-dependent immunological effector, such as rotavirus antibody, was required for complete resolution of EDIM shedding in BALB/c mice. To examine this possibility, the levels of rotavirus serum and fecal antibody were measured at weekly intervals for 7 weeks after EDIM infection of nondepleted and CD4-depleted mice in the experiment already described (Fig. 3). CD4 cell depletion had a dramatic effect on the levels of all rotavirus antibodies examined (i.e., serum IgG, serum IgA, and stool IgA) (Fig. 4). Rotavirus IgG titers in CD4-depleted mice were less than 10% of those found in nondepleted control mice, and almost no serum or stool rotavirus IgA was produced in these depleted animals. Therefore, the suppression of rotavirus antibody production in CD4-depleted BALB/c mice correlated with their inability to fully resolve virus shedding.

We next examined whether rotavirus antibody production correlated with the resolution of EDIM shedding in BALB/c mice depleted of CD8 cells. When nondepleted BALB/c mice were inoculated with 4×10^4 FFU of passage 9 EDIM, rotavirus shedding was fully resolved within 9 days (Fig. 5). However, complete resolution of rotavirus shedding in CD8-depleted BALB/c mice was delayed until day 14, and significantly ($P < 0.001$) more virus antigen was shed between days 7 and 13 in mice depleted of CD8 cells. When this experiment was repeated, shedding was resolved in control mice by day 10 and was extended an additional 3 days in CD8 cell-depleted animals, but unlike the observation made in mice depleted of CD4 cells, once shedding was resolved, it remained undetectable within the subsequent 2-week observation period (results not shown). Furthermore, when the CD8 cell-depleted mice were rechallenged with EDIM on day 28, they did not shed detectable amounts of rotavirus antigen. Therefore, depletion of CD8 cells delayed but did not prevent resolution of EDIM shedding in BALB/c mice. The time of resolution of viral shedding in the CD8 cell-depleted mice, as shown in Fig. 5, corresponded to the time after infection when stool rotavirus IgA titers began to rapidly increase (Table 1). CD8 cell depletion did not appear to affect antibody responses because a significant difference ($P = 0.04$) in the concentrations of stool rotavirus IgA between groups was found on only one day (day 9) after infection.

The potential importance of antibody in resolution of EDIM infection in BALB/c mice was further examined in mice depleted of both CD4 and CD8 cells. MAbs against both cell types was initiated 5 days before EDIM inoculation as before, and a final injection was performed 2 days after EDIM inoculation. Nondepleted mice inoculated with EDIM resolved shedding within the usual period of 9 days, and as expected, the depleted animals shed significantly ($P < 0.001$ after week 1) larger quantities of rotavirus for an extended period (Fig. 6). FACS analysis revealed that at the time of virus administration, the CD4 and CD8 cells in mesenteric lymph nodes had been depleted to 87 and 92%, respectively, of their normal concentrations. By day 49 after virus inoculation and 47 days after the last MAb injection, these levels had returned to within 23 and 67%, respectively, of normal. In accordance with the return of CD4 cells, serum and stool rotavirus IgA titers began to rise (Fig. 7), and virus shedding decreased to nearly undetectable levels by day 47 postinfection when stool collections were terminated (Fig. 6). These results show consistent relationships between the presence of CD4 cells, production of rotavirus antibody, and complete resolution of rotavirus shedding in a strain of normal mice.

DISCUSSION

Based on the inability of immunocompromised humans (23, 28) as well as mice with severe combined immunodeficiency (4) to resolve rotavirus shedding, normal resolution of rotavirus infection in the gut is clearly dependent on the development of active immune responses. The effector functions responsible for clearance of virus, however, have been poorly character-

