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Recovery from Chronic Rotavirus Infection in Mice with Severe Combined Immunodeficiency: Virus Clearance Mediated by Adoptive Transfer of Immune CD8$^+$ T Lymphocytes

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Severe combined immunodeficient (SCID) mice lack both functional T and B cells. These mice develop chronic rotavirus infection following an oral inoculation with the epizootic diarrhea of infant mice (EDIM) rotavirus. Reconstitution of rotavirus-infected SCID mice with T lymphocytes from immunocompetent mice allows an evaluation of a role of T-cell-mediated immunity in clearing chronic rotavirus infection. Complete rotavirus clearance was demonstrated in C.B-17/scid mice 7 to 9 days after the transfer of immune CD8$^+$ splenic T lymphocytes from histocompatible BALB/c mice previously immunized intraperitoneally with the EDIM-w strain of murine rotavirus. The virus clearance mediated by T-cell transfer was restricted to *H-2^d*-bearing T cells and occurred in the absence of rotavirus-specific antibody as determined by enzyme-linked immunosorbent assay, neutralization, immunohistochemistry, and radioimmunoprecipitation. Temporary clearance of rotavirus was observed after the transfer of immune CD8$^+$ T cells isolated from the intestinal mucosa (intraepithelial lymphocytes [IELs]) or the spleens of BALB/c mice previously infected with EDIM by the oral route. Chronic virus shedding was transiently eliminated 7 to 11 days after spleen cell transfer and 11 to 12 days after IEL transfer. However, recurrence of rotavirus infection was detected 1 to 8 days later in all but one SCID recipient receiving cells from orally immunized donors. The viral clearance was mediated by IELs that were both Thy1$^+$ and CD8$^+$. These data demonstrated that the clearance of chronic rotavirus infection in SCID mice can be mediated by immune CD8$^+$ T lymphocytes and that this clearance can occur in the absence of virus-specific antibodies.

Rotavirus infection represents the major cause of acute viral gastroenteritis in infants and young children worldwide. Significant morbidity and mortality, particularly in developing countries, are well documented. Recovery from rotavirus infection in normal hosts generally occurs within 1 week after the onset of symptoms and is followed at least in part by the development of immunity to subsequent infection (1, 7). In contrast, rotavirus infection in immunocompromised hosts can be prolonged up to 6 months, as has been reported for children with a variety of immunodeficiency diseases (20). Severe rotavirus infection in adults undergoing bone marrow transplantation has also been documented (25). Furthermore, experimental rotavirus infection in mice with severe combined immunodeficiency (SCID) resulted in a chronic persistent infection (19). These reports suggest an important role of the immune system in mediating recovery from rotavirus infection.

The fact that rotavirus replication is generally limited to the villus enterocytes of the small intestine (2) suggests that any immunologic determinants involved in protection and in disease resolution may need to be present at this mucosal site. Protection against acute rotavirus infection can be mediated by rotavirus-neutralizing antibody present in intestinal lumen but not in serum (12, 13). Nonneutralizing antibodies have not been shown to play a protective role in rotavirus infection, although they can play such a role in some other virus systems (10, 16). The role of antibody in recovery from acute infection is less well defined. Transient recovery from chronic rotavirus infection was reported following oral administration of serum immunoglobulins containing rotavirus-specific antibody to immunodeficient children suffering from chronic rotavirus infection. The treatment was able to eliminate virus shedding, but recurrence was observed after the treatment was discontinued (11). On the other hand, little information is available regarding a possible role of other immunity mediation mechanisms, including cell-mediated immunity in the recovery from acute or chronic rotavirus infection. The acute nature of most rotavirus infections as well as the age-restricted susceptibility to rotavirus illness, especially in mice (23), significantly restricted potential strategies to study and characterize the immunologic components of recovery from rotavirus infection. The availability of the SCID mouse model of chronic rotavirus infection offers an interesting opportunity to study a possible role of a particular part of the immune system (CD8$^+$ T lymphocytes) without interference from other immune factors such as antibody.

In this study, the SCID mouse model was employed to evaluate the role of T-cell-mediated immunity in the clearance of chronic rotavirus infection. The experiments involved adoptive transfer of CD8$^+$ T cells into SCID mice chronically infected with murine rotavirus. The experiments indicate that selected T cells can efficiently and completely eliminate rotavirus infection of the gut, even in the absence of any rotavirus antibody.

MATERIALS AND METHODS

Animals. Lactating homozygous C.B-17/scid females with 3-day-old pups, provided by the Radiation Biology Division, Stanford University, were housed in microisolator cages at the animal research facility of the Palo Alto Veterans Ad-

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ministration Medical Center. The pups were inoculated orally 1 to 2 days after arrival with 5 μl of epizootic diarrhea of infant mice rotavirus (EDIM) containing 200 50% mouse infectious doses. Inoculation with virus resulted in chronic infection in all mice. After weaning, each mouse was caged individually. Regular mouse chow was given ad libitum. Prophylactic sulfamethoxazole-trimethoprim (160 mg of sulfamethoxazole per 100 ml) was given in drinking water for half a week; acidified water (pH 2.7) was given for the rest of the week. SCID mice (6 to 10 weeks old) which were chronically infected with EDIM were used as adoptive transfer recipients.

T-cell donor, rotavirus antibody-negative BALB/c mice were obtained from Charles River Breeding Laboratories, Portage, Mich. For intraperitoneal (i.p.) immunization spleen cell transfer experiments, 6-week-old BALB/c females were injected twice i.p. with 50 μg of partially purified EDIM-w (3- to 4-week intervals between injections), and the spleens were collected 4 days after the second immunization. For oral (p.o.) immunization spleen cell and intraepithelial lymphocyte (IEL) transfer experiments, individual 5- to 7-day-old BALB/c mice were inoculated p.o. with 5 μl of EDIM containing 200 50% mouse infectious doses. Three to four weeks postinfection, the mice were boosted p.o. with 1 ml of EDIM-w (see below) at a dose of 10^3 to 10^4 PFU per mouse. Spleens or small intestines were collected 4 days after p.o. boosting.

