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Differing T-Cell Requirements for Recombinant Retrovirus Vaccines

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Friend murine leukemia virus is a retrovirus complex that induces rapid erythroleukemia and immunosuppression in susceptible strains of adult mice. Using this model, we directly examined the T-cell subsets required for a protective retrovirus vaccine. Paradoxically, recovery in mice immunized with a chimeric envelope containing only T-helper (T_H) and B-cell epitopes was dependent on $CD8^+$ T cells as well as $CD4^+$ T cells despite the fact that the vaccine contained no $CD8^+$ cytolytic T-lymphocyte (CTL) epitopes. However, the requirement for $CD8^+$ T cells was overcome by inclusion of additional T_H and B-cell epitopes in the immunizing protein. These additional epitopes primed for more rapid production of virus-neutralizing antibody which appeared to limit virus spread sufficiently to protect even in the absence of $CD8^+$ T cells. Inclusion of an immunodominant CTL epitope in the vaccine was not sufficient to overcome dependence on $CD4^+$ T cells. These data suggest that T_H priming is more critical for retrovirus immunity than CTL priming.

A basic understanding of the immunological requirements for induced retrovirus immunity is fundamental to the rational design of a human immunodeficiency virus (HIV) vaccine, yet little is known about these requirements for any retrovirus vaccine. We have used Friend virus (FV) in mice as a model to analyze characteristics of both the vaccine and the host immune responses which are necessary for protection from retroviral disease. FV is an immunosuppressive retrovirus complex that induces rapid erythroleukemia in susceptible strains of mice (5, 13, 33). In previous experiments, we demonstrated that mice could be protected from FV-induced erythroleukemia by immunization with vaccinia virus recombinants expressing the gag (20) or envelope proteins of the Friend helper virus (F-MuLV) (11). The present study focuses on the F-MuLV envelope protein which was previously demonstrated to contain at least two B-cell (4, 28) and T-helper (T_H) epitopes (12), as well as an immunodominant cytolytic T-lymphocyte (CTL) epitope (29, 30). In mice immunized with the envelope vaccine, recovery from challenge with FV was shown to be associated with the appearance of immunoglobulin G (IgG)-neutralizing antibodies and virus-specific T-cell responses (11, 21). Such correlations suggest that a successful vaccine might be required to stimulate both $CD4^+$ and $CD8^+$ T-cell subsets as well as B cells. We sought to directly test this hypothesis by studying the effects of removing certain immunological epitopes from the vaccine. The data demonstrate that a CTL epitope is not required for effective immunization, nor are the N-terminal B and T_H epitopes of the envelope protein required. In addition, we also examined the $CD4^+$ and $CD8^+$ T-cell requirements for recovery by studying vaccinated mice that were challenged with FV following antibody-mediated T-cell depletions. Interestingly, we found that the T-cell subset requirements for in vivo protection were highly dependent on the potency of the vaccine.

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MATERIALS AND METHODS

Mice. (B10.A × A.BY)F_1 mice were bred at the animal care facilities at Rocky Mountain Laboratories from mice obtained from the Jackson Laboratory. The mice used in the experiments were age-matched females. All animal experiments were done according to the guidelines of the Rocky Mountain Laboratories Animal Care and Use Committee.

Virus. The B-tropic, polycythemia strain of FV was produced as previously described (9) and used in all experiments. FV infectivity titers were determined by the spleen focus-forming assay for (B10.A × A.BY)F_1 mice (9).

Envelope genes and recombinant vaccinia virus vectors. The molecular clone of FMCF envelope from FMCF54B (23) and the vaccinia virus recombinant vvFMCF were generously provided by R. V. Srinivas of St. Jude Children's Research Hospital, Memphis, Tenn. F-MuLV *env* sequences were obtained from a molecular clone of F-MuLV 57 (24, 25) in pBR322 (clone 74-1). To construct Ch1, the *Bam*HI fragment of FMCF54B was subcloned into pSP72 (Promega). The *Avr*II-to-*Cla*I fragment was then replaced with the *Avr*II-to-*Cla*I fragment from F-MuLV 57. Ch1 was subcloned into the unique *Bgl*II site of pSC11SB, a modified version of pSC11 (20). Correct cloning and orientation in the vector were determined by restriction digest analyses. Vaccinia virus recombinants were produced, screened, and purified as described previously (11, 20).

