Characterization of a Live-Attenuated Retroviral Vaccine Demonstrates Protection via Immune Mechanisms

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Live-attenuated retroviruses have been shown to be effective retroviral vaccines, but currently little is known regarding the mechanisms of protection. In the present studies, we used Friend virus as a model to analyze characteristics of a live-attenuated vaccine in protection against virus-induced disease. Highly susceptible mice were immunized with nonpathogenic Friend murine leukemia helper virus (F-MuLV), which replicates poorly in adult mice. Further attenuation of the vaccine virus was achieved by crossing the Fv-1 genetic resistance barrier. The minimum dose of vaccine virus required to protect 100% of the mice against challenge with pathogenic Friend virus complex was determined to be 10^3 focus-forming units of attenuated virus. Live vaccine virus was necessary for induction of immunity, since inactivated F-MuLV did not induce protection. To determine whether immune cells mediated protection, spleen cells from vaccinated donor mice were adoptively transferred into syngeneic recipients. The results indicated that immune mechanisms rather than viral interference mediated protection.

Live-attenuated viruses are successfully used for preventing virus-induced diseases such as measles, mumps, rubella, and polio. Since the discovery of retroviruses such as human T-cell leukemia virus and human immunodeficiency virus (HIV) that cause diseases in humans, biomedical researchers have been interested in designing live-attenuated vaccines for retroviruses as well. In recent experiments, monkeys were protected by live-attenuated simian immunodeficiency virus (SIV) against challenge with pathogenic SIV isolates (1, 13, 36). However, the mechanism of protection is not understood, and there has even been a question regarding whether protection was immunologically based. While it is not theoretically necessary to understand how a vaccine works in order to use it, there are numerous safety concerns involved in the use of live-attenuated retroviruses as a vaccine. Thus, it would be beneficial to establish the basic parameters regarding protection by live-attenuated retroviruses, and the most useful animals for such studies are mice. Since there is currently no mouse model for HIV infection, we have initiated studies using Friend virus (FV), a murine retrovirus that causes immunosuppression and erythroleukemia in adult mice. Although there are major differences between the diseases caused by FV and HIV, it is possible that the basic requirements for vaccine protection against retroviruses are very similar.

FV is a retroviral complex comprised of a replication-competent helper virus, Friend murine leukemia virus (F-MuLV), and a replication-defective spleen focus-forming virus (SFFV) (22). In susceptible adult animals, FV induces rapid polyclonal erythroblast proliferation (19, 24), followed within 3 to 4 weeks by the immortalization of erythroid cells (12, 28, 30, 38). FV infections cause profound splenomegaly, abnormally high hematocrits (greater than 80%), and lethal erythroleukemias in most strains of mice. In several different vaccination experiments, protective immunity against FV has been achieved by using killed virus with adjuvants (21, 29), isolated viral proteins (20, 21), recombinant viral vectors expressing FV proteins (15, 17), and live-attenuated vaccines (15, 25). In contrast to vaccination with vaccinia virus vectors, live-attenuated virus is highly protective, even in mouse strains that have poor immunological responsiveness to FV because of their major histocompatibility complex (MHC) haplotype (15).

In previous experiments with live-attenuated FV, attenuation was achieved by crossing a host genetic resistance barrier called Fv-1. Successful replication of virus in Fv-1-resistant cells in vitro requires at least 100-fold more virus than in Fv-1-compatible cells (32). Likewise, induction of disease in Fv-1-incompatible mice requires much higher doses of virus than are required in Fv-1-compatible strains (25). Thus, inoculation of 1,500 focus-forming units (FFU) of N-tropic FV (FV-N) complex induces rapid erythroleukemia in DBA/2 (Fv-1’n/n) mice, but not in A.BY (Fv-1’b/b) mice. In addition, after infection with FV-N, Fv-1’b/b mice are subsequently protected from challenge with B-tropic FV (FV-B) complex (15, 25). Although such vaccination has been shown to generate virus-specific B-cells, cytotoxic T cells (CTLs), and helper T cells (TH) (15), the role of these immune cells in protection has not been established. In fact, evidence indicates that in Fv-1-compatible neonatal mice, protection by live-attenuated vaccines occurs through viral interference rather than immunological mechanisms (11, 12, 26).