For major histocompatibility complex (MHC) restriction experiments, adult C57Bl/6 female mice (Charles River Breeding Laboratories) and congenic BALB.B ($H\text{-}2^b$), BALB.K ($H\text{-}2^k$) and B10.D2 ($H\text{-}2^d$) mice (kindly provided by I. Weissman, Stanford University) were immunized i.p. with 50 μg of partially purified EDIM-w and spleens were collected as described above.

Virus. EDIM-5099, originally obtained from R. Wyatt (National Institutes of Health, Bethesda, Md.), was used to infect mice. The virus stock, prepared from clarified intestinal homogenates (2×10^7 50% mouse infectious doses per ml), was kindly provided by M. Talty (The Children's Hospital of Buffalo, N.Y.). Tissue culture-adapted EDIM (EDIM-w) was propagated in MA-104 (fetal monkey kidney cell line) cells in M-199 medium, extracted with fluorocarbon, overlayered onto a 1.30 g/cm^3 sucrose cushion, and pelleted by centrifugation at 100,000 × *g* for 1.5 h (6).

Antibodies. Rat-mouse hybridomas 53.67 (anti-CD8) and 30-H12 (anti-Thy1.2) were obtained from the American Type Culture Collection (Rockville, Md.), and 53.21 (anti-Thy1.2) was kindly provided by E. Butcher (Stanford University). Specificities and characterizations of these hybridomas were described previously (9). The hydridomas were grown in a serum-free medium, HB101 (Hana Biologics, Inc., Alameda, Calif.). The monoclonal antibodies were purified from culture supernatant fluid after ammonium sulfate precipitation by protein G affinity chromatography (Protein G Sepharose 4 Fast Flow; Pharmacia LKB) according to the instructions of the manufacturer. Purified 53.67 and 53.21 were conjugated with fluorescein isothiocyanate (FITC) and biotin, respectively, as described previously (24).

T-cell preparation. Spleens were aseptically removed from the mice and kept in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin-streptomycin. Each cell suspension was prepared by perfusing the spleen with medium and then teasing it into small fragments and allowing large clumps to settle; the cell suspension was collected from the supernatant fluid. Erythrocytes were removed by lysis with ACK buffer (0.16 M NH_4Cl, 0.1 mM EDTA, 0.01 M $KHCO_3$). The preparation was then depleted of B cells by panning the preparation onto petri dishes precoated with 10 μg of goat anti-mouse immunoglobulin G (IgG) (Sigma Chemical Co., St. Louis, Mo.) per ml at 4°C for 30 min. After two panning steps, the nonbound cells were collected, washed, and stained with FITC-53.67. The $CD8^+$ population was selected by sorting twice for the FITC-positive population present in the lymphocyte gate by using a FACStar (Becton-Dickinson Immunocytometry Systems, San Jose, Calif.). Approximately 10^6 purified $CD8^+$ cells were then transferred to each SCID mouse by i.p. injection.

IELs were isolated from the small intestines, as described by Petit et al. (18), with some modifications. In brief, the epithelial layer was released from the intestinal tissue by incubation with 1 mM EDTA in Ca^{2+}-Mg^{2+}-free Hanks balanced salt solution with constant stirring. The liberated epithelial cells and IELs were collected and washed with Hanks balanced salt solution containing 2% fetal bovine serum. IELs were then enriched by pelleting in 35% Percoll (Sigma) and purified on a discontinuous 40 to 75% Percoll gradient. The IELs were collected from the interface, stained with FITC-53.67, and sorted on the FACStar as above. 1.5×10^6 $CD8^+$ IELs were transferred to each SCID-infected mouse.

Depletion of $Thy1^+$ cells from IELs was performed by complement-mediated lysis as follows. An IEL preparation recovered from 35% Percoll was incubated with 30-H12 for 30 min at 0°C and then incubated for 45 min with low-tox-M rabbit complement (Accurate Chemical & Scientific Corp., Westbury, N.Y.) at 37°C. The live IELs were then purified on the discontinuous 40 to 75% Percoll as above. This treatment reduced the yield of $CD8^+$ cells approximately 40 to 50%, whereas an incubation of IELs with low-tox-M rabbit complement alone, without prior exposure to 30-H12, did not significantly reduce the yield of recovered $CD8^+$ IELs.

Detection of $Thy1^+$ cells and $CD8^+$ cells in intestinal mucosa of SCID mice. Immunoperoxidase staining was performed on cryostat sections of duodenal tissues after fixation with methanol. Culture supernatant fluid of 53.21 (anti-Thy1) and 53.67 (anti-CD8) were used as first antibody, followed by appropriate dilutions of biotin-labeled mouse anti-rat IgG (Accurate Chemical & Scientific Corp.) and horseradish peroxidase-labeled Streptavidin (Kirkegaard & Perry, Gaithersburg, Md.), as previously determined by titration staining of intestinal tissue sections obtained from BALB/c mice. Peroxidase reaction was developed with 3-amino 9-ethyl carbazole (0.02% in 0.1 M acetate buffer [pH 5.2] containing 5% *N*,*N*-dimethylformamide and 0.03% hydrogen peroxide). Peroxidase-stained cells were not identified in intestinal tissue from untreated SCID mice when the first antibody (anti-Thy1 or anti-CD8) used in the above staining procedure was replaced with normal rat serum or rat monoclonal antibody GK 1.5 (anti-CD4).