Vaccination, virus challenge, and recovery experiments. Vaccinations were done by tail scratching with 10^7 PFU of vaccinia virus in 1.0 μl of phosphate-buffered saline as previously described (11, 20). Four weeks postvaccination, the mice were infected by intravenous inoculation of 1,000 spleen focus-forming units of virus in 0.5 ml of phosphate-buffered balanced salt solution (PBBS) with 2% fetal bovine serum (6). The development and progression of leukemia were monitored by weekly serial palpations for splenomegaly. Mice with obvious splenomegaly had spleens that were greater than or equal to 0.5 g and were considered leukemic. Spleen sizes as determined by palpation versus actual spleen weights were periodically cross-checked. Previous experiments have shown that serial palpations of individual mice over the course of the recovery period provide an accurate predictor of recovery as determined by more than 1 year of survival (3, 9).

Viremia and neutralizing antibody assays. Plasmas from immunized mice were assayed for FV-specific IgG by neutralization assays (20) and for viremia by plaque-forming assays (8) as described elsewhere.

CTL assays. CTL assays were performed essentially as previously described (29), except that the cells were collected in PBBS–2% fetal bovine serum (6) before transfer to RPMI, and the assay mixtures were incubated at 34°C for 16 h. EL4-Ch1 targets were produced in the same manner as the EL4-Fr57 and EL4-HPrP targets (29). Briefly, EL4 cells were transduced with the Ch1 gene in the pSFF retrovirus expression vector. Envelope-positive cells were sorted by flow cytometry for high-level expression, cloned, and reanalyzed following expansion. EL4-Ch1 targets had levels of envelope expression equivalent to those of EL4-Fr57 targets, as determined by flow cytometry with biotin-labeled monoclonal antibody 720 (28) (data not shown). Background levels were determined by using effector cells from uninfected mice.

Epitopes and restriction elements. The CTL epitope, which is restricted by H-$2D^b$, was identified (30) as amino acids 318 to 334 of F-MuLV *env* (AGTG DRLLNLVQGAYQA). The N-terminal T_H1 epitope, amino acid residues 125 to 136, is restricted by H-$2A^b$ and has a core sequence of LTSLTPRCNTAWN (12, 32). The T_H2 epitope (amino acids 462 to 479) is restricted by H-$2E^{b/d}$ and has

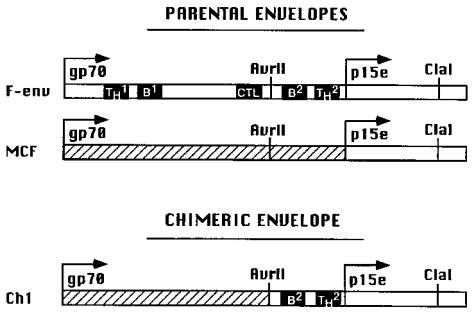

FIG. 1. Schematic diagram of the envelope genes expressed by the vaccinia virus recombinants used in protection experiments. Fr57 (F-env) is the F-MuLV envelope gene from molecular clone 57 (25). Black boxes designate the locations of known immunological epitopes specific for F-MuLV. MCF is the Friend MCF recombinant envelope gene from molecular clone 54-B (23). Ch1 is a molecular recombinant containing the *Avr*II-to-*Cla*I fragment from Fr57 in the FMCF background.

the sequence SPSYVYHQFERRAKYK (12). The B-cell epitopes are known only by the general locations shown in Fig. 1.