The present paper establishes that only the F-MuLV helper component of the FV-N complex is required for effective vaccination and demonstrates that protection by F-MuLV in adult mice is primarily mediated by immune cells rather than viral interference.

MATERIALS AND METHODS

Mice. (B10.A × A/Wy)F1 mice 3 to 6 months of age at experimental onset were used. F1 parental strain mice were obtained from the Jackson Laboratories. Breeding of F1 strains was done at Rocky Mountain Laboratories. All mice were treated in accordance with National Institutes of Health regulations and the guidelines of the Animal Care and Use Committee of Rocky Mountain Laboratories.

Virus vaccination and virus challenge. The FV-B complex used in these experiments was from uncloned virus stocks obtained from 10% spleen cell
homogenates from BALB/c mice infected 9 days previously with polyethylenimine-inducing FV stocks originally obtained from Frank Lilly (10, 15). The N-tropic F-MuLV (stock 29-51N) (6) was a 24-h supernatant from infected Mus dunni cells (23). Mice were vaccinated by intravenous injection of 0.5 ml of phosphate-buffered, balanced salt solution (PBBS) (9) containing 2% fetal bovine serum and 10^4 FFU of N-tropic F-MuLV vaccine virus. Heat inactivation of the F-MuLV vaccine was performed by 1 h of incubation in a 56°C water bath. In virus challenge experiments, mice were injected intravenously with 0.5 ml of PBBS containing 2% fetal bovine serum and 1,500 spleen FFU (SFFU) of FV-B complex.

**Splenomegaly as a measure of Friend disease.** Palpation for splenomegaly is the standard procedure used to monitor the progression of Friend disease (10, 15, 31) and was used in the following manner. At weekly intervals, each individual animal under general anesthesia was palpated in a blinded fashion and rated on a scale of 1+ to 4+ according to its spleen size. Normal (1+) spleen weights range from 0.1 to 0.25 g. Spleens greater than twice normal size (more than 0.4 g), but not large enough to reach the ventral midline, were rated as 2+. Spleens that weigh between 0.25 and 0.4 g were still rated as 1+. If spleens were large enough to reach the ventral midline, they were rated as 3+ (weight of between 0.63 and 1.0 g). Spleens which extended across the abdominal midline and caused protrusion of the abdominal wall were rated as 4+ (weight greater than 1.6 g). Cross-checking of actual spleen weights with spleen sizes determined by palpations has demonstrated consistency in differentiation of spleins weighing in the normal range from those weighing greater than 0.4 g (2+) (reference 5 and our unpublished data). Mice that have sustained splenomegaly by 8 weeks postinfection begin to die about 10 weeks postinfection, and most are dead by 16 weeks postinfection. In contrast, mice which recover from splenomegaly live normal life spans (5, 10).

**Viremia and virus-neutralizing antibody assays.** For viremia assays, freshly frozen plasma samples were titrated by focal infectivity assays (35) on susceptible M. dunni cells pretreated with 4 μg of Polybrene per ml. Cultures were incubated for 5 days, fixed with ethanol, stained with F-MuLV envelope-specific monoclonal antibody 720 (33), and developed with goat anti-mouse peroxidase-conjugated anti-rat sera (Cappel, West Chester, Pa.) and aminoethylcarbazol to detect foci. To test plasma samples for virus-neutralizing antibodies, heat-inactivated (56°C, 10 min) samples at titrated dilutions were incubated with virus stock in the presence of complement at 37°C as previously described (29). The samples were then plated as described for the viremia assay to determine the dilution at which 75% of the virus had been neutralized.