Detection of rotavirus antigen. A sandwich enzyme-linked immunosorbent assay (ELISA) method was used. In brief, 5% suspensions of fecal material were added to Immulon II microtiter plate wells precoated with a polyclonal goat (goat930) serum hyperimmune to the D strain of human rotavirus (dilution, 1:10,000 in carbonate-bicarbonate buffer [pH 9.6]). After incubation and washing, virus antigens were detected with a guinea pig serum hyperimmune to EDIM-w (dilution 1:10,000), followed by 50 ng of horseradish peroxidase-conjugated goat anti-guinea pig IgG (Kirkegaard & Perry) per ml. Dilutions of fecal material and subsequent antibodies were made in phosphate-buffered saline contain-

ing 0.05% Tween 20 and 5% fetal bovine serum. *o*-Phenylenediamine hydrochloride (0.4 mg/ml in citrate-phosphate buffer [pH 5.0] containing 0.03% H_2O_2) was used as enzyme substrate. The reaction was terminated with the addition of 2 M H_2SO_4 and then optical density at 490 nm was measured with an enzyme immunoassay autoreader EL310 (Bio-Tek Instruments, Winooski, Vt.).

Detection of rotavirus-specific IgA. An ELISA was used with EDIM-w (captured on the Immulon II plate precoated with the guinea pig antiserum to EDIM-w) as a detection antigen. Fecal suspensions (5%) from treated mice were then added to the wells and the presence of IgA bound to EDIM-w was detected by using horseradish peroxidase-conjugated goat anti-mouse IgA (50 ng/ml; Kirkegaard & Perry). The peroxidase reaction was developed as described above.

Immunoprecipitation. A [^{35}S]methionine-labeled lysate of rhesus rotavirus (RRV)-infected MA-104 cells was prepared as described previously (5) and used as a surrogate antigen for immunoprecipitation studies. In summary, MA-104 cells were infected with trypsin-activated RRV at 10 PFU per cell. At 4 h p.i., after methionine starvation, infected cells were labeled with 0.05 mCi of [^{35}S]methionine (Translabel; ICN Pharmaceuticals, Irvine, Calif.) per ml until 10 h p.i. The cell lysate was prepared using RIPA buffer (0.1 M Tris [pH 7.5], 0.3 M NaCl, 1% deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) without sodium dodecyl sulfate, and cell debris and virus particles were removed by ultracentrifugation. The labeled virus proteins were immunoprecipitated sequentially with undiluted mouse sera, rabbit anti-mouse immunoglobulins, and protein A-Sepharose beads and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) as described previously (5).

Detection of rotavirus-specific plasma cells. A sandwich immunofluorescence technique was described previously (3). In brief, the procedure involved a sequential incubation of the cryostat sections of duodenal tissue with the following: EDIM-w, guinea pig anti-EDIM-w, rhodamine-conjugated rabbit anti-guinea pig IgG (Accurate Chemical & Scientific Corp.), and fluorescein-conjugated rabbit anti-mouse IgA (Sigma). The cells that were positive for both fluorochromes were detected by using a Nikon Labophot epifluorescence microscope.

Focus reduction neutralization. The sera from SCID mice as well as from their BALB/c donors were inactivated at 56°C for 30 min and tested at a dilution of 1:50 against EDIM-w in a focus reduction neutralization assay as described by Shaw et al. (21).

RESULTS

Rotavirus infection in SCID mice used in adoptive transfer studies. All SCID mice inoculated with EDIM at 4 to 5 days of age ($n = 32$) developed diarrhea and shed rotavirus in their stools until the time of cell transfer, 6 to 10 weeks after infection. However, diarrhea was no longer detected after the mice were 15 days old. These chronically infected mice were apparently healthy throughout the experiments.

Surface characteristics of transferred cells. $CD8^+$ T cells were selected by labeling with FITC anti-CD8 antibody and sorting with FACStar. $CD8^+$ cells represented 5 to 10% of unsorted spleen cells and about 20% (mean ± standard deviation, 20.1 ± 1.0; $n = 5$) of spleen cells recovered after panning. After sorting twice with FACStar, the transferred cell preparations contained 99.7% ± 0.1% ($n = 5$) $CD8^+$ cells. Virtually all (>99%) of the $CD8^+$ sorted spleen cells also expressed Thy1 antigen, as detected by immunoperoxidase staining with biotin-labeled anti-Thy1 antibody (53.21). Isolated IELs contained higher percentages (72.5% ± 10.2%) of $CD8^+$ cells than the spleen and after sorting twice 99.8% ± 0.2% of the IELs were $CD8^+$. However, only half of the IEL population expressed both Thy1 and CD8, the other half were $Thy1^-$ but $CD8^+$.

Migration of transferred cells to rotavirus-infected intestinal mucosa. The intestinal tissue from rotavirus-infected SCID mice that did not receive transferred cells contained moderate numbers of $CD8^+$ cells in the IEL compartment, as shown in Fig. 1A by immunoperoxidase staining. However, none of the $CD8^+$ IELs were $Thy1^+$, although small numbers of $Thy1^+$ cells were seen in the villus lamina propria (Fig. 1B). Significant increases in $Thy1^+$ and $CD8^+$ cells were detected in the intestinal mucosa of recipient mice following immune cell transfer (Fig. 1C and D). A significant increase in the number of $Thy1^+$ cells was observed in the intestinal tissue of SCID recipients after transfer of i.p. immunized and p.o. immunized spleen cells, as well as after transfer of unimmunized spleen cells, compared with chronically infected SCID mice which did not receive transferred cells (Table 1). A significant increase in $Thy1^+$ cells was also noted for the recipients of spleen cells from orally immunized donors compared with the recipients of spleen cells from unimmunized donors. The number of $Thy1^+$ cells was compared among recipients receiving immune IELs, nonimmune IELs, and no transferred cells (Table 1). $Thy1^+$ IELs were significantly increased in the guts of immune IEL recipient mice compared with levels in recipients of immune $Thy1^-$ $CD8^+$ cells or nonimmune IELs and in mice which did not receive a cell transfer. On the other hand, a significant increase in $CD8^+$ cells was observed in the guts of animals receiving immune spleen cells but not in animals receiving nonimmune spleen cells or IELs or in animals not receiving a cell transfer. Background levels of residential $CD8^+$ IELs in SCID mice may have obscured increases in this cell population after cell transfer (Table 1).

