Immunization with Baculovirus-Expressed Recombinant Rotavirus Proteins VP1, VP4, VP6, and VP7 Induces CD8⁺ T Lymphocytes That Mediate Clearance of Chronic Rotavirus Infection in SCID Mice

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Clearance of chronic murine rotavirus infection in SCID mice can be demonstrated by adoptive transfer of immune CD8⁺ T lymphocytes from histocompatible donor mice immunized with a murine homotypic rotavirus (T. Dharakul, L. Rott, and H. B. Greenberg, *J. Virol.* 64:4375–4382, 1990). The present study focuses on the protein specificity and heterotypic nature of cell-mediated clearance of chronic murine rotavirus infection in SCID mice. Heterotypic cell-mediated clearance was demonstrated in SCID mice infected with EDIM (murine) rotavirus after adoptive transfer of CD8⁺ T lymphocytes from BALB/c mice that were immunized with a variety of heterologous (nonmurine) rotaviruses including Wa (human, serotype 1), SA11 and RRV (simian, serotype 3), and NCDV and RF (bovine, serotype 6). This finding indicates the serotypic independence of T-cell-mediated rotavirus clearance. To further identify the rotavirus proteins that are capable of generating CD8⁺ T cells that mediate virus clearance, donor mice were immunized with SF-9 cells infected with a baculovirus recombinant expressing one of the following rotavirus proteins: VP1, VP2, NS53 (from RF), VP4, VP7, NS35 (from RRV), VP6, and NS28 (from SA11). SCID mice stopped shedding rotavirus after receiving CD8⁺ T cells from mice immunized with VP1, VP4, VP6, and VP7 but not with VP2, NS53, NS35, NS28, or wild-type baculovirus. These results suggest that heterotypic cell-mediated clearance of rotavirus in SCID mice is mediated by three of the major rotavirus structural proteins and by a putative polymerase protein.

Rotaviruses are the most important etiologic agent of severe gastroenteritis in infants and young animals. Identifying the determinants of protective immunity as well as the factors that lead to the resolution of rotavirus infection is crucial to the development of an effective rotavirus vaccine. Both virus-specific antibodies and cell-mediated immunity are generated following rotavirus infection (9, 26, 30). Although antibodies to most rotavirus proteins can be detected after a rotavirus infection (33), a protective effect has been reproducibly demonstrated only with antibodies to the two outer capsid proteins, VP4 and VP7 (15, 20, 21, 27). The principle regions on each surface protein where heterotypic and homotypic neutralization epitopes are located have been identified (12, 18, 19, 34), and the minimal peptides necessary to encode neutralizing epitopes on VP4 have been characterized (19).

The determinants of cell-mediated immunity to rotavirus are less well understood. The appearance of rotavirus-specific major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes (CTLs) and their location following rotavirus infection have been described by Offit et al. (26). A protective effect of virus-specific CTLs in newborn mice (25) and of CD8⁺ T-cell-mediated clearance of chronic rotavirus infection in SCID mice has recently been demonstrated (11). However, the protein specificities of

CD8⁺ T cells that confer such effects in vivo have not been identified.

We have previously described the use of an SCID mouse model to evaluate the role of CD8⁺ T lymphocytes in mediating clearance of chronic rotavirus infection (11). Immune CD8⁺ T lymphocytes from histocompatible mice immunized with the homologous murine rotavirus can mediate rotavirus clearance in the absence of virus-specific antibodies. Immune lymphocytes elicited after systemic immunization were somewhat more effective than lymphocytes induced after intestinal infection at clearing chronic rotavirus infection (11). In the present study, we extended our studies by using the SCID mouse model to investigate the serotypic specificity of the cell-mediated immune response which clears chronic murine rotavirus infection and to identify which rotavirus proteins are capable of inducing such a response.

