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# Long-Term Production of Rotavirus Antibody and Protection against Reinfection Following a Single Infection of Neonatal Mice with Murine Rotavirus

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It has been found that mice infected with murine rotavirus can be protected against subsequent murine rotavirus infection for up to 2 months. It was also reported that protection against rotavirus infection in adult mice correlated with serum and stool rotavirus IgA titers. The present study was conducted to determine the duration of rotavirus antibody production and protection against rotavirus infection in this mouse model and its possible correlation with rotavirus antibody titers. It was found that protection of mice against subsequent infection following a single oral immunization with the murine rotavirus strain EDIM was 100% effective for at least 14 months, most of the lifetime of a mouse. During this period, serum and stool rotavirus antibody titers which included serum IgA, IgG, and neutralizing antibody to EDIM, as well as stool IgA, remained elevated. Of particular note, stool rotavirus IgA titers gradually decreased to levels that were approximately 10% of their peak at 1 month after infection but did not decrease further, while serum rotavirus IgG titers continuously increased during the 14 months of the study. Serum rotavirus IgA titers varied from month to month but overall remained relatively constant throughout the 14-month period. Thus, both serum and stool rotavirus antibody was retained at substantial levels long after a single rotavirus immunization in the absence of reexposure, and mice remained protected against reinfection. 

## INTRODUCTION

Vaccination and natural infection of infants with rotavirus has been correlated with protection against subsequent rotavirus disease, but the mechanism of protection has not been determined. Evidence has indicated that rotavirus antibody may play a role in that adults with higher serum and intestinal rotavirus antibody titers were found to be more resistant to infection and illness when challenged with a virulent rotavirus than were subjects with lower titers (Kapikian *et al.*, 1983; Ward *et al.*, 1989). Furthermore, children with higher serum rotavirus antibody titers were also found to be more resistant to a subsequent rotavirus illness than were those with lower titers (Chiba *et al.*, 1986; Clemens *et al.*, 1992; O'Ryan *et al.*, 1994), but the serotype specificity of protection associated with rotavirus antibody remains controversial (Chiba *et al.*, 1986; Ward *et al.*, 1992a; Ward and Bernstein, 1995).

To circumvent some of the limitations in studying the mechanism of protection against rotavirus disease in humans, animal models have been developed. In a mouse model of passive protection, it has been reported that infant mice suckled on dams previously immunized with rotavirus were protected against rotavirus disease in a serotype-specific manner (Offit *et al.*, 1986). However, following development of a model to study active immunity in adult mice (Ward *et al.*, 1990), protection against rotavirus infection could not be associated with serotype-specific serum or intestinal neutralizing antibody (Ward *et al.*, 1992b). Furthermore, protection against rotavirus disease in neonatal mice was reported following passive transfer of splenic lymphocytes from immunized mice in the absence of detectable rotavirus-neutralizing antibodies (Offit and Dudzik, 1990) and clearance of chronic rotavirus infection in SCID mice by $CD8^+$ T lymphocytes following adoptive transfer was not serotype-specific (Dharakul *et al.*, 1990, 1991).

Very recently, however, active immunity against rotavirus infection in mice was correlated with levels of serum and intestinal rotavirus IgA (McNeal *et al.*, 1994; Feng *et al.*, 1994). To determine whether these relationships are retained over an extended time period, neonatal mice were immunized by infection with a murine rotavirus and groups were periodically challenged with this same virus during a 14-month period. In this way, the duration of protection against reinfection in the absence of intervening exposure to rotavirus was determined. During the same time, monthly analyses of serum rotavirus IgA and IgG and stool rotavirus IgA levels were conducted along with periodic analyses of serum-neutralizing antibody titers. Thus, we simultaneously monitored rotavirus antibodies and the duration of protection to determine their possible correlation over most of the lifetime of a mouse.