Effect on chronic rotavirus infection in SCID mice of transferring immune $CD8^+$ spleen cells from parenterally immunized BALB/c mice. Chronically infected SCID mice were treated with $CD8^+$ spleen cells isolated from BALB/c mice immunized i.p. with partially purified EDIM-w. All of the SCID mice recipients ($n = 5$) stopped shedding rotavirus 7 to 8 days after cell transfer, and rotavirus remained undetectable for at least 10 days (Fig. 2). No effect on rotavirus shedding was observed for SCID mice that received nonimmune $CD8^+$ spleen cells ($n = 5$) (Fig. 2).

Effect on chronic rotavirus infection in SCID mice of transferring $CD8^+$ spleen cells and IELs from orally immunized BALB/c mice. In order to investigate whether cells that mediate clearance of rotavirus infection in SCID mice are generated following infection of the intestine, we studied lymphocytes obtained from mice immunized orally rather than systemically. $CD8^+$ spleen cells and $CD8^+$ IELs recovered from BALB/c mice 3 to 4 weeks after intestinal infection with EDIM virus and 4 days after an oral boost with EDIM-w were transferred into SCID mice. The results are shown in Fig. 3. Rotavirus shedding dropped to undetectable levels in individual mice 7 to 11 days after transfer of spleen cells from orally immunized mice ($n = 5$), similar to the decline observed after transfer of immune spleen cells from parenterally immunized mice (Fig. 2). However, low levels of virus shedding recurred 1 to 8 days after the decline (Fig. 3). The levels of rotavirus shedding seen in Fig. 3 do not drop

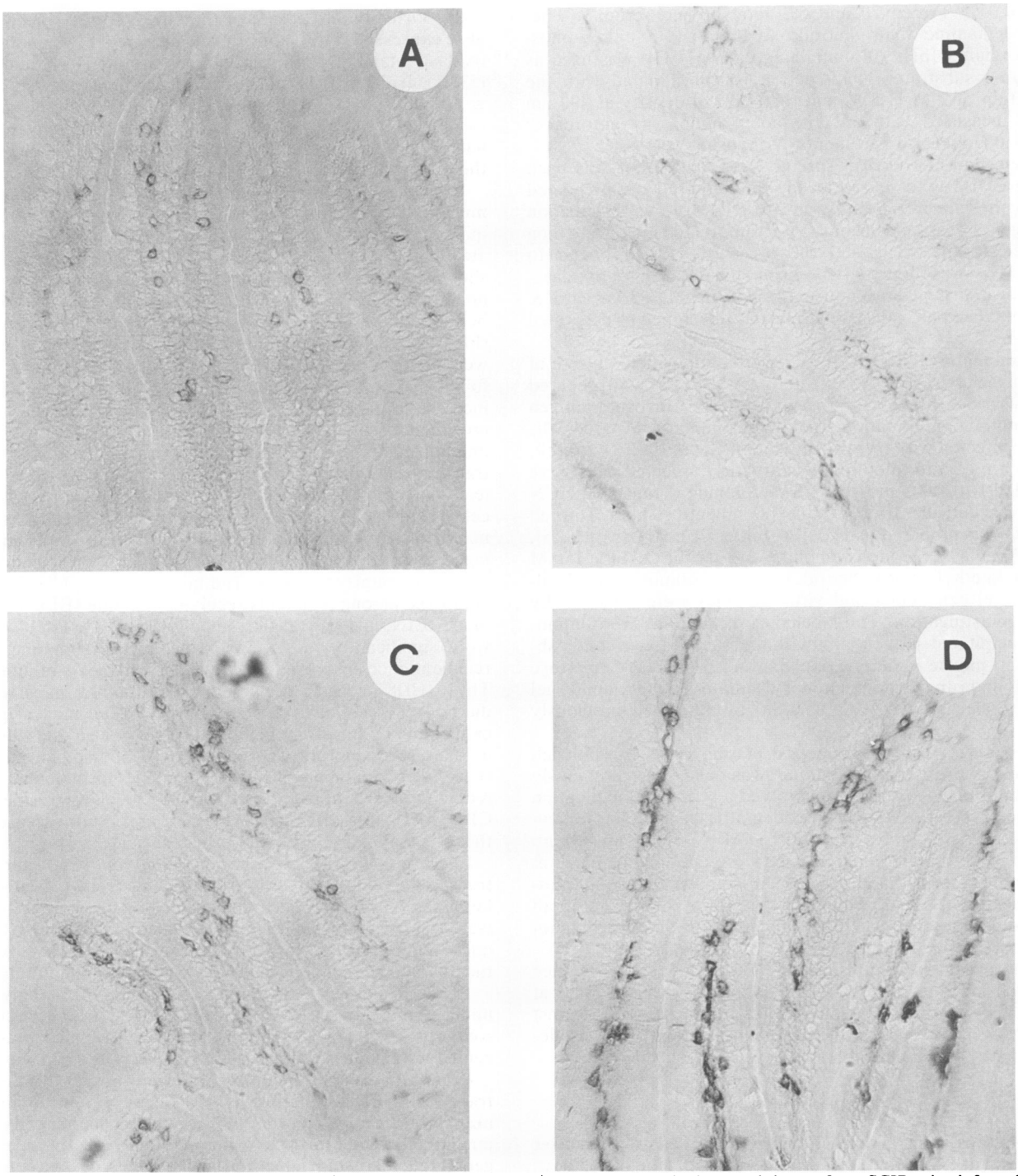

FIG. 1. Immunoperoxidase staining of $CD8^+$ cells (A and C) and $Thy1^+$ cells (B and D) in duodenal tissues from SCID mice infected with rotavirus. SCID mice were examined before (A and B) and 3 weeks after (C and D) adoptive transfer of immune $CD8^+$ spleen cells.

to zero, because they represent the average daily values for five mice. The specific day on which virus shedding stopped and the day on which it reoccurred varied (from day 8 to 22) among the mice.