**Infectious center assays.** Titration of single-cell suspensions from persistently infected mouse spleens were plated onto susceptible M. dunni cells (23), cocultivated for 5 days, fixed with ethanol, stained with F-MuLV envelope-specific monoclonal antibody 720 (33), and developed with peroxidase-conjugated goat anti-mouse antibodies and aminoethylcarbazol to detect foci.

**Adaptive spleen cell transfer.** For the transfer experiments, B10.A × A/Wy)F1 mice (Fv-1b/b) were vaccinated by infection with 10^4 FFU of live-attenuated N-tropic F-MuLV vaccine virus. After 30 days, spleen cells from these mice were adoptively transferred to naive syngeneic animals via tail vein injection. Each mouse received 7.5 × 10^7 spleen cells in 0.75 ml of PBBS. Cell suspensions were depleted of erythroid cells and filtered through a nylon screen to remove chunks. The PBBS was supplemented with 15 U of heparin per ml. Cultures were incubated with virus stock in the presence of complement at 37°C as previously described (10). The mice were then vaccinated with live F-MuLV (Fig. 2A). In contrast, animals vaccinated with inactivated virus had no detectable virus-neutralizing antibody titers. Thus, vaccination prevented the spread of virus to the blood.

**Protection of Fv-1b/b mice with N-tropic F-MuLV.** Previous studies describing a live-attenuated FV vaccine used the FVN complex composed of N-tropic F-MuLV helper virus and SFFV (15). However, FV-N is pathogenic, depending on the virus dose and mouse strain used, while F-MuLV alone is nonpathogenic in adult mice. We wished to determine if F-MuLV alone could be used to provide protection, thereby reducing the pathogenic potential of the vaccine and eliminating SFFV as an immunological variable. To this end, highly susceptible (B10.A × A/Wy)F1 mice (Fv-1b/b) were vaccinated by intravenous injection with 10^4 FFU of N-tropic F-MuLV alone. A control group of animals was inoculated with the same dose of heat-inactivated virus. At 1 month postvaccination, the mice were challenged with pathogenic FV-B complex, and the animals were examined weekly for virus-induced splenomegaly. All 18 animals that were vaccinated with live F-MuLV were protected from splenomegaly during the 8-week observation period after FV-B challenge (Fig. 1). Furthermore, they were also protected against a second FV-B challenge 3 months later (data not shown). In contrast, all 16 mice immunized with inactivated F-MuLV developed splenomegaly within 2 weeks after infection, and more than 80% of the animals had to be euthanized within 8 weeks because of FV-induced erythroleukemia (Fig. 1). Thus, the helper component alone was sufficient for protection against FV-induced disease. Furthermore, in vivo infection and, perhaps, replication were required to induce protective immunity.

**Viremia and virus-neutralizing antibodies in vaccinated mice.** To determine if vaccination had limited the challenge infection sufficiently to prevent viremia, plasma samples from immunized animals were tested for the presence of free virus. Infectious virus was detected 7 days after FV-B challenge in the blood of unvaccinated animals and animals inoculated with inactivated virus (Fig. 2A). In contrast, animals vaccinated with live F-MuLV had no measurable viremia at the same time point.

Since it has been previously shown that viremia in FV infection is controlled by virus-neutralizing antibodies (4, 8), the titers of neutralizing antibodies in the groups vaccinated with live or inactivated virus were determined. Virus-neutralizing antibody titers ranging from 1/40 to 1/160 were found in the sera from mice that had received the live vaccine virus (Fig. 2B). In contrast, mice immunized with inactivated virus had no detectable virus-neutralizing antibody titers. Thus, vaccination with live F-MuLV induced production of virus-neutralizing antibody and prevented the spread of virus to the blood.