RESULTS

Protection with a chimeric envelope gene. We wished to understand the epitope requirements for a protective FV vaccine as well as the immunological responses involved in protection. To this end, we constructed a chimeric envelope vaccine which contained only a subset of the known antigenic epitopes from the F-MuLV envelope. Mice that had been vaccinated with the chimeric envelope and challenged with FV were then analyzed for protection and immune responsiveness. The chimeric envelope was constructed by inserting a gene fragment from the protective F-MuLV envelope (Fr57) into a nonprotective Friend mink cell focus-inducing virus (FMCF) envelope background. The resulting chimeric envelope (Ch1) lost some previously described T_H (12), B-cell (4), and CTL epitopes (30) contained in the full-length F-MuLV envelope but retained the gp70 C-terminal T_H (12) and B-cell epitopes (28) (Fig. 1). A vaccine was produced by introducing the Ch1 gene into vaccinia virus by homologous recombination.

To test for protection, mice were immunized by tail scratching with the vaccinia virus recombinant expressing the chimeric envelope protein (vvCh1). Vaccinia virus recombinants expressing the parental envelopes, vvFr57 and vvFMCF, were used as positive and negative controls, respectively. At 1 month postvaccination, the mice were challenged with a high dose of FV and were monitored for induction and progression of leukemia. By 6 weeks postchallenge, more than 90% of the mice that received the vvFMCF vaccine were leukemic or had been euthanized because of severe leukemia (Fig. 2). In contrast, fewer than 10% of the mice vaccinated with vvCh1 or vvFr57 showed any signs of leukemia. The protective Ch1 chimeric envelope and the nonprotective FMCF envelope differ by only 23 amino acids. Thus, one or more of those differences determine immunological epitopes sufficient to confer protection. However, the protection provided by vvCh1 did not appear to be as good as that provided by vvFr57, because fewer of the mice in the vvFr57 group became leukemic following challenge (Fig. 2), and they developed IgG-neutralizing antibody significantly faster than mice in the vvCh1 group (Fig. 3A). Furthermore, the vvFr57 group had no detectable viremia at 7 days postinfection (Fig. 3B). Thus, the additional T_H, B-cell, and/or CTL epitopes present in Fr57 but not in Ch1 were also important in anti-FV protection.

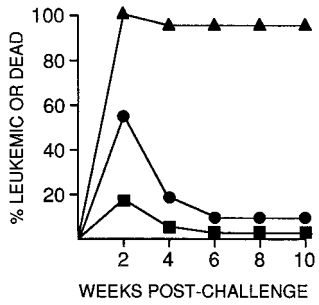

FIG. 2. Recovery from Friend disease in vaccinated mice. (B10.A × A.BY)F_1 age-matched female mice were vaccinated with vaccinia virus recombinants and challenged with 1,000 spleen focus-forming units of FV 1 month after vaccination. The symbols for each group and the number of mice per group (*n*) are as follows: ▲, vvFMCF vaccinated (n = 17); ●, vvCh1 vaccinated (n = 16); ■, vvFr57-vaccinated (n = 20). The development and progression of leukemia in individual mice were monitored by sequential weekly palpations for splenomegaly. Mice with obvious palpable splenomegaly (spleens greater than or equal to 0.5 g) were considered leukemic (3, 9).

Analyses of FV-specific CTLs. Previous studies had shown that CTL responses were not detectable in vaccinated mice until challenge with FV but that there was a good correlation between CTL activity and recovery following challenge with