MATERIALS AND METHODS

Immunogens and immunization of donor mice. Rotavirus-free, T-cell donor BALB/c mice (Charles River Breeding Laboratories, Portage, Mich.) were immunized intraperitoneally with 100 µg of the indicated purified double-shelled heterologous rotaviruses. Each of the following viruses was used for immunization: Wa (human, serotype 1), RRV (simian, serotype 3), SA11 (simian, serotype 3), NCDV (bovine, serotype 6), and RF (bovine, serotype 6). For immunization with recombinant proteins, baculovirus

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recombinant VP1, VP2, and NS53 proteins were derived from the RF strain of rotavirus, as described by Cohen et al. (10), Labbe et al. (16), and Brottier et al. (4), respectively. Recombinant VP4, VP7, and NS35 proteins were derived from the RRV strain of simian rotavirus. Construction and characterization of baculovirus recombinant VP4 and NS35 proteins have been described by Mackow et al. (17) and Bass et al. (1), respectively. Recombinant VP7 protein was constructed as above from RRV gene 7 segment (12a). Recombinant VP6 and NS28 proteins were derived from the SA11 strain of rotavirus and cloned into the baculovirus transfer vector pAc RP23 (28a). This vector possesses upstream sequences identical to vector pAc yM1 (22). Wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus was used as the control antigen. SF-9 cells were infected with individual recombinant baculovirus, and the cell pellet was harvested 3 days after infection and was sonicated. The total amount of appropriate recombinant protein in the cell pellet was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining with protein standards. T-cell donor mice were injected intraperitoneally with approximately 100 μ g of the indicated individual recombinant protein as a crude cell sonicate and then received three weekly booster injections beginning 3 weeks after the primary injection. Spleens were collected 4 days after the last injection to use for the transfer experiments.

Chronic rotavirus infection of SCID mice. T-cell-recipient SCID mice were infected at 3 days of age with the EDIM strain of murine rotavirus (31). The serotype classification of this strain remains unclear, but most investigators conclude that it is not a serotype 1, 2, 3, 4, 5, or 6 rotavirus (13, 37). Infected SCID mice were shown to shed murine rotavirus chronically for at least 6 weeks before the study.

Adoptive cell transfer. Splenic lymphocytes were isolated, and CD8⁺ T lymphocytes were purified by labeling with fluorescein isothiocyanate-anti-CD8 monoclonal antibody and sorting twice with a FACStar (Becton Dickinson) as previously described (11). Groups of four to six SCID mice each received 10⁶ CD8⁺ T cells from donor mice immunized with the indicated individual antigen or whole virus. The transferred cells were administered by intraperitoneal injection. Prior studies using this purification and transfer protocol demonstrated that 99.7% $\pm$ 0.1% of the purified spleen cells were CD8⁺ and virtually all expressed Thy1 antigen (11). In addition, prior studies demonstrated that clearance of chronic rotavirus infection by immune spleen cells prepared in this manner was MHC restricted (11).

Evaluation of viral clearance. Before and up to 3 weeks after cell transfer, shedding of rotavirus in stools of SCID mice was monitored by a captured antibody enzyme-linked immunosorbent assay (ELISA) as previously described (11). The sensitivity of the ELISA was standardized with purified double-shelled rhesus rotavirus. Optical density values greater than 1.0 reflect plaque titers greater than 1.2×10^7 PFU/ml. Optical density values less than 0.2 (negative) reflect plaque titers less than 1.4×10^4 PFU/ml.

Evaluation of humoral immune status in donor and recipient mice. Sera from donor mice immunized with recombinant protein were tested by radioimmunoprecipitation of a [³⁵S]methionine-labeled lysate of MA104 cells infected with the rotavirus strain from which the recombinant protein was derived, as previously described (11). Sera from mice immunized with recombinant VP7 were also studied by an ELISA in which microtiter plates were coated with purified double-shelled RRV or control antigen and antibody response was

FIG. 1. Heterotypic clearance of murine rotavirus (EDIM) infection in SCID mice after adoptive transfer of 10⁶ CD8⁺ T lymphocytes from mice immunized with nonmurine rotaviruses RRV (□), NCDV (◆), RF (■), SA11 (●), and Wa (◇). Four SCID mice received cell transfers in each group. All values after day 12 are negative. Standard error for each value is shown with bars. OD, optical density.

detected with an affinity-purified peroxidase conjugate of goat anti-mouse immunoglobulin G (Kirkegaard & Perry). All recipient SCID mouse sera were tested for the presence of antibody to rotavirus by radioimmunoprecipitation as above. The presence of rotavirus-specific immunoglobulin A antibody was also investigated in stools of recipient mice by ELISA as previously described (11).