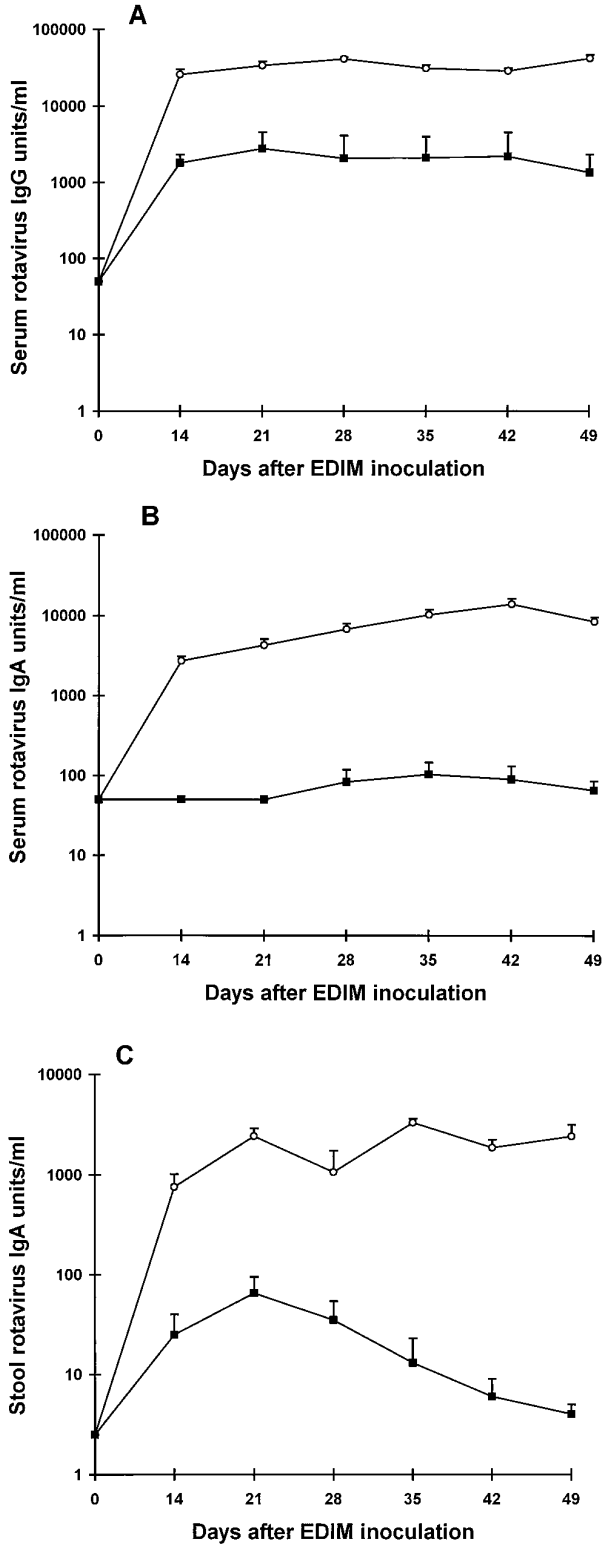

FIG. 4. Effect of CD4 cell depletion on rotavirus antibody titers following EDIM infection of BALB/c mice. BALB/c mice were either not depleted (six mice) or depleted of CD4 cells only (five mice) on days −4, −3, −2, −1, 1, 4, 8, 11, 14, 18, 21, and 25 and inoculated with 4×10^4 FFU of EDIM (passage 9) on day 0. Blood and stool specimens collected on the days specified were analyzed for levels of serum rotavirus IgG (A) or IgA (B) or stool rotavirus IgA (C). The values presented are the geometric mean titers for mice in the nondepleted (○) and CD4 cell-depleted (■) groups. The bracketed line above each data point represents the magnitude of the standard error of the mean.