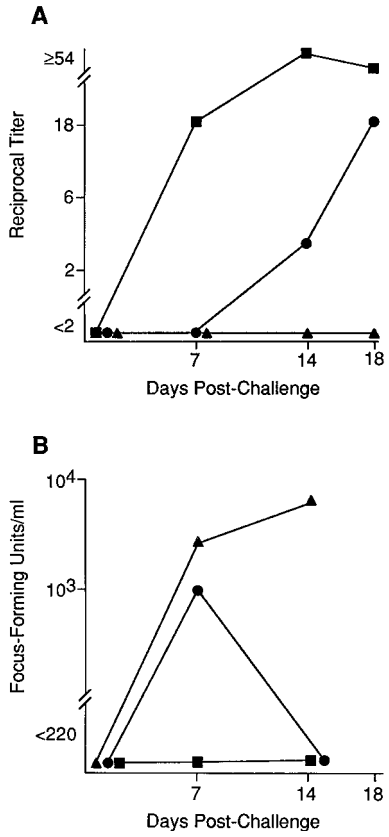

FIG. 3. (A) Kinetics of FV-specific-neutralizing IgG production following FV challenge of vaccinated mice. Methods for determining virus neutralization and the antibody subclass of neutralizing antibodies have been described elsewhere (20). Values are geometric means from group sizes ranging from four to nine mice per point. Vaccination with vvFr57 (■), vvCh1 (●), and vvFMCF (▲), is indicated. (B) Plasma viremia levels in vaccinated mice at three time points following challenge with FV. Values are geometric means from group sizes ranging from four to nine mice per point. Symbols are the same as for panel A.

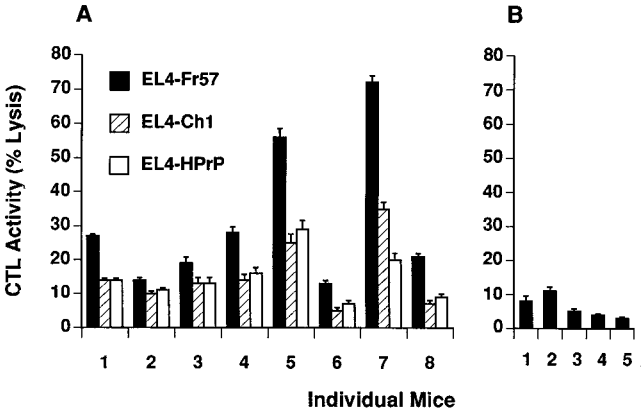

FIG. 4. CTLs from immunized and FV-challenged mice. At 15 to 16 days postinfection, spleen cells were used in direct CTL assays at an E-to-T of 200:1 ratio with ^{51}Cr-labeled EL4 targets as indicated. Standard errors were calculated from triplicate wells. (A) Mice vaccinated with vvCh1. Solid bars, EL4 cells expressing the Fr57 envelope protein; cross-hatched bars, EL4 cells expressing the Ch1 envelope protein; open bars, EL4 cells expressing the unrelated HprP protein. On any given day, it is common to see a range of CTL activities because of the transient nature of the CTL responses and the individual differences in immunological responses and recovery (29). The amount of specific lysis is similar to what has been observed for vvFr57-vaccinated mice (11). (B) Mice vaccinated with vvFMCF and tested on the same days as those in panel A. Only the reactivities against EL4 cells expressing the Fr57 envelope protein are shown; however, lysis results against EL4-Ch1 and EL4-EL4-HprP targets were similarly low.

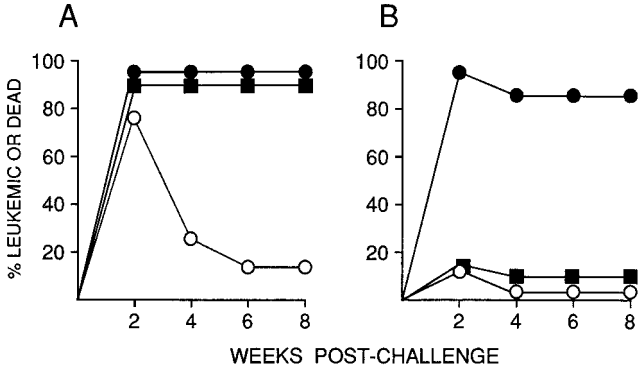

FIG. 5. Effect of T-cell subset depletion on protection of vaccinated mice. (A) vvCh1-vaccinated mice were depleted for T-cell subsets as described in Materials and Methods, infected with 1,000 spleen focus-forming units of FV, and monitored for leukemia induction and recovery. For the nondepleted (○), CD4-depleted (●), and CD8-depleted (■) groups, n = 13, 15, and 16, respectively. (B) vvFr57-vaccinated mice were treated as described above. For the nondepleted (○), CD4-depleted (●), and CD8-depleted (■) groups, n = 19, 13, and 13, respectively. Antibodies used for depletions were both of the IgG2b isotype. Results are compiled from two separate experiments.