RESULTS

Serotypic specificity of cell-mediated immunity in clearance of chronic rotavirus infection in SCID mice. Complete clearance of EDIM rotavirus as measured by ELISA was demonstrated in SCID mice that received T cells transferred from histocompatible BALB/c mice immunized with all the nonmurine rotavirus strains tested (Fig. 1). Virus clearance was not observed until 9 to 12 days posttransfer, compared with clearance 7 to 9 days posttransfer in mice receiving CD8⁺ T cells from donors immunized with the homologous, homotypic EDIM strain of murine rotavirus (11). Permanent clearance for up to 3 weeks was observed in all groups (Fig. 1). This finding indicates that clearance of rotavirus infection mediated by CD8⁺ T cells can be induced by heterologous (nonmurine) rotaviruses, although somewhat less quickly than the homologous virus. Clearance appears to be independent of serotype since serotype 1, 3, and 6 viruses were all capable of mediating clearance of the EDIM strain of murine rotavirus.

Protein specificity of cell-mediated immunity in clearance of chronic rotavirus infection in SCID mice. The finding that heterotypic clearance of rotavirus infection in SCID mice could be mediated by CD8⁺ T lymphocytes stimulated us to investigate the ability of individual recombinant rotavirus proteins to induce CD8⁺ T cells that could mediate the same effect. T-cell donor mice were immunized with SF-9 cell homogenates containing baculovirus-expressed VP1, VP2, VP6, VP4, VP7, NS53, NS35, and NS28, as described in the

FIG. 2. Clearance of murine rotavirus infection in SCID mice after adoptive transfer of 10^6 CD8⁺ T lymphocytes from mice immunized with baculovirus recombinant rotavirus proteins VP1 (□), VP6 (■), VP4 (●), and VP7 (◆) as described in the text. Four SCID mice received cell transfers in each group. Standard error for each value is shown with bars. OD, optical density.

Materials and Methods. After adoptive transfer of 10^6 CD8⁺ T lymphocytes, rotavirus shedding in stools of SCID mice was monitored by ELISA, as shown in Fig. 2 and 3.

Resolution of chronic rotavirus shedding occurred at 12 days after the transfer of VP1-, VP4-, VP6-, and VP7-immune CD8⁺ T cells (Fig. 2) but not after the transfer of VP2-, NS53-, NS35-, NS28-, or wild-type baculovirus-immune CD8⁺ T cells (Fig. 3). Of note, prolonged clearance was observed only after VP1-immune T-cell transfer. Rota-

FIG. 3. Rotavirus shedding in SCID mice after adoptive transfer of 10^6 CD8⁺ T lymphocytes from mice immunized with baculovirus recombinant rotavirus proteins VP2 (□), NS53 (◆), NS35 (■), NS28 (●), and wild-type *A. californica* nuclear polyhedrosis virus control (▲). Six, four, three, four, and two SCID mice received cell transfers in each group, respectively. Standard error for each value is shown with bars. OD, optical density.