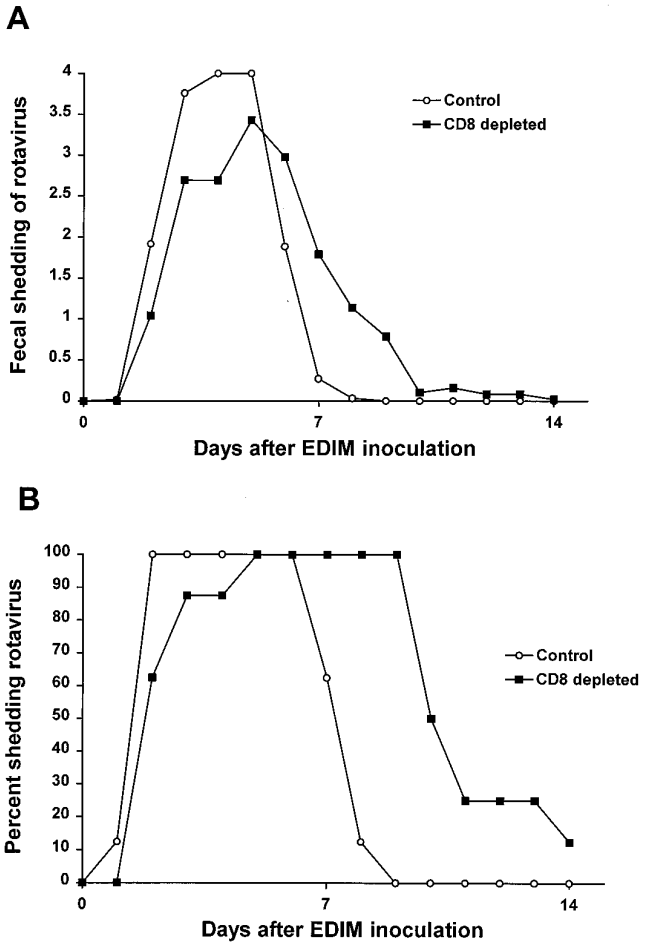

FIG. 5. Effect of CD8 cell depletion on fecal rotavirus shedding in BALB/c mice following oral inoculation of EDIM. Mice were depleted of CD8 cells by i.p. injection with MAb 2.43 on days −4, −3, −2, −1, and 3. Following inoculation with 4×10^4 FFU of EDIM (passage 9) on day 0, mice were monitored daily for fecal shedding of rotavirus. The values presented represent either the mean shedding (A_{490}) of eight control and eight CD8 cell-depleted mice (A) or the percentage of mice in either group with detectable rotavirus shedding (B).

ized. Recent studies performed with genetically altered B-cell-deficient mice strains have suggested that at least two different immune components other than antibody can be responsible for rotavirus resolution (13). In J_HD mice, the presence of CD8 cells correlated with resolution of shedding; in μMt mice, where depletion of CD8 cells had only a limited effect on virus clearance, a second unidentified effector function appeared to play a role. The purpose of this study was to determine whether the activity of this unidentified effector was dependent on the presence of CD4 cells and whether CD4 and CD8 cells both played roles in resolution of rotavirus infection in normal mice.

Depletion of CD4 cells with MAb GK1.5 prior to inoculation of B-cell-deficient μMt mice with the EDIM strain of murine rotavirus resulted in chronic shedding of large amounts of rotavirus. This occurred even though CD4 cells did not appear to be completely depleted. This high level of shedding in the CD4 cell-depleted mice did not abate until MAb depletion of CD4 cells had been stopped for >4 weeks and CD4 cells were repopulated. CD4 cells are believed to affect virus resolution by several possible mechanisms which include production of cytokines that provide helper function for B-cell

TABLE 1. Concentrations of stool rotavirus IgA in normal and CD8 cell-depleted BALB/c mice following EDIM administration

Days after infection	Geometric mean titer of rotavirus IgA (U/ml)[a]	
	Nondepleted mice	CD8 cell-depleted mice
0	<5 (0)	<5 (0)
4	<5 (0)	<5 (0)
5	6 (2)	5 (2)
6	55 (25)	48 (21)
7	250 (113)	154 (86)
8	616 (191)	401 (87)
9	1,399 (293)	766 (162)
10	1,064 (555)	1,065 (417)

[a] CD8 cell depletion, virus inoculation, and collection of stools are described in the legend to Fig. 5. Stools collected on days 0 and 4 to 10 were analyzed for rotavirus IgA, and the geometric mean titers and standard errors of the mean (in parentheses) were calculated for the eight mice in each group. No significant differences in rotavirus IgA concentrations were found between groups except on day 9 ($P = 0.04$).

expansion and maturation as well as CD8 cell expansion and development into active CTLs (3, 24). In addition, they can produce cytokines with direct antiviral activity (11, 16, 22) and can themselves develop into effector CTLs (5). Because μMt mice were B-cell deficient and made no detectable antibody, providing helper function for B-cell development into plasma cells could be eliminated as the mechanism by which CD4 cells affect virus resolution in this strain of mice. Furthermore, depletion of CD8 cells slowed but did not prevent resolution of shedding in μMt mice, while depletion of CD4 cells appeared to completely block the resolution of rotavirus shedding in these mice (Fig. 2). Therefore, providing help for CD8 cell expansion and development into CTLs appeared to be one but not the only effector mechanism through which CD4 cells resolve rotavirus shedding in μMt mice. This left production of antiviral cytokines or development of CD4 cell CTL activity as other possible mechanism. Regardless of which mechanisms are involved, they may be relatively inefficient because detectable viral shedding in nondepleted μMt mice was not fully resolved for at least several weeks after EDIM infection in three of the four times this experiment was performed.