**Determination of the minimal viral dose for efficient F-MuLV vaccination.** To determine the minimal live-attenuated F-MuLV viral dose required to induce protection, (B10.A × A/Wy)F1 mice were inoculated with various amounts of the vaccine virus. Groups of animals vaccinated with either 10^3 from splenomegaly.
or $10^3$ FFU of F-MuLV were completely protected against splenomegaly induced by challenge with 1,500 FFU of FV-B (Fig. 3). In contrast, at 8 weeks postchallenge, mice vaccinated splenomegaly induced by challenge with 1,500 FFU of FV-B determined by measuring infectious centers (ICs) in the MuLV.

To address the question of whether F-MuLV-induced protection was due to an immunological mechanism, we performed an adoptive cell transfer experiment. Spleen cells ($7.5 \times 10^7$) from F-MuLV-vaccinated or naive (B10.A $\times$ A/Wy)F1 mice were transferred into naive, syngeneic animals. One day later, the recipients were challenged with pathogenic FV-B. Six of nine animals receiving cells from vaccinated mice were protected against FV-induced early splenomegaly (Fig. 4). In addition, one of the three animals which had early splenomegaly later recovered from Friend disease. In contrast, all nine animals that received spleen cells from naive mice developed severe splenomegaly, and none of the mice recovered.

The current results demonstrate that the primary mechanism of protection by live-attenuated retroviruses is viral interference. This mechanism appeared unlikely, given that there was no detectable vaccine virus in the immune spleen cells that were transferred at 30 days post-F-MuLV vaccination (Table 1). However, as a control for passage of an interfering virus, spleen cells were transferred at 3 days postvaccination. At this time point, there was detectable vaccine virus in the spleen cells, but the immune response had not yet had time to develop. None of the mice that received spleen cells from donors at 3 days postvaccination were protected from FV-induced splenomegaly (Fig. 4). Likewise, mice that received the F-MuLV vaccine virus 1 day prior to FV-B challenge also were not protected. These results indicate that immune cells rather than viral interference mediated protection by the F-MuLV live-attenuated retroviral vaccine.

**TABLE 1. Detection of vaccine virus at various time points after F-MuLV inoculation**

<table>
<thead>
<tr>
<th>No. of ICs/10^7 spleen cells at postvaccination day</th>
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<tr>
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<tr>
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</tr>
<tr>
<td>0</td>
<td>9</td>
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<td>0</td>
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* Determined after inoculation with $10^4$ FFU of F-MuLV.

DISCUSSION

The current results demonstrate that the primary mechanism of protection by live-attenuated FV vaccination is immunologically mediated. There was no indication of an effect from viral interference, since vaccine virus without any immune cells did not protect adult mice against FV-induced disease. This was found for the inoculation of free virus 1 day prior to challenge, the same time point used to establish viral interference as the mechanism of protection in neonatal mice (11). To rule out the possibility that transfers of infected cells might
transfer vaccine virus more efficiently than infection with free virus, we transferred cells at 3 days postvaccination. At this time point, there was detectable virus in the transferred cells, but significant numbers of immune cells had not been generated. There was no protection conferred by transfers of infected, but nonimmune spleen cells. In contrast, immune cells that harbored no detectable F-MuLV were protective. Although these cells might have harbored an undetectable low-level infection, the results from our control groups make it unlikely that this contributed significantly to their protective effect. It has been shown for Fv-1 induction of resistance of mice against FV that high but not low levels of FV envelope expression induced protection by an interference mechanism (26). Thus, large numbers of vaccine virus-infected target cells seem to be necessary to induce resistance against FV infection via interference. However, a minor additive effect of interference together with the immune-mediated protection by live-attenuated retroviruses cannot be ruled out.

Our results are significant in that they suggest it may be possible to achieve similar protection with vectors believed to be safer than live-attenuated viruses. However, this will require much more detailed knowledge about the immunological mechanisms required for protection. For example, previous attempts to vaccinate mice against FV with recombinant vaccinia virus vectors expressing viral proteins have resulted in only limited protection dependent on the MHC type of the mouse (7, 15, 18). Recombinant vaccinia virus vectors have also been relatively unsuccessful in the SIV model (14, 16), possibly because of the nonresponsiveness of some MHC types (3). Likewise, peptide vaccines in the FV model have shown only limited protection in certain MHC types (27). Any vaccine useful for human immunization against retroviral infections must be broadly protective in multiple MHC types, as are the live-attenuated viruses. Thus, it is important to determine which characteristics of the live-attenuated viruses account for their efficacy and differentiate them from less-effective vaccines, such as vaccinia virus vectors.