FIG. 4. Radioimmunoprecipitation of metabolically labeled rotavirus-infected cell lysates with sera from T-cell donor mice. Lanes: 1, mice immunized with double-shelled RF virus, ³⁵S-lysate prepared from cells infected with RF strain; 2, mice immunized with recombinant VP1, ³⁵S-lysate prepared from cells infected with RF strain; 3, mice immunized with recombinant VP2, ³⁵S-lysate prepared from cells infected with RF strain; 4, mice immunized with recombinant NS53, ³⁵S-lysate prepared from cells infected with RF strain; 5, mice immunized with double-shelled RRV virus, ³⁵S-lysate prepared from cells infected with RRV strain; 6, mice immunized with recombinant VP4, ³⁵S-lysate prepared from cells infected with RRV strain; 7, mice immunized with recombinant VP7, ³⁵S-lysate prepared from cells infected with RRV strain; 8, mice immunized with recombinant NS35, ³⁵S-lysate prepared from cells infected with RRV strain; 9, mice immunized with double-shelled SA11 virus, ³⁵S-lysate prepared from cells infected with SA11 strain; 10, mice immunized with recombinant VP6, ³⁵S-lysate prepared from cells infected with SA11 strain; 11, mice immunized with recombinant NS28, ³⁵S-lysate prepared from cells infected with SA11 strain.

virus shedding reappeared in mice receiving VP4-, VP6-, and VP7-immune T cells, as evidenced by low-level rotavirus antigen shedding at later time points (Fig. 2). As demonstrated in our previous studies (11), antibodies to rotavirus were not detected in the stools or sera of any of the recipient SCID mice, indicating that the effect on rotavirus shedding was not being mediated by antibody (data not shown).

Because immunization with some recombinant rotavirus proteins failed to induce CD8⁺ T cells that mediated viral clearance, we sought to determine whether all donor mice demonstrated an immune response to the recombinant protein used for immunization. Sera from mice immunized with recombinant VP1, VP4, VP6, and NS35 precipitated single bands from [³⁵S]methionine-labeled viral lysates (Fig. 4, lanes 2, 6, 7, and 8 respectively). Mice immunized with NS28 (lane 11) precipitated the 28-kDa gene 10 product glycoprotein plus another band of approximately 56-kDa which probably represents NS28 dimers. Mice immunized with recombinant VP2 immunoprecipitated a 94-kDa band as well as several bands of slightly smaller size. The smaller bands presumably represent proteolytic breakdown products of VP2 as previously described (5). Mice immunized with recombinant VP7 did not produce antibody that could immunoprecipitate VP7 or other rotavirus proteins (Fig. 4, lane 7); however, an immune response to purified double-shelled RRV rotavirus was detected by ELISA (data not shown). The mice immunized with recombinant NS53 and wild-type baculovirus had no detectable immune response to rotavirus

(Fig. 4, lane 4, and data not shown). This finding demonstrates that the donor mice were exposed to only the immunizing protein and were not contaminated with intercurrent rotavirus infection during the study.

DISCUSSION

In the present study, we demonstrated that clearance of chronic rotavirus infection in SCID mice can be mediated by heterotypic cell-mediated immunity. Chronic EDIM rotavirus infection was eliminated by CD8⁺ spleen cells obtained from mice immunized with serotype 1, 3, or 6 rotavirus strains. Elimination was somewhat delayed compared with cells from mice immunized with EDIM, but it was complete and long lasting as measured by ELISA. This demonstration of heterotypic immunity may partially explain the reduced severity of rotavirus illness in humans observed after prior natural exposure to heterotypic rotavirus infection or to heterotypic rotavirus vaccination (7, 8, 36). This finding also confirms and extends the previous report of an in vitro heterotypic CTL response to rotavirus (26).

Clearance of chronic rotavirus infection in SCID mice can also be mediated by CD8⁺ T cells after immunization with selected individual recombinant rotavirus proteins. Our findings indicated that either major or minor viral structural proteins can mediate clearance and that VP1 (a putative polymerase protein) is the most effective protein in this system. The three nonstructural proteins we tested did not induce CD8⁺ T cells that cleared infection, although both NS35 and NS28 can readily induce specific antibody responses in the immunized animals. Our inability to demonstrate an immune response to NS53 is most likely due to the low antigenicity of this protein (9a). The possibility that other rotavirus proteins including VP3, NS34, NS26, and NS20 (not available at the time of this study) could mediate viral clearance after immunization cannot be ruled out. In this study, we demonstrated that CD8⁺ T lymphocytes generated in BALB/c mice by immunization with rotavirus proteins VP1, VP6, VP4, and VP7 can eliminate a heterologous rotavirus infection. Of note, proteins of similar function (polymerase, major capsid protein, and hemagglutinin) have been shown to be the target of CTLs in the influenza virus system (29). The applicability of these findings to studies in humans or other strains of mice remains unclear, since studies with influenza virus have demonstrated that the selection of specific peptides for CTL targeting is dependent on the specific MHC class I molecules being studied (29).