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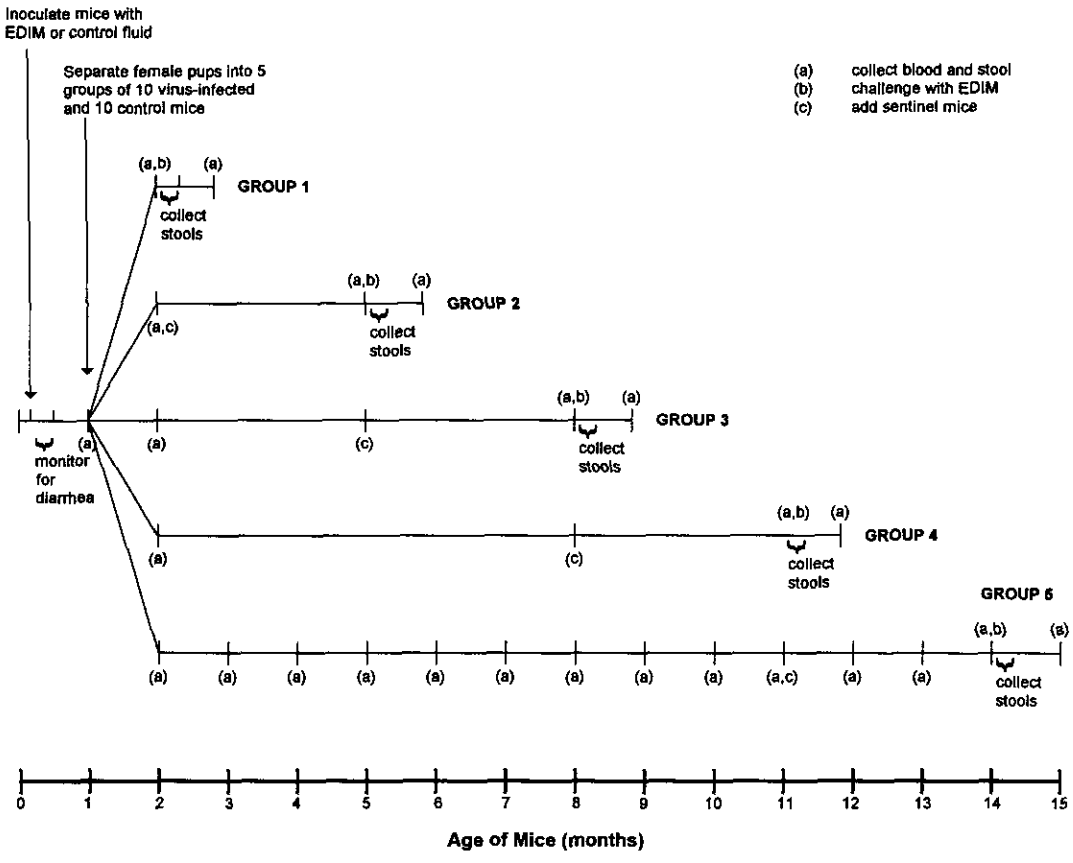


FIG. 1. Experimental plan for immunization of neonatal mice with EDIM and subsequent specimen collection and viral challenge. A detailed explanation is given in the text.

## MATERIALS AND METHODS

### Rotavirus strain

The murine rotavirus strain EDIM used for these studies was culture-adapted from a stool specimen of an infected mouse as previously described (Ward *et al.*, 1990). The ninth passage of this virus, containing $2 \times 10^6$ focus forming units (FFU)/ml, was used as the challenge pool. Initial immunizations of the neonatal mice were performed with a triply plaque-purified preparation of the passage 9 pool.

### Mice

Pregnant pathogen-free BALB/c mice were purchased from Harlan–Sprague–Dawley (Indianapolis, IN) and housed individually in sterile microisolation cages containing autoclaved food and water where they delivered their litters. Only the pups were used for this study.

### Study design

The experimental plan for this study is outlined in Fig. 1. Litters of 4-day-old mice were orally inoculated with $4 \times 10^4$ FFU of the plaque-purified EDIM or uninfected cell lysate processed in the same manner as the virus preparation. Inoculated mice were observed for a period of 9 days for diarrheal illness as determined by excretion of liquid stool after gentle palpation of the abdomen. At 1 month of age, male mice were eliminated from the experiment, and 100 female pups were distributed into Groups 1–5, each group containing 10 mice inoculated with virus and 10 inoculated with control fluid (housed 5 mice/cage). Blood specimens were collected from female pups on this same day using retroorbital capillary plexus puncture. Finally, stool specimens were collected on this date as well [two pellets/mouse into 0.5 ml of Earles balanced salt solution (EBSS)]. The blood and stool specimens were used to measure levels of rotavirus antibodies. At 2 months of age, blood and stool specimens were collected from all 100 mice in the five groups. Ten mice inoculated with EDIM and 10 inoculated with control fluid (Group 1) were challenged by oral inoculation with $4 \times 10^4$ FFU of EDIM. Stool specimens were then collected daily from each mouse for a period of 7 days to be examined for rotavirus antigen. Blood and stool specimens were again collected at 21 days after challenge to be examined for rotavirus antibody levels. As was done previously (McNeal *et al.*, 1994), EDIM in-

fection was determined by viral shedding during the 7-day period following challenge or by rises of ≥ fourfold in serum IgA.