FV (11). Other studies have demonstrated the importance of CTLs in the elimination of FV-induced tumors (14, 15). Therefore, it was somewhat surprising that the vvCh1 vaccine worked so well, since it contained no known CTL epitopes. This suggested that the CTL response might not be critical for protection or that the Ch1 envelope contained unidentified epitopes which could prime for CTL activity. To determine if FV-specific CTL responses were generated in vvCh1-vaccinated mice following challenge with FV, spleen cells were tested in a CTL assay against EL4 targets expressing F-MuLV envelope (EL4-Fr57). This target expresses the major epitopes recognized by CTL effectors from mice during spontaneous recovery from FV (29). Spleen cells from five of eight vvCh1-vaccinated mice showed CTL reactivity with greater than 20% specific chromium release against EL4-Fr57 target cells (Fig. 4A, black bars). In contrast, all chromium release values from vvFMCF-vaccinated mice were at or below background levels (Fig. 4B, black bars). Pretreatment of the CTL effectors with anti-CD8 antibody plus complement reduced CTL activity to background levels, while the same treatment with anti-CD4 antibodies had no significant effect on CTL activity (data not shown). Thus, the effectors appeared to be classical $CD8^+$ CTLs. These data suggested that vvCh1 vaccination primed for a virus-specific $CD8^+$ CTL response and that Ch1 might contain a CTL epitope.

To test for such an epitope, the same CTL effectors were tested against EL4 targets expressing the Ch1 protein (EL4-Ch1). CTLs from vvCh1-vaccinated mice generally had no better reactivity against EL4-Ch1 targets (Fig. 4, cross-hatched bars) than against a negative control EL4 line expressing the unrelated gene, HPrP (Fig. 4, open bars). Thus, it appeared that the vvCh1 vaccine did not contain a CTL epitope. A possible explanation for stimulation of the CTL response in vvCh1-vaccinated mice is that upon challenge with FV, unprimed CTLs specific for Friend envelope received help from vvCh1-primed $CD4^+$ T cells to stimulate an effective CTL response (10).

Differential effect of T-cell subset depletion on protection by vvFr57 and vvCh1. These data implicated both $CD4^+$ and $CD8^+$ T cells as important mediators of protection in vvCh1-vaccinated mice. To directly examine the in vivo importance of T-cell subsets in protection, we depleted vvCh1-vaccinated mice of either $CD4^+$ or $CD8^+$ T cells and challenged them with FV. $CD4^+$ T cells played a critical role in protection, since CD4-depleted mice had dramatically reduced recovery (Fig. 5A). Both cellular immunity and humoral immunity were reduced in CD4-depleted mice, since no CTL responses were detected in six mice tested, and the mice failed to switch isotype from IgM- to IgG-neutralizing antibody (data not shown). $CD8^+$ T cells were also essential for recovery, since CD8-depleted mice rapidly progressed to end-stage disease (Fig. 5A). Because of the importance of $CD8^+$ CTLs in recovery, it remains unclear whether the negative effects of CD4 depletion were due to lack of $CD4^+$ T-cell help for $CD8^+$ CTLs or for B cells.

We next examined whether the type of vaccine used to immunize the mice could alter the dependence on T cells for recovery. In contrast to vvCh1-vaccinated mice, animals vaccinated with the full-length Friend envelope protein (vvFr57) were not dependent on $CD8^+$ T cells for recovery (Fig. 5B). Thus, by priming with multiple B-cell and T_H epitopes, the dependence on $CD8^+$ T cells was overcome. However, the expression of the immunodominant CTL epitope in the vvFr57 vaccine was not sufficient to overcome the requirement for $CD4^+$ T cells, since depletion of $CD4^+$ T cells eliminated protection in animals immunized with vvFr57 (Fig. 5B).

