Differential Requirements for T Cells in Viruslike Particle- and Rotavirus-Induced Protective Immunity[▽]

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Correlates of protection from rotavirus infection are controversial. We compared the roles of B and T lymphocytes in protective immunity induced either by intranasally administered nonreplicating viruslike particles or inactivated virus or by orally administered murine rotavirus. We found that protection induced by nonreplicating vaccines requires CD4⁺ T cells and CD40/CD40L. In contrast, T cells were not required for short-term protective immunity induced by infection, but both T-cell-dependent and -independent mechanisms contributed to long-term maintenance of protection. Our findings indicate that more than one marker of protective immunity exists and that these markers depend on the vaccine that is administered.

Rotavirus causes dehydrating diarrhea in children, resulting in over 600,000 deaths worldwide each year (21). Correlates of protection from rotavirus infection are controversial. Immunoglobulin A (IgA) levels moderately correlate with protection from severe disease in humans (14, 17, 24), but studies with the extensively used murine model of rotavirus infection indicate that IgA is dispensable for protection (20, 23). Both replicating homologous and heterologous rotaviruses and nonreplicating protein and subunit vaccines induce protection in mice against a rotavirus challenge (7). Complete protection against reinfection is induced by replicating murine rotaviruses in the absence of T cells (10), while protection induced by vaccination with the capsid protein VP6 depends solely on the presence of CD4⁺ T cells (16). These data suggest that although antigenically similar, replicating virus and nonreplicating protein vaccines trigger different pathways of protective immunity against rotavirus infection with differential requirements for T cells. Here, we examine the contributions of B and T lymphocytes to protective immunity induced by the intranasal administration of nonreplicating viruslike particles (VLPs) or inactivated rotavirus and the oral administration of replication-competent wild-type murine rotavirus. Understanding and comparing the requirements for the induction of protective immunity against rotavirus infection will provide critical information to ascertain the correlates of protection from rotavirus infection.

Protection induced by a live viral infection, not VLPs, is maintained long-term. CD-1 mice (Charles River, Wilmington, MA) were vaccinated intranasally on days 0 and 14 with 10 μ g of VLPs plus 5 μ g of mutant *Escherichia coli* heat-labile toxin R192G (LT-R192G); they were orally challenged after 6 weeks with the wild-type murine strain of rotavirus (EC_{wt}), and the percentage of protection was calculated (1, 6, 8). High

levels of protection (~60 to 100%) are achieved 6 weeks after the administration of rotavirus VLPs composed of proteins VP2 and VP6 (2/6-VLPs) but are significantly lower than the levels of protection induced by EC_{wt} infection (18, 19). Orally administered EC_{wt} induces complete protection from infection (100%) at 6 weeks that is maintained at 6 months (11). To determine if VLP-induced protection persists beyond 6 weeks, mice were vaccinated with VLPs and challenged with EC_{wt} 6 months later. As expected, the mice vaccinated with VLPs exhibited a significantly lower level of protection than the mice that received a primary EC_{wt} infection (Fig. 1). Unlike what has been observed with a soluble recombinant VP6 protein vaccine (16), the level of protection induced by VLPs was not maintained over time, as it was significantly lower after 6 months than it was after 6 weeks (Fig. 1). This could be attributed to inherent differences between the soluble recombinant protein vaccine and the subunit particulate vaccine or to the differences in the strains of murine rotavirus used as a challenge. Unlike VLP-induced protection, the high level of protection induced by EC_{wt} infection was maintained over time (Fig. 1). Therefore, VLP-mediated protection results from the induction of pathways different from those induced by a live viral infection.

B cells contribute to, but are not essential for, protection from rotavirus infection. To assess the contribution of B cells to rotavirus protective immunity, JhD^{-/-} mice [C;129(B6)-IgH-J^{tm1Dhu} N?+N2] intercrossed to homozygosity on a BALB/c background (Taconic, Germantown, NY) or BALB/c mice were vaccinated or administered EC_{wt}. Mice were also vaccinated intranasally with 10 μ g of inactivated rhesus rotavirus (4) plus adjuvant to address whether replication was an important component in the induction of protective immunity. Vaccinated JhD^{-/-} mice exhibited significantly lower levels of protection than vaccinated BALB/c mice (Fig. 2A). As reported previously for C57BL/6 mice expressing the JhD mutation (9, 15), protection established in BALB/c mice expressing the JhD^{-/-} mutation by EC_{wt} infection was less than that established in BALB/c mice (Fig. 2B). However, protection induced by EC_{wt} infection in JhD^{-/-} mice was significantly higher than that induced in VLP-treated or inactivated-virus-

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[▽] Published ahead of print on 9 January 2008.