The importance of CD4 cells in resolution of rotavirus shed-

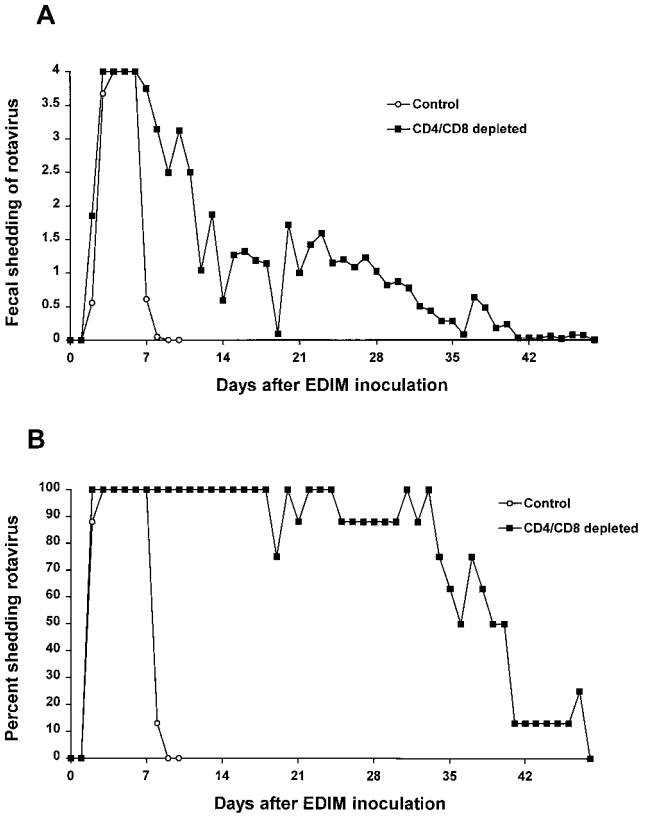

FIG. 6. Effect of CD4 and CD8 cell depletion prior to EDIM inoculation of BALB/c mice on subsequent virus shedding. Mice either were not depleted or were depleted of both CD4 and CD8 cells by i.p. injection of MAbs GK1.5 and 2.43 on days −4, −3, −2, −1, and 2 and inoculated with 4×10^4 FFU of EDIM (passage 9) on day 0. Two stool pellets collected daily up to day 48 were monitored for rotavirus antigen. The values represent the mean rotavirus shedding (A_{490}) of eight mice in each group (A) or the percentage of mice shedding detectable amounts of rotavirus (B).

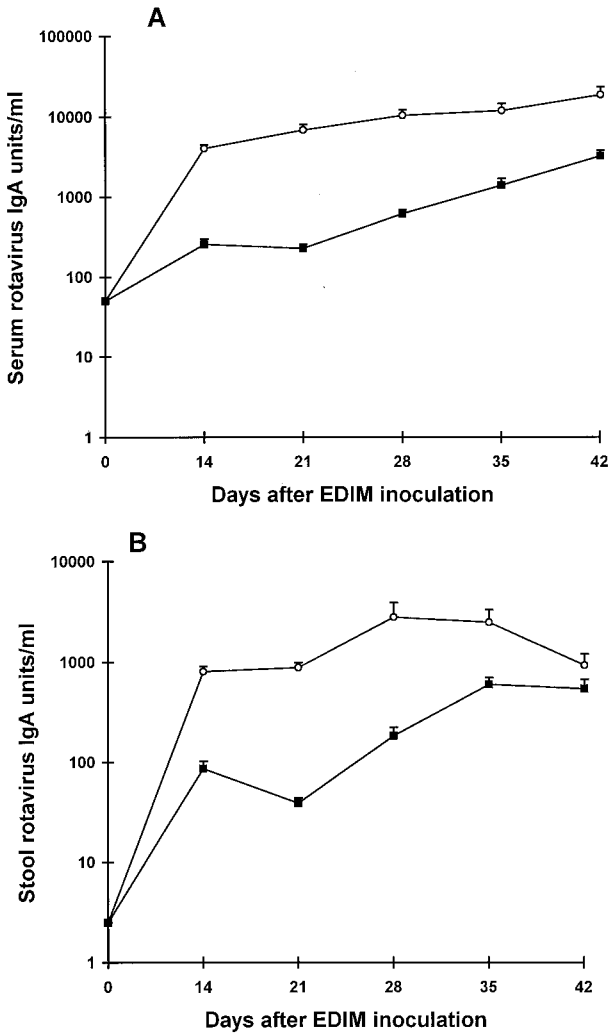

FIG. 7. Concentrations of rotavirus serum and stool IgA as a function of time after EDIM inoculation of nondepleted or CD4/CD8 cell-depleted BALB/c mice. The conditions of depletion and virus inoculation are given in the legend to Fig. 5. Serum (A) and stool (B) specimens collected on the days specified were monitored for rotavirus IgA, and the values given are the geometric mean titers of the nondepleted (○) and CD4/CD8 cell-depleted (■) groups. The bracketed line above each data point shows the standard error of the mean.

ding was also evident in normal mice. BALB/c mice depleted of CD4 cells before EDIM inoculation rapidly resolved most virus shedding. However, low levels of shedding continued for at least 48 days after infection. When a portion of these mice were also depleted of CD8 cells at 18 days after infection, continuous excretion of virus occurred in significantly ($P < 0.001$) larger amounts. This finding demonstrated that CD8 cells were responsible for the reduced level of shedding. Elimination of infection by other viruses is also typically completed within 7 to 10 days after primary infection, which parallels the CD8 cell CTL development and localization to target organs (3).

