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Approach to a retrovirus vaccine: Immunization of mice against Friend virus disease with a replication-defective Friend murine leukemia virus

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ABSTRACT **In an initial attempt to test the ability of replication-defective retroviruses to immunize against immunologically related pathogenic viruses, we have worked with the erythroleukemogenic Friend retrovirus complex (FV), which consists of a replication-competent helper component, Friend murine leukemia virus (FMuLV), and a related defective pathogenic component, spleen focus-forming virus (SFFV). An 81-base-pair deletion was introduced into the p15E-encoding region of the *env* gene of an otherwise replication-competent molecular clone of the FMuLV provirus. After transfection of this clone into cells that package the viral RNA in MuLV coats, infectious virus was released into the culture medium. Mouse fibroblasts infected with this virus, here called ΔFMuLV, expressed the truncated viral *env* gene products in their cytoplasm but not on cell surfaces, and culture fluids from these cells did not transmit the infection to fresh mouse fibroblasts. In preliminary experiments, immunization of mice of *H-2*-congenic BALB/c strains with ΔFMuLV conferred levels of immunity to FV disease ranging from weak to relatively strong. Immunized mice developed anti-FV IgM and IgG antibodies and cytotoxic T cells. Mice observed for 15 weeks after the first of two immunizations showed no detectable pathology, but ΔFMuLV DNA was detectable in livers of some immunized mice for at least 3–6 weeks. These results suggest that our approach to development of retrovirus vaccines may be a useful one.**

Friend virus (FV) is a murine retrovirus complex that induces erythroleukemia and immunosuppression in adult mice of many strains (1). It consists of a replication-competent helper virus, Friend murine leukemia virus (FMuLV), that is rarely pathogenic in adult mice and an immunologically related pathogenic component, spleen focus-forming virus (SFFV), derived from FMuLV (2). Because many strains of murine retrovirus are pathogenic only when administered soon after birth, FV is one of the few that can be used to develop models for retroviral vaccines.

Immunity to FV disease in susceptible mice can be attained by several immunization protocols, most of which are not attractive as models for adaptation to use in humans. In one system, mice are immunized with FV-infected cells, either live, irradiated, or chemically fixed, and usually syngeneic with the recipient (ref. 3; D. Polsky and F.L., unpublished results). In another system, mice receive low doses of FV of a substrain that replicates poorly in the recipient—e.g., N-tropic FV into relatively resistant *Fv-1*^{b} hosts (4). Recombinant vaccinia virus expressing FMuLV genes can also confer anti-FV resistance (5, 6). The induced resistance can be adoptively transferred to syngeneic hosts using either serum (7) or T cells (3), but T cells—CD4$^+$ or CD8$^+$, and preferably both together—are considerably more effective than serum. It appears that an effective anti-FV vaccine should generate humoral and cellular immune responses to the virus.

We now report an initial altered version of FMuLV, here called ΔFMuLV, that is infectious but cannot generate infectious progeny virions due to an in-frame deletion of 81 base pairs (bp) from the p15E-coding region of the viral *env* gene. In theory, immunization with such a virus will lead to infection of a limited number of host cells, and expression by these cells of viral proteins, including those encoded by the truncated *env* gene, might then induce an immune response protective against challenge with pathogenic FV. The results from our preliminary studies with ΔFMuLV indicate some promise for the approach and some problems that will need to be overcome, if possible.

MATERIALS AND METHODS

Mice and Cells. *H-2*-congenic strains BALB/cAn (*H-2*^{d}), BALB.B (*H-2*^{b}), and BALB.K (*H-2*^{k}) were bred in our colony. Cell lines used were FRE (Fischer rat embryo) (8), and its derivatives SFFV-FRE/FMuLV, expressing complete FV (SFFV and FMuLV) (9), clone Df45, expressing complete FV plus the *H-2D*^{b} restriction element, and clone B2, expressing the *H-2D*^{b} gene only (10); SC-1 mouse fibroblasts (11); ψ-2, Moloney MuLV retrovirus packaging cells (12); PA12, amphotropic MuLV packaging cells (13); and F_{201}NIH, a line of NIH 3T3 cells expressing a molecularly cloned NB-tropic FMuLV genome (14).