In the FV model, one of the known differences between vaccination with live-attenuated viruses and that with vaccinia virus vectors is that the live-attenuated viruses elicit immunological effectors, including CTLs, virus-neutralizing antibody, and CD4+ T cells, while vaccinia virus vectors generally only prime for CTL and antibody responses, but do elicit CD4+ T cells (15). The reasons for this remain unclear, but may be related to the duration of antigen available for immune stimulation. In the present studies, which were done with highly susceptible animals with poorly responsive MHC haplotypes, it is possible that vaccine virus persisted at levels below the limit of detection of our assay and contributed to immune protection. With sensitive PCR techniques, persistent vaccine viruses have been found in macaques vaccinated with live-attenuated SIV (37), and a chronic source of antigen may be an essential aspect of long-term protection. It will be important to address this issue more carefully in future studies.

Other attributes which may contribute to the efficacy of live-attenuated viruses as vaccines include the wide range of viral proteins which are expressed, the types of immune cells responding to the infection, the specific location of viral antigen within the immunological architecture, and the degree of vaccine virus replication. These issues have broad implications for the rational design of safe and effective retroviral vaccines and must be addressed. The system described here offers the opportunity to examine these basic issues and could lead to novel vaccination methods which exploit the advantages of live-attenuated retroviruses while reducing or eliminating potential dangers. While this is obviously the preferred outcome, it may also be found that only live-attenuated retroviruses stimulate the specific types of immune responses uniquely required for retroviral protection. The critical issue then becomes how to make a safe live-attenuated retrovirus. Many of the safety issues, such as reversion to virulence, insertional mutagenesis, and recombination with endogenous or exogenous retroviral sequences, are frequency dependent and are related to levels of infection and virus replication. Thus, it may be possible to reduce the risk to acceptable levels by determining the proper dose and precisely attenuating the vaccine virus such that replication is only high enough to stimulate protective immunity. Our data give a hint about what those minimum levels might be. In some animals, doses as low as 10 FFU of F-MuLV induced protection (Fig. 3). It is possible that a booster administration of the same dose might significantly improve protection while restricting replication levels.

In the current experiments, only live vaccine virus induced protection. However, it remains possible that virus replication and spread were not requirements for protection and that there were only requirements for infection and protein synthesis. Future experiments with live replication-defective viruses may clarify this issue, but previous experiments have indicated that without replication, protection is limited by the MHC haplotype of the animal (34). Since we have now established immune cells as the effectors of protection, it will be possible in future studies to dissect which types of immune cells are necessary. Such experiments may reveal whether it is feasible to focus on a single lymphocyte subset alone to provide protection from retroviral infection.

FIG. 4. Transfer of F-MuLV-primed spleen cells into syngeneic mice. Spleen cells (7.5 × 10^7) from F-MuLV-vaccinated or naive (B10.A × A/Wy)F1 mice were transferred to age-matched syngeneic animals. Nine animals in each group received either spleen cells from naive mice (●) or spleen cells from mice vaccinated with F-MuLV 30 days prior to transfer (○). One day after transfer, the recipients were challenged with FV-B complex and monitored for virus-induced splenomegaly. Eight controls were inoculated with the F-MuLV vaccine virus 1 day prior to FV-B challenge (□). In addition, nine animals received spleen cells from animals which were F-MuLV vaccinated only 3 days prior to the transfer (◇). The difference between the groups of animals that received spleen cells 3 days postvaccination and those who received them 30 days postvaccination was statistically very significant (P = 0.009, by Fisher's exact test of chi-square analysis).
REFERENCES


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