Subsequently, additional mice (Groups 2–5) inoculated with virus or control fluid as neonates were challenged with EDIM at 3-month intervals up to 14 months of age (mice were 5, 8, 11, or 14 months when challenged), and specimens were collected as described for Group 1 challenged at 2 months of age. In addition, two unimmunized (naive) sentinel mice of the corresponding age were added to the cages of the previously infected mice in each group at 3 months before they were challenged to detect possible rotavirus infections within the cages during this 3-month period. These mice were challenged at the appropriate times along with the other mice in the group. Finally, blood and stool collections were obtained monthly for rotavirus antibody analyses from all mice in Group 5, whether inoculated as neonates with virus or control fluid. This group was retained for the entire experiment and challenged with EDIM at 14 months of age.

### Detection of rotavirus shedding

Stools collected into 0.5 ml of EBSS, either for 9 days following the initial inoculation of neonatal mice with EDIM or for 7 days following EDIM challenge of adult immunized or naive mice, were analyzed for shedding of rotavirus antigen by ELISA as previously described (McNeal *et al.,* 1994).

### Determination of rotavirus antibody titers

Serum rotavirus IgA and IgG titers were measured as previously described (McNeal *et al.,* 1994). In short, 96-well microdilution plates were coated with rabbit IgG against single-shelled rotavirus strain SA11 followed by addition of an EDIM rotavirus lysate or a mock-infected cell lysate (control wells). Serial twofold dilutions of pooled sera from EDIM-infected mice (serum standards) assigned concentrations of 160,000 or 10,000 U/ml of rotavirus IgG or IgA, respectively, were made and added to duplicate wells coated with EDIM or control fluid to generate a standard curve. Appropriate dilutions of mouse sera to be tested were also added to duplicate wells containing virus or control fluid. This was followed by sequential addition of biotin-conjugated goat anti-mouse IgG or IgA, peroxidase-conjugated avidin–biotin, and substrate (*o*-phenylenediamine plus $H_2O_2$). Color development was stopped with 1 *M* $H_2SO_4$ and $A_{490}$ was measured. Units/per milliliter of rotavirus IgG or IgA were determined from a standard line plot of the control antisera after subtraction of the average $A_{490}$ values of duplicate wells coated with control fluid from those coated with EDIM lysate. Stool rotavirus IgA was measured in the same manner as serum IgA following collection of two pellets into 0.5 ml of EBSS, homogenization, and centrifugation (1500 *g,* 5 min). Stool specimens were kept frozen (−20°) until analyzed, and all procedures prior to IgA analysis were conducted at 4° to retard enzymatic digestion of antibody. Neutralizing antibody to EDIM was performed by an antigen reduction assay described previously (Knowlton *et al.,* 1991). The EDIM virus used in this neutralization assay was passaged 40 times and triply plaque purified twice during these passages to select virus with the best growth properties in cell culture.

TABLE 1

Rotavirus Antibody Levels in 50 Female BALB/c Pups at 1 and 2 Months after Inoculation with EDIM[a]

| Rotavirus antibody | GMT (U/ml) | |
|---|---|---|
| | 1 month (range) | 2 months (range) |
| Serum IgG | 34,435 (16,000–76,000) | 37,879 (16,000–96,000) |
| Serum IgA | 5613 (2200–11,500) | 4373 (2350–8200) |
| Stool IgA | 2045 (555–7500) | 992 (140–4200) |

[a] No rotavirus antibody was detected in any of 50 mock-infected female mice, i.e., titers of <100, <50, and <10 U/ml for serum rotavirus IgG and IgA and stool rotavirus IgA, respectively.

## RESULTS

### Duration of rotavirus antibody following neonatal rotavirus infection

Following inoculation of neonatal mice with $4 \times 10^4$ FFU of triply plaque-purified EDIM, diarrheal disease began within 2 days and persisted in several mice until 9 days after inoculation (the last day mice were inspected for diarrhea). Although the number of days any one pup was ill was not determined, at least 86 of the 103 pups inoculated had diarrhea for at least 1 day based on the number of mice with diarrhea in each litter on each day. All 50 virus-inoculated female pups had serum rotavirus IgG and IgA as well as stool rotavirus IgA responses when analyzed at 1 month of age (males were not tested), and serum antibody levels remained elevated when again analyzed 1 month later (Table 1). By this time, however, there was a measurable decrease in the geometric mean titer (GMT) of stool rotavirus IgA titers. None of the 50 female pups inoculated with control fluid developed rotavirus antibody during this 2-month period.