FIG. 1. VLP-induced immunity against rotavirus infection is not maintained long-term. CD-1 mice were vaccinated intranasally with 2/6-VLPs (VLP) on days 0 and 14 or administered 10^5 50% infective doses (ID_{50}) of EC_{wt} (RV) on day 0. Mice were challenged on day 42 (short-term [ST]) or at 6 months (long-term [LT]) with 10^5 ID_{50} of EC_{wt} , and the percentages of protection were assessed. The mean values for each group are indicated by the bars and the percentages (five mice per group). *, $P < 0.05$, compared to the results for the ST mice vaccinated with VLPs as determined by the Mann-Whitney U test; #, $P < 0.05$, compared to the results for the respective RV group as determined by the Mann-Whitney U test.

treated JhD^{-/-} mice (Fig. 2B). In agreement with previous findings (12), protection induced by EC_{wt} was not maintained over time, as JhD^{-/-} mice challenged at 6 months exhibited significantly lower levels of protection than mice challenged at 6 weeks (Fig. 2B). These findings indicate that B cells contribute to the induction of complete protection from rotavirus infection in BALB/c mice but that substantial protection (>60%) persists after 6 months in the absence of antibody.

T cells are essential for protection induced by vaccines and long-term maintenance of protection. Although T cells are not required for protection induced by rotavirus infection (10), T cells are essential for protection induced by a soluble VP6 vaccine (5, 16). To further characterize the contribution of T cells to protection from rotavirus infection, $\beta\delta$ T-cell-receptor knockout (TCRKO) mice (B6.129P2-Tcrb^{tm1Mom} Tcrd^{tm1Mom}/J; Jackson Laboratory, Bar Harbor, ME) or the Jackson Laboratory-recommended control C57BL/6 mice were vaccinated with VLPs or inactivated virus or were orally administered EC_{wt} . Vaccinated TCRKO mice failed to develop detectable fecal or serum antibody, while infected TCRKO mice had detectable but significantly lower titers of both fecal and serum rotavirus-specific antibody than the C57BL/6 mice (data not shown). Vaccinated TCRKO mice exhibited limited protection from EC_{wt} 6 weeks after vaccination compared to that exhibited by the C57BL/6 mice (Fig. 3A). Virus-infected TCRKO mice were highly protected against a challenge at 6 weeks, but protection waned significantly by 6 months (Fig. 3B). Antibody titers were similar at 6 weeks and 6 months, indicating that the difference in protection was not due to waning antibody

FIG. 2. B cells contribute to the protective immunity induced by a live viral infection or by nonreplicating vaccines. (A) Wild-type BALB/c and B-cell-deficient JhD mice were vaccinated intranasally with 2/6-VLPs (VLP) or inactivated rotavirus (InV) on days 0 and 14 and challenged with 10^6 50% infective doses (ID_{50}) of EC_{wt} on day 42. Daily stool samples were analyzed by enzyme-linked immunosorbent assay for rotavirus antigen. The results are displayed as percentages of protection for the individual animals, and the mean values for each group are indicated by the bars and percentages (five mice per group). *, $P < 0.05$, compared to the results for the identically treated BALB/c mice as determined by the Mann-Whitney U test. (B) BALB/c and JhD mice were administered 10^6 ID_{50} of EC_{wt} on day 0 and challenged with 10^6 ID_{50} of EC_{wt} either on day 42 (short-term [ST]) or at 6 months (long-term [LT]). *, $P < 0.05$, compared to the results for the ST JhD mice as determined by the Mann-Whitney U test; #, $P < 0.05$, compared to the results shown in panel A for the VLP- or InV-vaccinated JhD^{-/-} mice as determined by the Mann-Whitney U test.

levels (data not shown). These results suggest that T cells are essential for protection induced by nonreplicating vaccines and contribute to the maintenance of long-term protection through a non-antibody-mediated process induced by rotavirus infection in C57BL/6 mice.

CD4⁺ T cells and the expression of CD40/CD40L are essential for protection induced by nonreplicating vaccines. We investigated whether protection could be attributed to ei-

FIG. 3. T cells are essential for protection induced by nonreplicating vaccines but only contribute to protection induced by a live viral infection. (A) Wild-type C57BL/6 or TCRKO mice were vaccinated intranasally with 2/6-VLPs (VLP) or inactivated rotavirus (InV) on days 0 and 14 and challenged on day 42 with 10^3 50% infective doses (ID_{50}) of EC_{wt} . Daily stool samples were analyzed by enzyme-linked immunosorbent assay for rotavirus antigen. The results are displayed as percentages of protection for the individual animals, and the mean values for each group are indicated by the bars and percentages (five mice per group). *, $P < 0.05$, compared to the results for the identically treated C57BL/6 mice as determined by the Mann-Whitney U test. (B) C57BL/6 and TCRKO mice were administered 10^3 ID_{50} of EC_{wt} on day 0 and challenged with 10^3 ID_{50} of EC_{wt} either on day 42 (short-term [ST]) or at 6 months (long-term [LT]). *, $P < 0.05$, compared to the results for the ST TCRKO mice as determined by the Mann-Whitney U test.

ther the CD4 or CD8 T-cell subsets. $CD4^{-/-}$ (B6.129S2- $CD4^{tm1Mak/J}$) and $\beta_2m^{-/-}$ (B6.129P2-B2 $m^{tm1Unc/J}$) mice (both on a congenic C57BL/6 background; Jackson Laboratory) were vaccinated with 2/6-VLPs or orally administered EC_{wt} . The $CD4^{-/-}$ and $\beta_2m^{-/-}$ mice infected with EC_{wt} were completely protected from a rotavirus challenge at 6 weeks (Fig. 4). The vaccinated $\beta_2m^{-/-}$ mice exhibited high levels of