Support for the conclusion that CD8 cells were responsible for suppression of rotavirus shedding in CD4 cell-depleted BALB/c mice was provided by an additional experiment whose purpose was to determine the effects of CD8 cell depletion alone in these mice prior to EDIM inoculation. Under these conditions, the normal resolution of virus shedding within 9 to 10 days was extended up to 14 days after inoculation, by which time stool rotavirus IgA titers had begun to appear. A similar result was reported by Franco and Greenberg (7) for β_2-microglobulin knockout mice. The rapid decrease in virus shedding in nondepleted BALB/c mice was not due to an earlier appearance of rotavirus antibody than occurred in the CD8 cell-depleted mice because antibody titers in the two groups of mice increased nearly simultaneously. Therefore, it is likely that CD8 cells are responsible for the initial rapid decrease in shedding. However, virus clearance was not completed for at least several weeks in the absence of CD4 cells.

Because of the simultaneous appearance of stool rotavirus IgA and resolution of shedding in CD8 cell-depleted BALB/c mice, and because of previous correlations made between the presence of serum and stool rotavirus IgA and protection against rotavirus infection (6, 14), it is possible that one mechanism by which CD4 cells resolved rotavirus infection in BALB/c mice was by providing help for B-cell development and antibody production. In CD4 cell-depleted BALB/c mice, serum rotavirus IgG titers were suppressed to levels that were less than 10% of their normal ranges, and both serum and stool rotavirus IgA production was almost completely blocked. Thus, the inability to resolve rotavirus shedding in CD4 cell depleted normal mice was directly correlated with low rotavirus antibody titers presumably due to the lack of helper function for B-cell maturation into antibody-producing plasma cells, thus indicating that rotavirus is a T-cell-dependent antigen. However, the possibility that CD8 cell activation or maturation into CTLs was also partially inhibited by a lack of CD4 cell helper function was not eliminated by this experiment. CD4 cell help for CD8 cell development into effector CTLs has been found to be required in some (9) but not other (1) viral infections. Likewise, it is possible that CD4 cell-dependent function other than CD8 cell activity or B-cell production of antibody was involved in resolution of rotavirus shedding in BALB/c mice as apparently occurred in μMt mice.

The importance of antibody as the effector needed for complete resolution of shedding in CD4 cell-depleted BALB/c mice was supported by the results of the final experiment in which both CD4 and CD8 cells were depleted prior to EDIM inoculation. In this case, depletion of both cell types was halted after the final MAb injection at 2 days after rotavirus inoculation. After experiencing a phase of high-level, chronic shedding, these mice began to gradually resolve their infections, and as virus shedding decreased, rotavirus IgA levels rose. FACS analysis revealed that CD4 cells were also being repopulated during this time. Thus, resolution of rotavirus shedding in these doubly depleted mice correlated with the return of CD4 cells and the production of rotavirus serum and intestinal IgA. Since CD8 cells also began to repopulate several weeks after MAb depletion was halted in this experiment, but at a slower rate than CD4 cells, they may also have played a role in resolution of EDIM shedding.

The only other study in which both CD8 and CD4 cells were depleted in order to determine their importance in resolution of rotavirus infection was conducted in calves by Oldham et al. (20). These authors reported that CD8 cells were involved in limiting primary rotavirus infection, while in contrast to results found with mice, CD4 cells were not. Because of the multiple differences in these experimental models, it is not possible to reconcile the results or predict which will be most applicable to humans.

If the importance of rotavirus antibody in the resolution of shedding predicted by the results of this study is validated by subsequent experimentation, it will be important to determine the mechanisms by which this occurs. We have previously reported that protection of mice did not correlate with serotype-specific neutralizing antibody titers (27). Furthermore, an anti-VP6 MAb without neutralizing activity was reported to be protective against rotavirus infection in mice (2). Therefore, the mechanisms by which rotavirus antibody results in protection and, possibly, resolution of infection remain to be determined.

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