Ten mice inoculated with virus and 10 administered control fluid were kept for the duration of the study and not reexposed to rotavirus until 14 months of age (Group 5). During this time, monthly blood and stool specimens were collected from all 20 mice and analyzed after each collection period for rotavirus antibody to determine the duration of these antibodies following a single rotavirus infection as neonates. Stool rotavirus IgA titers de-

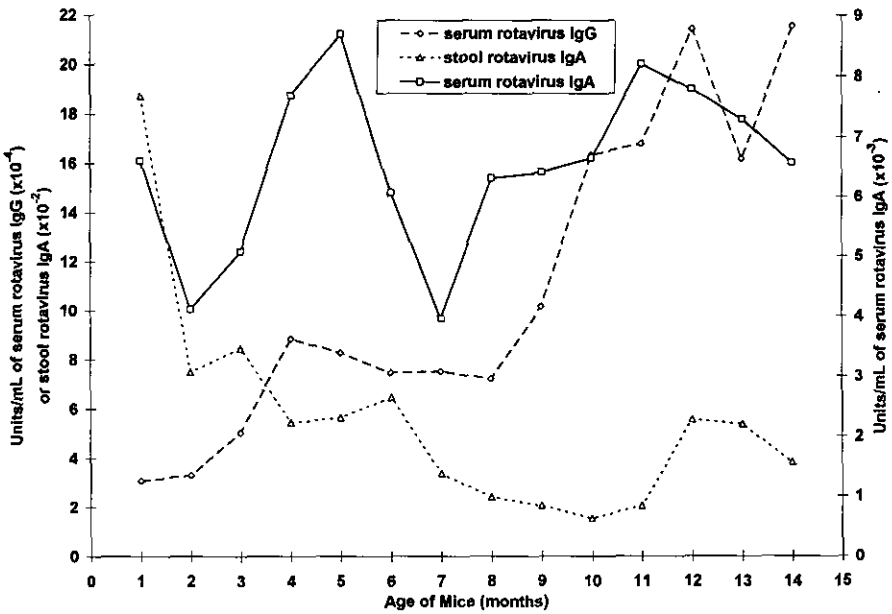


FIG. 2. Persistence of serum rotavirus IgG and IgA and stool rotavirus IgA following a single immunization of mice with murine rotavirus at 4 days of age. Mice were immunized with EDIM as neonates and monthly specimens were collected for the next 14 months and analyzed for rotavirus antibody. Values presented represent the GMT of the 10 virus-infected mice in Group 5. Units per milliliter were determined relative to a standard mouse serum specimen. For stool IgA, the values were from two stool pellets collected into 0.5 ml of EBSS. Significant changes ($P < 0.05$, determined by an unpaired Student's $t$ test) between any two collection periods were found for serum rotavirus IgG (Months 3–4, 9–10), serum rotavirus IgA (Months 1–2, 3–4, 5–6, 6–7, 7–8), and stool rotavirus IgA (Months 1–2, 11–12).

creased between 1 and 2 months of age for this group, as was found for all mice, but titers then leveled off and remained elevated for the duration of the study (Fig. 2). The overall GMT of serum rotavirus IgA for this group changed little in mice between 1 and 14 months of age, and the GMT of serum rotavirus IgG increased throughout the study period. Elevated antibody titers appeared to be retained in the absence of reexposure to rotavirus since all mice inoculated with control fluid remained negative for rotavirus antibody until challenged, and naive mice placed in cages with infected animals at 3 months before challenge (sentinel mice) also remained negative for rotavirus antibody until challenged.

In addition to the mice in Group 5 that were challenged at 14 months of age, mice in Groups 1–4 were also challenged with EDIM at 2, 5, 8, and 11 months of age, respectively. Antibody titers in these mice determined at the time of challenge were comparable to those measured in Group 5 at the same age (Table 2 and Fig. 2). Also, as found in Group 5, mice administered control fluid remained negative for rotavirus antibody until challenged, and naive sentinel mice placed in cages of immunized mice of Groups 2–4 3 months prior to challenge remained rotavirus antibody negative until challenged.