DISCUSSION

These results clearly demonstrate a critical role for $CD4^+$ T cells in protective immunity from FV-induced disease. It was not possible to achieve protection in CD4-depleted mice, even following vaccination with the full-length Friend envelope which contains an immunodominant CTL epitope. One possible explanation for this result is that the development of FV-specific CTL effectors at the time of challenge was dependent on $CD4^+$ T cells. This issue has not been well studied in vaccinated animals; however, in experiments addressing spontaneous recovery from virus infections, animals generally do

not show dependence of CTLs on $CD4^+$ T cells. For example, CD4-depleted mice develop $CD8^+$ CTL responses to acute infections with ectromelia virus (1), vaccinia virus (1), herpes simplex virus (22), LP-BM5 (MAIDS) virus (16), and lymphocytic choriomeningitis virus (17). Alternatively, $CD4^+$ independent CTL responses might have been elicited but not detected by our assays. However, even if such effectors were elicited, they were not protective.

Interestingly, CTLs were more important in the protection of mice that were not vaccinated with a CTL epitope (vvCh1 group) than in those that were (vvFr57 group). Most likely, protection in vvFr57-vaccinated mice could be achieved independently of $CD8^+$ T cells because the vaccine contained multiple T_H and B-cell epitopes and stimulated a very rapid anamnestic virus-neutralizing IgG response (Fig. 3A). In contrast, the vvCh1 vaccine contained fewer T_H and B-cell epitopes, and immunized animals developed virus-neutralizing IgG with significantly slower kinetics (Fig. 3A). Thus, in vvCh1-vaccinated mice, the FV infection might have spread for a longer time after challenge, as evidenced by a greater percentage of the animals becoming splenomegalic (Fig. 2) and viremic (Fig. 3B). $CD8^+$ T-cell effectors might then have been necessary to clear the higher numbers of infected cells.

T-cell requirements for protection in vvCh1-vaccinated $H\text{-}2^{a/b}$ mice were quite similar to previous results from genetically resistant $H\text{-}2^{b/b}$ mice that spontaneously recover from FV infection without vaccination. That is, spontaneous recovery in $H\text{-}2^{b/b}$ mice was also dependent on both $CD4^+$ and $CD8^+$ T cells (29). The kinetics of FV-neutralizing antibody production between vvCh1-vaccinated mice and mice which spontaneously recover from FV infection is also very similar (7). Detectable levels of neutralizing antibody do not appear until about 2 weeks postinfection in either case. This is consistent with our interpretation that dependence on $CD8^+$ T cells for recovery may be due to the relatively slow kinetics of virus-neutralizing antibody production.

Previous results demonstrated that not all mouse strains can be successfully immunized with vaccinia virus recombinants expressing F-MuLV envelope protein (11). Only mice that express certain major histocompatibility complex (MHC) class II alleles such as $H\text{-}2A^b$ can be protected (19, 21). Since MHC class II molecules are involved in antigen presentation to $CD4^+$ T cells, these data are consistent with our present findings showing that protective immunization is dependent on $CD4^+$ T cells. Recently, it was shown that a peptide from the T_H epitope contained in vvCh1 (Fig. 1) could be used with Freund's adjuvant as a vaccine to protect mice from FV infection (18). This finding is consistent with our data demonstrating the importance of $CD4^+$ T cells in protection and also with our data showing that CTL epitopes are not required for a protective vaccine. Furthermore, it indicates that B-cell epitopes are also not required.

When these findings are considered in the context of the development of an HIV vaccine, the absolute requirement for $CD4^+$ T cells for protection is somewhat disconcerting. Because $CD4^+$ T cells are major targets for HIV, it has been suggested that vaccination-induced priming of HIV-specific $CD4^+$ T cells might actually contribute to amplification and spread of HIV (31). To avoid this possibility, a $CD4^+$ T-cell-independent retrovirus vaccine would be desirable. While CTLs are probably critical for anti-HIV immunity (2, 26, 27, 34, 35), our data suggest that a $CD8^+$ T-cell response alone is probably insufficient for retrovirus protection, even when an immunodominant CTL epitope is expressed by a live vector in a situation which favors MHC class I presentation of viral peptides to CTLs. An alternative approach would be to determine the right combination of epitopes that would prime for a secondary immune response which would develop fast enough to control virus spread. While CTL epitopes would undoubtedly be part of such a vaccine, current results suggest that T_H epitopes are more important than CTL epitopes for rapid immune response kinetics.

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