Friend Virus. Stocks of FV originated from culture fluids from F_{201}NIH and SFFV-FRE/FMuLV cells mixed in a 1:3 ratio and passed twice in BALB/c mice. They were used as dilutions of clarified supernatants from 10% (wt/vol) homogenates of the greatly enlarged spleens of mice infected 12–15 days earlier as described (15). Stocks were titrated by the spleen focus method (16) in BALB/c mice [usual titer, ≈10^4 spleen focus-forming units (SFFU)/ml].

Antibodies. Goat antisera to Rauscher MuLV gp70^{env} and Pr65^{gag} were from the Viral Oncology Division, National Cancer Institute (Bethesda, MD). Monoclonal antibodies 55 (anti-FMuLV gp70) and 34 (anti-FMuLV p15^{gag}) (17) were used in the form of mouse ascites fluid for detection of cell-surface expression of viral antigens by fluorescence-activated cell sorting (FACS).

Production of Defective Virus ΔFMuLV. The *Hin*dIII insert from vector pKR1 (18) containing the FMuLV *env* gene with deletion of the 81-bp *Stu* I fragment in the p15E coding region was subcloned into the *Hin*dIII site of pGEM3Z (Promega) to produce vector pKR3. The *Sph* I fragment of pKR3 then replaced the homologous fragment of the replication-

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Abbreviations: FV, Friend erythroleukemia virus; FMuLV, Friend murine leukemia virus; SFFV, spleen focus-forming virus; SFFU, spleen focus-forming units; FRE, Fischer rat embryo; vRNA, viral RNA; FACS, fluorescence-activated cell sorting; CTL, cytolytic T lymphocyte.

competent FMuLV proviral genome in clone 2-1a1c (10) to produce pKR4 (see Fig. 1). The defective ΔFMuLV genome of pKR4 and the pSV2neo selection marker were cotransfected into either ψ-2 cells or 1:1 cocultures of ψ-2 and PA12 cells (19) by the $Ca_3(PO_4)_2$ precipitation method (20), and G418-resistant cell clones were selected.

Efficiency of packaging of defective viral RNA (vRNA) was estimated by analysis of virions collected from filtered media from subconfluent cultures of pKR4-transfected ψ-2 clones by comparison with those from FMuLV-producer F_{201}NIH cells. Dot blots of vRNA extracted by proteinase K and phenol/chloroform treatment were hybridized with the ^{32}P-labeled intact FMuLV *env* gene insert from pSV2Fe/gpt-1 (10).

Infectivity of the helper-free, ψ-2-packaged ΔFMuLV was examined by adding filtered culture fluids to subconfluent cultures of SC-1 or FRE cells in the presence of 4 μg of Polybrene per ml. After fixation with acetone, the attached cells were incubated in mouse monoclonal anti-gp70 (no. 55) or anti-p15^{gag} (no. 34) antibody-containing ascites fluid (1:100) for 1 hr, washed and incubated again in fluorescence-conjugated goat anti-mouse IgG Fc (1:1000) for 30 min, washed, and examined by fluorescence microscopy.

Cytoplasmic and cell-surface expression of viral proteins in pKR4-transfected FRE cells was monitored by immunoprecipitation and gel electrophoresis and by FACS analysis as described (18).

Studies of Response to ΔFMuLV *in Vivo*. Mice 6–14 weeks old received two 2-ml immunizations i.p. in 2 weeks with filtered fresh ΔFMuLV-containing culture fluids from pKR4-transfected ψ-2 cells or ψ-2/PA12 cocultures. Three weeks after the second injection they were challenged with 1 ml of the desired dilution of FV into the tail vein. Spleen size was monitored by palpation twice weekly from day 7 to day 28 and once weekly thereafter for at least 3 months. Mice were deemed susceptible if the spleen attained ≥0.5 g, although at low virus doses the enlarged spleens of some mice regressed to a near normal size.