In addition to serum rotavirus IgG and IgA, serum-neutralizing antibody to EDIM was measured for the 20 mice in Group 5 at 1, 9, and 14 months of age. Titers were quite low as found previously following EDIM infection (Ward *et al.*, 1992b), but, in agreement with the results found for serum rotavirus IgG, the GMTs gradually increased during the 14 months of the study (Table 3). The increase was not as great as that found for serum rotavirus IgG, however, suggesting that neutralization epitopes on VP4 and VP7 proteins may have been less immunogenic than other rotavirus epitopes on these and other proteins. In any case, it is clear that all rotavirus antibody levels measured remained elevated for at least 14 months following EDIM infection of neonatal mice.

## Duration of protection against EDIM reinfection

It was shown previously that active protection against rotavirus infection correlated with serum (McNeal *et al.*, 1994) and stool (Feng *et al.*, 1994) IgA titers when mice were challenged within 2 months of their initial infection. Since it has now been found that these antibodies, as well as serum IgG and serum-neutralizing antibody to EDIM, remain elevated for at least 14 months following neonatal infection with EDIM, we determined whether full protection against EDIM reinfection was maintained during this time. Mice infected with EDIM as neonates and previously uninfected age-matched control mice were challenged with EDIM at 2, 5, 8, 11, or 14 months of age (Table 4). No previously infected mouse became infected as determined by viral shedding or rises in serum rotavirus IgA (both used as indicators of protection). All but 7 of 58 control mice, including the sentinel animals, became infected when challenged as determined

TABLE 2

Prechallenge Rotavirus Antibody Titers of Mice Inoculated with EDIM as Neonates

| Group | Age at challenge | GMT of rotavirus antibody at the time of challenge | | |
|---|---|---|---|---|
| | | *Serum IgG* | *Serum IgA* | *Stool IgA* |
| 1 | 2 months | 35,047 (16,000–60,000)[a] | 4382 (2350–7400) | 705 (15–1600) |
| 2 | 5 months | 70,678 (32,000–174,000) | 5385 (2475–10,500) | 352 (85–470) |
| 3 | 8 months | 135,513 (40,900–326,000) | 5462 (2750–9125) | 488 (115–1280) |
| 4 | 11 months | 140,041 (35,000–700,000) | 7590 (4000–19,800) | 394 (80–2480) |

[a] Ranges of titers are shown in parentheses.

by the same criteria. (There was 99% agreement between the two methods used to detect infection). No resistance to rotavirus infection was found as the mice aged based on the quantity of rotavirus antigen shed per mouse following EDIM challenge of the control animals (Table 4) and, in fact, shedding was significantly ($P \leq 0.003$) greater in the oldest group challenged than in any other group. None of the previously infected mice had ≥ fourfold rises in serum rotavirus IgG or stool rotavirus IgA when challenged with EDIM, and all control mice that were infected when challenged did have ≥ fourfold rises in the levels of these two antibodies. Thus, retention of rotavirus antibody in serum and stool correlated with continued protection against rotavirus infection.

## DISCUSSION

Neonatal infection of mice with the murine rotavirus strain EDIM was found to provide complete protection against reinfection with EDIM for a period of at least 14 months, which is a large portion of the lifetime of a laboratory mouse. This protection correlated with the maintenance of elevated titers of serum and intestinal antibodies to the immunizing virus in the absence of reexposure to rotavirus. This result supports the concept that levels of rotavirus antibody correlate with protection against reinfection in this mouse model. Since all rotavirus antibody titers remained elevated during this study, however, none could be singled out as a correlate of protection as was done previously for serum rotavirus IgA (McNeal *et al.*, 1994) and stool rotavirus IgA (Feng *et al.*, 1994). Further experiments will be required to determine whether any of these antibodies were actually responsible for protection.

Evidence for persistent production of rotavirus antibody and protection following a single immunization in animals has been reported by others. Shaw *et al.* (1993) found that the frequency of murine intestinal antibody-secreting cells after 1 year following infection as neonates with a heterologous rotavirus remained at approxi-

TABLE 3

Persistence of Serum-Neutralizing Antibody to EDIM Following Neonatal Infection

| Age of mice | GMT of neutralizing antibody[a] | |
|---|---|---|
| | Infected mice | Control mice |
| 1 month | 19.3 (13–32)[b] | 9.4 (5–32) |
| 9 months | 26.2 (5–121) | 5.7 (5–9) |
| 14 months | 31.3 (8–179) | 6.4 (5–14) |

[a] Sera from the 20 Group 5 mice were examined at the times specified for the presence of neutralizing antibody to EDIM.

[b] Ranges of titers are shown in parentheses. Titers of ≤5 were considered as 5 for determination of GMT and range.