Transient clearance of virus shedding was also observed after the transfer of immune $CD8^+$ IELs obtained from orally immunized donors ($n = 5$). The effect of immune IELs on virus shedding was not observed until 11 to 12 days after transfer (Fig. 3). In addition, recurrence was observed 3 to 8 days later in four of five SCID mice treated with immune IELs; however, one SCID mouse had no recurrence for at least 12 days. Transfer of nonimmune IELs ($n = 3$) did not have an effect on rotavirus shedding (data not shown).

Two subpopulations of T cells were present in the IEL preparation, $Thy1^+$ $CD8^+$ cells and $Thy1^-$ $CD8^+$ cells. To identify the type of T cells that are responsible for mediating viral clearance, the IELs were depleted of $Thy1^+$ cells prior to sorting and transfer. Virus shedding was not affected for

TABLE 1. Distribution of Thy1$^+$ and CD8$^+$ cells in the intestinal mucosa of the duodenum of recipient SCID mice following adoptive cell transfers

Immunization route (origin of donor cells)	No. of SCID mice[a]	Days post-transfer[b]	No. of cells/villus (mean ± SD)[c]	
			Thy1	CD8
i.p. primed (spleen)	5	18–20	9.6 ± 3.5***	12.3 ± 2.0***[***]
p.o. primed (spleen)	5	22–25	10.1 ± 2.5***[*]	12.6 ± 3.0**[**]
Unprimed (spleen)	5	21–46	6.4 ± 2.5***	7.4 ± 2.2
p.o. primed (IEL)	5	18–27	6.8 ± 3.4**(**)	8.5 ± 5.3
p.o. primed (Thy1$^-$ CD8$^+$ IEL)	4	23–46	1.7 ± 0.7	6.2 ± 1.6
Unprimed (IEL)	3	30–46	1.3 ± 0.4	7.7 ± 2.7
No transfer	5		1.4 ± 1.6	7.1 ± 2.3

[a] Number of SCID mice which received cell transfers.
[b] Intestinal tissues examined were collected from SCID mice at specified days after adoptive cell transfers.
[c] The number of Thy1$^+$ and CD8$^+$ cells per villus for each mouse (calculated from an average of 20 to 40 villi) was detected in cryostat sections of duodenal tissue by using an immunoperoxidase technique, as described in Materials and Methods. One-tailed t test statistical significance is indicated as follows. ** ($P < 0.01$) and *** ($P < 0.001$) are comparisons with no-transfer SCID mice. [*] ($P < 0.05$), [**] ($P < 0.01$), and [***] ($P < 0.001$) are comparisons with unprimed spleen cell recipients. (**) ($P < 0.01$) is a comparison with unprimed IEL transfer recipients.

the SCID mice ($n = 4$) that received immune Thy1$^-$ CD8$^+$ IELs (Fig. 3).

Detection of rotavirus-specific antibodies. Sera from SCID mice reconstituted with immune CD8$^+$ spleen cells or IELs were tested for antibody to rotavirus by ELISA, focus reduction neutralization assay, and radioimmunoprecipitation assay. Rotavirus-specific antibodies were not detected by the sandwich ELISA with EDIM-w as captured antigen. None of the SCID recipient sera were able to neutralize EDIM-w at a serum dilution of 1:50, whereas complete neutralization (100% focus reduction) was accomplished by the donor sera at the same dilution. In addition, these sera did not immunoprecipitate any ^{35}S-labeled RRV proteins, whereas sera from EDIM-w-immunized BALB/c donor mice were able to immunoprecipitate viral proteins (VP1, VP2, VP6, VP7, and NS35) from the same lysate (Fig. 4). Fecal materials and intestinal tissues were also tested for the presence of antibody to rotavirus by ELISA and immunohistochemistry, respectively. Rotavirus-specific IgA was not detected in feces, and rotavirus-specific plasma cells were not detected in the intestinal tissues.

MHC restriction. The C.B-17/scid and BALB/c mice used

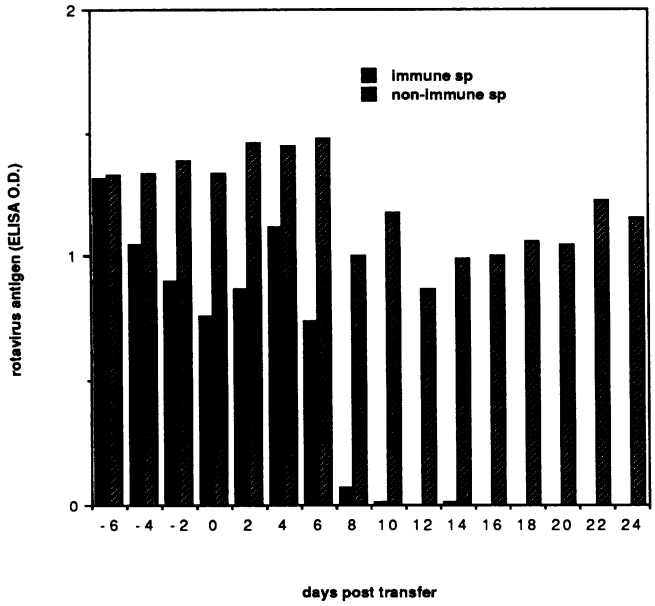

FIG. 2. Rotavirus shedding in stools of SCID mice before and after the transfer of immune and nonimmune CD8$^+$ spleen (sp) cells obtained from BALB/c mice immunized intraperitoneally with rotavirus. Rotavirus in stools was detected by ELISA, as described in Materials and Methods. Each point represents a mean optical density of the data collected from five mice. For the immune cell transfer group, the standard error of the mean for values on days −6, −2, 0, 2, and 4 ranged from 9 to 15%; on days 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 it ranged from 19 to 32%. For the nonimmune cell transfer group, the standard errors of the mean for all values were less than 10%.