Rotavirus shedding recurred at a low level after transfer of CD8⁺ T cells directed at VP4, VP6, and VP7. A similar finding was observed after transfer of immune intraepithelial lymphocytes or spleen cells obtained from mice after an oral inoculation with EDIM (11). The reason for the reappearance of viral shedding is unclear and requires further study. It may simply reflect quantitative differences in the number of actual immune cells being transferred in each instance. On the other hand, it is possible that the reappearance of viral shedding reflects the selection of a variant viral population not reactive with the transferred cells. Recently, CTL-selected viral variants in the lymphocytic choriomeningitis virus system have been described (28).

The ability of baculovirus recombinant proteins to generate a CD8⁺ T-cell response in vivo has not, to our knowledge, been previously described. Exogenously administered antigens are generally not capable of stimulating MHC class I-restricted CD8⁺ CTLs by the classical mechanism that requires processing of endogenous antigens in a distinct

cytoplasmic compartment before being displayed in association with MHC class I molecule on the cell surface (3, 35). However, in vivo induction of class I-restricted CTLs by exogenous antigen has been previously described for the influenza hemagglutinin expressed in *Escherichia coli* (38) and for ovalbumin (6). These findings support our observation of in vivo priming of rotavirus protein-specific CD8⁺ T cells by baculovirus recombinant proteins. The mechanism of this phenomenon has been studied by Rock et al. (32), who demonstrated that specialized antigen-presenting cells in the spleen can present exogenous antigen in association with class I MHC molecules to class I-restricted CTLs. Alternatively, induction of class II-restricted CD8⁺ CTLs by the baculovirus recombinant proteins and lysis of rotavirus-infected enterocytes by an MHC class II-dependent pathway (2) may have occurred. Induction of such T cells and their role in viral clearance in vivo have been demonstrated in influenza system (14, 23).

This study confirms and extends our prior investigations and demonstrates that passively transferred, highly purified CD8⁺ cells can eliminate or reduce chronic rotavirus infection in SCID mice. This effect is heterotypic and can be induced by a variety of recombinant proteins and occurs in the absence of systemic or local antibody to rotavirus. The mechanism by which the CD8⁺ cells accomplish this effect in vivo has not yet been studied. CD8⁺ cells frequently possess cytotoxic activity in vitro. However, we did not directly determine whether this was true for the cells transferred in our study. The presence of in vitro cytotoxic activity in our transferred cells would not prove that this activity was responsible for the in vivo effects we observed. Of note, CD8⁺ cells are also capable of mediating the release of a variety of lymphokines, including gamma interferon (24). The in vivo effects we observed could potentially be caused by either the direct antiviral activity of gamma interferon or its effects on macrophage activation.

Irrespective of the mechanism by which the immune response was generated, we demonstrated that systemic immunization with heterotypic rotaviruses or individual recombinant proteins can stimulate CD8⁺ T cells that modulate rotavirus infection in the gut. It will be interesting to determine whether similar phenomenon might occur in normal mice or, in fact, in humans. This information and our recent finding that rotavirus-specific immunoglobulin A-producing B cells, generated by systemic immunization, are capable of clearing chronic rotavirus infection in SCID mice (10a) suggest that systemic immunization with either whole virion or recombinant proteins is potentially useful for attenuating rotavirus infection and offers an alternative approach for rotavirus vaccination.

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