TABLE 4

Duration of Protection against EDIM Infection in Mice Immunized as Neonates

| Age at challenge | Number of mice infected | | |
|---|---|---|---|
| | Immunized mice | Control mice | Sentinel mice |
| 2 months | 0/10 | 9/10 (1.32)[a] | — |
| 5 months | 0/10 | 8/10 (1.28) | 2/2 (1.31) |
| 8 months | 0/10 | 8/10 (1.26) | 1/2 (1.18) |
| 11 months | 0/10 | 9/10 (1.49) | 2/2 (1.94) |
| *14 months* | *0/10* | *10/10 (2.48)*[b] | *2/2 (2.30)* |
| Total | 0/50 (0%) | 44/50 (88%) | 7/8 (88%) |

[a] Average $A_{490}$ values above background determined for viral shedding by ELISA for each mouse during each day for the 7 days following EDIM challenge are shown in parentheses.

[b] Shedding ($A_{490}$ values) was significantly ($P \leq 0.003$) greater for this group than any other group as determined by an unpaired Student's *t* test.

mately 17% of the level found 1 month after infection. Similarly, Conner and Estes (1994) reported that rabbits immunized with a rabbit rotavirus strain maintained anti-rotavirus serologic and mucosal antibody titers for 708 days postinoculation. Furthermore, Burns *et al.* (1995) reported that infection of 6- to 8-week-old-mice with murine rotavirus provided protection against reinfection for 1 year. The present report, however, was the first to show the correlation between long-term protection and maintenance of elevated serum and intestinal antibody levels.

It has been suggested that B-cell memory and long-term antibody production requires persistent presence of antigen (Gray, 1993). If this occurs without reexposure to an external source of antigen, then the source must arise from within. Possible sources following viral infection could be low-level persistent viral replication or trapped antigen within follicular–dendritic cells. Alternatively, plasma cells may be long-lived and produce antibody without continuous antigen exposure to memory B-cells (Slifka *et al.*, 1995). Regardless of the mechanism, there are many instances where long-term antibody production has been found following acute viral infections in the absence of reexposure to the virus. Based on the results of this and previous (Shaw *et al.*, 1993; Conner and Estes, 1994) studies, rotavirus can be added to this list.

The relative amounts of serum rotavirus IgG, serum rotavirus IgA, and stool rotavirus IgA had considerable variation over the 14-month period in which they were analyzed following the initial EDIM infection of neonatal mice. Stool rotavirus IgA titers decreased dramatically between 1 and 2 months following EDIM infection, as is typical of mucosal antibody (McGhee and Kiyono, 1993), but then changed little during the remainder of the study. Serum rotavirus IgA titers fluctuated considerably from one month to the next but overall changed little during the 14-month period. Serum rotavirus IgG titers were the most unusual. These increased nearly 10-fold during the 14-month period and appeared to do so in a series of steps. Since comparable changes were not seen in titers of the other rotavirus antibodies, inadvertant reexposure to an external source of antigen was highly unlikely. Therefore, the stepwise increases in serum rotavirus IgG appeared to be due to stimulation by an internal source such as trapped antigen. The shorter half-life of IgA coupled with possible differences in its inductive and effector sites (Jackson *et al.*, 1993; McNeal *et al.*, 1994) could explain why serum rotavirus IgA titers did not change in parallel with those of IgG.

The mechanisms by which oral immunization with live rotavirus induces protection against rotavirus infection and disease in humans have not been clearly established, and one purpose of establishing the mouse model of active immunity was to elucidate these mechanisms. Differences in the immune systems of mice and humans, however, will limit extrapolation of the results found in mice directly to humans. For example, mice were found to be totally protected against rotavirus infection following neonatal infection while rotavirus infection of human neonates was clearly less protective (Bishop *et al.*, 1983). Although correlations between the levels of serum and intestinal antibody versus protection against rotavirus disease have been observed in humans as well as in mice, the levels of neither total nor serotype-specific rotavirus antibody have been consistently associated with protection of humans (Ward *et al.*, 1992a; Ward and Bernstein, 1995). Thus, it has not yet been possible to use either type of rotavirus antibody as a surrogate marker of immunity following vaccination. It appears, therefore, that even though serum and intestinal IgA titers have been correlated with protection against rotavirus infection in mice over the short-term (McNeal *et al.*, 1994; Feng *et al.*, 1994) and now over the long-term, rotavirus antibody is probably not the only immunological component responsible for protection against rotavirus infection and disease in humans.

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