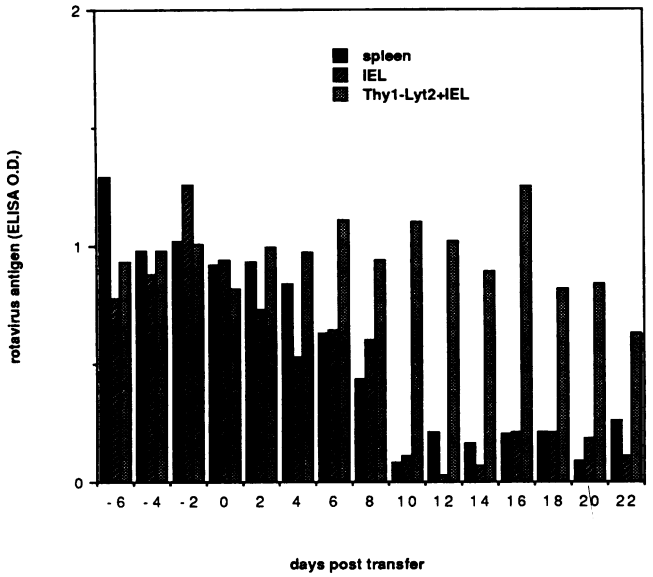

FIG. 3. Rotavirus shedding in stools from SCID mice before and after the transfers of CD8$^+$ spleen cells, CD8$^+$ IELs, and CD8$^+$ IELs depleted of Thy1$^+$ cells. Stool samples were from BALB/c mice 3 to 4 weeks after oral infection and 4 days after oral boosting with EDIM. Rotavirus was detected by ELISA. Each point represents a mean optical density of the data collected from four to five mice. For the spleen cell transfer group, the standard error of the mean of the values for days −6, −4, −2, 0, 2, 4, 6, 12, 14, 18, 20, and 22 ranged from 8 to 23%; on days 8 and 10, it ranged from 34 to 35%. For the IEL transfer group, the standard error of the mean of the values for days −6, −4, −2, 0, 2, 4, 6, 8, 10, 18, 20, and 22 ranged from 5 to 25%; on days 12, 14, and 16, it ranged from 33 to 36%. For the Thy1$^-$ IEL transfer group, the standard errors of the mean for all values ranged from 2 to 11%.

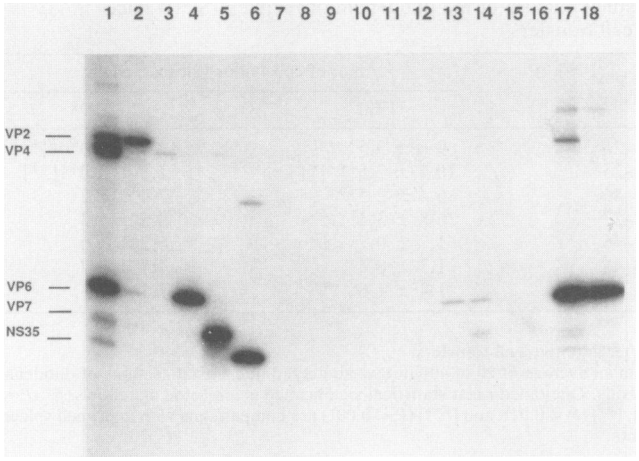

FIG. 4. Immunoprecipitation of metabolically ^{35}S-labeled RRV lysate by sera from i.p. and p.o. immunized BALB/c donors and recipient SCID mice after cell transfers. Lanes: 1, guinea pig serum hyperimmune to RRV; 2, monoclonal antibody (MAb) 3A8 against VP2; 3, MAb 1A9 against VP4; 4, MAb 4H10 against VP6; 5, MAb 60 against VP7; 6, MAb 191 against NS35; 7 and 8, sera from SCID mice which did not receive transferred cells; 9 and 10, sera from SCID mice which received spleen cells from orally immunized donors; 11 and 12, sera from SCID mice which received IELs from orally immunized donors; 13 and 14, sera from orally immunized BALB/c donor mice; 15 and 16, sera from SCID mice which received spleen cells from parenterally immunized donors; 17 and 18, sera from parenterally immunized BALB/c donor mice.

in the above experiments expressed the same *H-2* haplotype, *H-2*^{d}. To investigate whether the CD8$^+$ T-cell-mediated clearance of rotavirus in SCID mice is MHC restricted, different strains of mice, including C57Bl/6 (*H-2*^{b}), B10.D2 (*H-2*^{d}), BALB.B (*H-2*^{b}), and BALB.K (*H-2*^{k}), were immunized with EDIM-w and immune CD8$^+$ spleen cells were transferred into SCID mice. The results are summarized in Table 2. Immune spleen cells from B10.D2 and BALB/c mice expressing *H-2*^{d} were able to clear rotavirus infection in C.B17/scid mice, whereas immune spleen cells from BALB.K and BALB.B mice were not able to clear the infection. MHC restriction of IELs was partially studied. Immune IELs from BALB/c mice (*H-2*^{d}) were able to clear the virus infection (n = 5), but immune IELs from C57Bl/6 (*H-2*^{b}) were not able to clear rotavirus infection (n = 3). Unfortunately, attempts to breed other congenic strains in order to obtain newborn pups to orally immunize were not successful.