FIG. 4. T-cell-mediated protection induced by nonreplicating vaccines requires CD4 but not CD8 T cells. $\beta_2m^{-/-}$, $CD4^{-/-}$, $CD40^{-/-}$, and $CD40L^{-/-}$ mice were vaccinated with 2/6-VLPs (VLP) on days 0 and 14 or administered 10^3 50% infective doses (ID_{50}) of EC_{wt} (RV). The mice were challenged on day 42 with 10^3 ID_{50} of EC_{wt} , and daily stool samples were analyzed by enzyme-linked immunosorbent assay for rotavirus antigen. The results are displayed as percentages of protection for the individual mice, and the bars and percentages indicate the means for each group (five mice per group). *, $P < 0.05$, compared to the results for the C57BL/6 mice shown in Fig. 3 as determined by the Mann-Whitney U test; #, $P < 0.05$, compared to the results for the respective strain administered EC_{wt} as determined by the Mann-Whitney U test.

protection (~89%); however, these levels of protection were significantly lower than those induced by EC_{wt} infection (Fig. 4). The vaccinated $CD4^{-/-}$ mice had significantly lower levels of protection and geometric mean titers of fecal antibody than the vaccinated wild-type $\beta_2M^{-/-}$ mice and the EC_{wt} -infected $CD4^{-/-}$ mice (Fig. 4 and data not shown). Therefore, protection induced by 2/6-VLPs appears to be modulated by $CD4^+$ T cells, with a small contribution from $CD8^+$ T cells.

T cells mediate specific antibody production through CD40L signaling (13). To determine if CD40/CD40L expression is critical to VLP- or infection-induced protective immunity, $CD40L^{-/-}$ (B6.129S2- $CD40lg^{tm1Imx}$) and $CD40^{-/-}$ (B6.129P2- $CD40^{tm1Kik/J}$) mice (both congenic on a C57BL/6 background; Jackson Laboratory) were vaccinated with 2/6-VLPs or administered EC_{wt} . The vaccinated $CD40^{-/-}$ or $CD40L^{-/-}$ mice failed to develop fecal or serum antibody (data not shown) and had much lower levels of protection against rotavirus challenge at 6 weeks (Fig. 4) than the C57BL/6 mice (Fig. 3B) and the $CD40^{-/-}$ and $CD40L^{-/-}$ mice receiving the EC_{wt} infection (Fig. 4). In contrast, all infected mice developed serum and fecal antibody titers similar to those of TCRKO and $CD4^{-/-}$ mice, which were significantly reduced compared to those of the wild-type mice (data not shown), and were completely protected after 6 weeks (Fig. 3B and 4). These data indicate that CD40/CD40L signaling is an important component of nonreplicating-vaccine-induced protective immunity to rotavirus infection. Although CD40/

CD40L plays an essential role in antibody production, the interaction also mediates antigen-presenting cell activation and influences CD4 or CD8 cellular immunity (22). More studies are necessary to determine whether one or both of the CD40/CD40L signaling functions are critical to VLP-induced immunity.

The contribution of T cells to rotavirus protective immunity. Clearly, subunit vaccines induce protection that requires CD4⁺ T cells. Neither the VLPs or inactivated virus examined above nor a soluble VP6 vaccine (16) induce protection in the absence of CD4⁺ T cells. In contrast, CD4⁺ T cells contribute little to short-term protective immunity induced by replicating virus, which supports previous work indicating that rotavirus infection induces a strong T-cell-independent response (2, 10). However, after examination of the maintenance of protection in the absence of T cells, we find that T cells support and enhance long-term immunity against rotavirus infection. Interestingly, although the lack of T cells causes a decrease in protective immunity, substantial protective immunity is still present after 6 months, indicating that T-cell-independent immunity persists longer than anticipated. Therefore, rotavirus immunity is induced through both T-cell-dependent and -independent pathways. One caveat to examining the effect that lymphocyte subsets have on the induction of rotavirus protective immunity is the influence of the genetic background on the mutation phenotype. The ability of SCID mice to clear rotavirus infection differs based on whether the defect is present in BALB/c or C57BL/6 mice (10). Our own work has indicated significant differences between the two strains in susceptibility to rotavirus infection, as well as in protection, induced by VLPs (3). More studies are necessary to tease out the specific features that are unique to the inbred mouse strains that influence rotavirus immunity.

We thank John Clements for providing the LT-R192G; David Keeland, Ann Robertson, Natalie Dang, Jillian Pennington, Erin Sargent, and John Dawson for technical assistance; and the Texas Gulf Coast Digestive Disease Center for helpful advice.

This work was supported by NIH AI10604 (S.E.B.), NIH AI24998 (K.L.W., M.K.E., and M.E.C.), and NIH DK56338 (M.K.E.) and by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs (S.E.B. and M.E.C.).

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