TABLE 2. MHC-restricted, T-cell-mediated clearance of rotavirus infection

Source of transferred spleen cells[a]	*H-2* haplotype	Virus clearance in recipient SCID mice (no. cleared/ no. transferred)
BALB/c	*d*	5/5
B10.D2	*d*	5/5
C57Bl/6	*b*	0/3
BALB.B	*b*	0/5
BALB.K	*k*	0/4

[a] Immune CD8$^+$ spleen cells (10^6) from different strains of mice were transferred into rotavirus-infected C.B-17/scid mice (*H-2*^{d}), and excretion of rotavirus in stool was monitored by ELISA for up to 4 weeks after cell transfer.

DISCUSSION

It has been difficult to define clearly the individual roles of humoral and cellular immunity in mediating both protection from and resolution of a variety of acute viral diseases of humans. This has been especially true for infections that take place primarily at the mucosal surface. In the case of rotavirus, the observation that several animal species, including mice, become maturationally resistant to disease and infection has made analysis of the immunologic determinants of viral irradication even more difficult. Although a number of studies have clearly implicated antibody as a potent mediator of protection from rotavirus illness, the immunologic determinants of rotavirus clearance have not been well characterized.

In order to evaluate the potential of cytotoxic T cells to eliminate rotavirus infection from the intestine in the absence of antibody, we have taken advantage of the SCID mouse model. This combined immunodeficient mouse becomes persistently infected with rotavirus and hence enabled us to characterize the selective effects of passively transferred CD8$^+$ T cells on this chronic infection.

As has been demonstrated previously by Reipenhoff-Talty et al., when SCID mice are infected with a murine rotavirus early in life, they become infected indefinitely (19). This infection is characterized by chronic shedding of virus in the stool but not by overt signs of gastrointestinal illness.

In this study, two cell populations were detected in SCID mice that did not receive transferred cells: Thy1$^-$ CD8$^+$ cells in the intraepithelial layer and a small number of Thy1$^+$ CD8$^-$ CD4$^-$ cells in the lamina propria. The first cell type probably represents the nonlymphoid cells found in IELs from athymic nude mice as well as in normal mice, as previously described (8, 17). The finding of Thy1$^+$ cells in SCID lamina propria is unexpected but appears to be specific since these cells did not stain with normal rat serum (see method). These cells might represent intestinal natural killer cells previously described in normal mice (22). SCID mice appear to have functional natural killer cells as well (4).

Following adoptive transfer of CD8$^+$ spleen cells and IELs, the migration of transferred Thy1$^+$ CD8$^+$ cells to the intestinal mucosa of SCID mice was monitored by using Thy1 and CD8 antigen as a marker. The presence of significant numbers of CD8$^+$ cells in the guts of untreated SCID mice created a background which might have curtailed our ability to accurately measure trafficking of the transferred cells with this marker. Significant increases in both Thy1$^+$ and CD8$^+$ cells were observed following the transfer of both immune and nonimmune spleen cells, although higher numbers of these cells were detected in the gut mucosa after immune-cell transfer (Table 1). The migration of transferred IELs appears to differ from that of spleen cells; a significant increase in Thy1$^+$ cells was detected only after immune (versus nonimmune) IEL transfer. The basis of these findings is unknown, the increase might be a result of preferential migration to the gut of immune IELs or of decreased exit of transferred cells from this region. Alternatively, transferred immune IELs might expand more efficiently in the gut mucosa.

In this study, adoptive transfer of i.p. primed, CD8$^+$-immune splenocytes into SCID mice chronically infected with murine rotavirus completely and reproducibly eliminated viral infection from the intestine. This effect was not mediated by nonimmune spleen cells and appeared to be MHC restricted. Although we did not directly prove that the BALB.B and BALB.K immunized mice developed a cyto-

toxic T-cell response following immunization, this seems highly likely given the previous results of Offit and Dudzik (14, 15) using C57Bl/10, *H-2*^{b} mice, as well as the results of our own studies using BALB/c *H-2*^{d} mice. It is highly unlikely that this effect was mediated by adoptive transfer of antibody-producing cells, since no rotavirus antibody could be detected by ELISA in the sera or feces of the SCID mice, no antibody-producing plasma cells could be identified in frozen sections of the intestinal mucosa of the SCID mice, and antibody-neutralizing activity was not detected in the sera of the SCID mice. Although we have not demonstrated directly that the Thy1$^+$ CD8$^+$ splenocytes mediated their effect by a cytotoxic mechanism, this seems most likely. Offit and Dudzik have demonstrated that MHC-restricted, Thy1$^+$ cells in both the spleen and IEL compartments have cytotoxic activity against rotavirus-infected cells and that these cytotoxic cells can be generated by either oral or systemic immunizations with rotavirus (14, 15). Alternatively, this effect could be mediated, at least in part, by local production of gamma interferon by CD8$^+$ cells.

Both IELs and spleen cells generated after oral immunization were also evaluated in adoptive transfer studies. Neither group was as effective as systemically primed, immune spleen cells in eliminating chronic infection. In both cases, viral excretion was transiently but not permanently suppressed. This effect may be due to the fact that we have not optimized the time of harvest of IELs or splenocytes following oral immunization. In addition, quantitative comparison of transferred rotavirus-specific effector cells after systemic and oral immunization was not determined in this study. It seems likely that fewer specific immune cells may be generated following oral infection. Since only Thy1$^+$ CD8$^+$ IELs appear to participate in viral eradication, it may simply be a relative difference in transferred cell numbers that accounts for the difference in effect seen after IEL transfer.

This study clearly demonstrates a potential biologic role of CD8$^+$-immune lymphocytes from both spleen and intestine in mediating rotavirus clearance from chronically infected animals. It would seem likely that such a role might also occur during the course of acute rotavirus infection in mice and humans. The antigenic targets of the immune T cells and the role of these cells in preventing and clearing acute infection remain to be determined.

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LITERATURE CITED

1. **Chiba, S., S. Nakata, T. Urasawa, S. Urasawa, T. Yokoyama, Y. Morita, K. Taniguchi, and T. Nakao.** 1986. Protective effect of naturally acquired homotypic and heterotypic rotavirus antibodies. Lancet **ii**:417–421.
2. **Davidson, G. P., and G. L. Barns.** 1979. Structural and functional abnormalities of the small intestine in infants and young children with rotavirus enteritis. Acta Paediatr. Scand. **68**:181–186.
3. **Dharakul, T., M. Riepenhoff-Talty, B. Albini, and P. L. Ogra.** 1988. Distribution of rotavirus antigen in intestinal lymphoid tissues: potential role in development of the mucosal immune response to rotavirus. Clin. Exp. Immunol. **74**:14–19.
4. **Dorshkind, K., S. B. Pollack, M. J. Bosma, and R. J. Phillips.** 1985. Natural killer (NK) cells are present in mice with severe combined immunodeficiency (scid). J. Immunol. **134**:3798–3801.
5. **Greenberg, H. B., J. Valdesuso, K. van Wyke, K. Midthun, M. Walsh, V. McAuliff, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47**:267–275.
6. **Greenberg, H. B., P. T. Vo, and R. Jones.** 1986. Cultivation and characterization of three strains of murine rotavirus. J. Virol. **57**:585–590.
7. **Grimwood, K., J. C. S. Lund, B. S. Coulson, I. L. Hudson, R. F. Bishop, and G. L. Barnes.** 1988. Comparison of serum and mucosal antibody responses following severe acute rotavirus gastroenteritis in young children. J. Clin. Microbiol. **26**:732–738.
8. **Klein, J. R.** 1986. Ontogeny of Thy1$^-$ Lyt2$^+$ murine intestinal intraepithelial lymphocyte. Characterization of a unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa. J. Exp. Med. **164**:309–314.
9. **Ledbetter, J. A., and L. A. Herzenberg.** 1979. Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev. **47**:63–90.
10. **Lefrancois, L.** 1984. Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: distinct mechanisms of action in vivo. J. Virol. **51**:208–214.
11. **Losonsky, G. A., J. P. Johnson, J. A. Winkelstein, and R. H. Yolken.** 1985. Oral administration of human serum immunoglobulin in immunodeficient patients with viral gastroenteritis. J. Clin. Invest. **76**:2362–2367.
12. **Matsui, S. M., P. A. Offit, P. T. Vo, E. R. Mackow, D. A. Benfield, R. D. Shaw, L. Padilla-Noriega, and H. B. Greenberg.** 1989. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and the VP8 fragment of VP4. J. Clin. Microbiol. **27**:780–782.
13. **Offit, P. A., and H. F. Clark.** 1985. Protection against rotavirus-induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. J. Virol. **54**:58–64.
14. **Offit, P. A., and K. I. Dudzik.** 1988. Rotavirus-specific cytotoxic T lymphocytes cross-react with target cells infected with different rotavirus serotypes. J. Virol. **62**:127–131.
15. **Offit, P. A., and K. I. Dudzik.** 1989. Rotavirus-specific cytotoxic T lymphocytes appear at the intestinal mucosal surface after rotavirus infection. J. Virol. **63**:3507–3512.
16. **Offit, P. A., R. D. Shaw, and H. B. Greenberg.** 1986. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to surface proteins vp3 and vp7. J. Virol. **58**:700–703.
17. **Parrott, D. M. V., C. Tait, S. MacKenzie, A. M. Mowat, M. D. J. Davies, and H. S. Micklem.** 1983. Analysis of the effector functions of different populations of mucosal lymphocytes. Ann. N.Y. Acad. Sci. **409**:307–320.
18. **Petit, A., P. B. Ernst, A. D. Befus, D. A. Clark, K. L. Rosenthal, T. Ishizaka, and J. Bienenstock.** 1985. Murine intestinal intraepithelial lymphocytes. I. Relationship of a novel Thy1$^-$, Lyt1$^-$, Lyt2$^+$, granulated subpopulation to natural killer cells and mast cells. Eur. J. Immunol. **15**:211–215.
19. **Riepenhoff-Talty, M., T. Dharakul, E. Kowalski, D. Sterman, and P. L. Ogra.** 1987. Persistent rotavirus infection in mice with severe combined immunodeficiency. J. Virol. **61**:3345–3348.
20. **Saulsbury, F. T., J. A. Winkelstein, and R. H. Yolken.** 1980. Chronic rotavirus infection in immunodeficiency. J. Pediatr. **97**:61–65.
21. **Shaw, R. D., D. L. Stoner-Ma, M. K. Estes, and H. B. Greenberg.** 1985. Specific enzyme-linked immunoassay for rotavirus serotypes 1 and 3. J. Clin. Microbiol. **22**:286–291.
22. **Tagliabue, A., A. D. Befus, D. A. Clark, and J. Bienenstock.** 1982. Characteristics of natural killer cells in the murine intes-

tinal epithelium and lamina propria. J. Exp. Med. **155**:1785–1796.

23. **Wolf, J. L., G. Cukor, N. R. Blacklow, R. Dambrauskas, and J. R. Trier.** 1981. Susceptibility of mice to rotavirus infection: effect of age and administration of corticosteroids. Infect. Immun. **33**:565–574.
24. **Wolsy, L.** 1983. Methods and applications of hapten-sandwich labeling. Methods Enzymol. **92**:472–488.
25. **Yolken, R. H., C. A. Bishop, T. R. Townsend, E. A. Bolyard, J. Bartlett, G. W. Santos, and R. Saral.** 1982. Infectious gastroenteritis in bone-marrow transplant recipients. N. Engl. J. Med. **306**:1009–1112.