Production of IgM and IgG antibodies to FMuLV *env* gene products by ΔFMuLV-immunized mice was determined using an ELISA. Antigen was an extract from a clone of pKR1-transfected FRE cells expressing high levels of ΔFMuLV^{env} proteins. Second antibodies were peroxidase-conjugated anti-mouse IgM or IgG antibodies (Sigma). Reactions were evaluated spectrophotometrically at 405 nm.

Cytolytic T-lymphocyte (CTL) responses were determined using ^{51}Cr-labeled Df45 cells as targets as described (18).

Persistence of ΔFMuLV provirus in immunized mice was studied in high molecular weight DNA extracted from livers using the polymerase chain reaction (PCR). Oligonucleotide primers used (18) spanned the 81-bp deletion in the ΔFMuLV *env* gene and produced a 1.4-kilobase (kb) product from positive samples.

RESULTS

Vector Construction. pKR1 is an expression vector containing a modified *Hin*dIII fragment from the replication-competent FMuLV provirus clone 2-1a1c (10, 18). The pKR1 insert comprises the FMuLV *env* gene from which an 81-bp *Stu* I restriction fragment within the p15E coding region has been deleted and a long terminal repeat. To obtain a clone of ΔFMuLV, a complete provirus with this same *Stu* I deletion, the native *env* gene of vector 2-1a1c was replaced with the *env* gene from pKR1 to create the new vector pKR4 (Fig. 1).

Viral Gene Expression in pKR4-Transfected Cells. We have previously reported that FRE cells transfected with pKR1 express abundant FMuLV *env* gene products in the cytoplasm but not on the cell surfaces (18). Clones of FRE cells transfected with pKR4 were analyzed for cytoplasmic and cell-surface expression of *gag* and *env* gene products. These cells expressed products of both viral genes in the cytoplasm, as revealed by electrophoresis of proteins immunoprecipitated from extracts of metabolically radiolabeled cells (Fig. 2). However, FACS studies of the cells showed that only *gag* products, and not the *env* gene gp70 product, were detectable on cell surfaces (Fig. 3). Confirmation that the vRNA in pKR4-transfected FRE cells was indeed that bearing the 81-bp deletion came from sizing the product of a reverse transcriptase PCR reaction using primers spanning the deletion site by comparison with the homologous product from cells expressing the intact FMuLV *env* gene. Only PCR fragments of the smaller size were detected (data not shown).

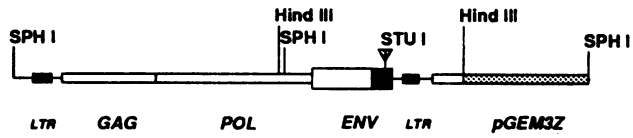

FIG. 1. Diagram of the 13.2-kb ΔFMuLV-encoding vector, pKR4. LTR, long terminal repeat.

Transfection of pKR4 into ψ-2 Cells. To obtain infectious ΔFMuLV particles, 13 insert-positive cell clones were obtained from cells of the ψ-2 retroviral packaging line after transfection with pKR4. Culture supernatants from 10 of the clones were tested for sedimentable RT activity as a measure of virion production and showed activity averaging 63% of that detected in a supernatant from F_{201}NIH cells, a clone of NIH 3T3 cells expressing the biologically cloned replication-competent FMuLV from which vector 2-1a1c was derived (data not shown). Levels of vRNA in particles released from pKR4-transfected ψ-2 clones were determined by RNA dot blotting and compared with those in serial dilutions of an F_{201}NIH cell supernatant in order to obtain an estimate of the relative levels of RNA-containing particles. vRNA levels detected in the ψ-2 clone supernatants ranged from ≈0.1 to 0.01 of those in the F_{201}NIH supernatant (Fig. 4). Thus, only a small fraction of ΔFMuLV particles produced by the ψ-2 clones contained vRNA.

Infectivity of the virions produced by pKR4-transfected ψ-2 cells was examined by infecting SC-1 cells with a ΔFMuLV-containing culture supernatant and examining the fixed cells 2 days later by immunofluorescence using antibodies to FMuLV *gag* and *env* gene products. Approximately 5% of the cells stained brightly with either antibody. SC-1

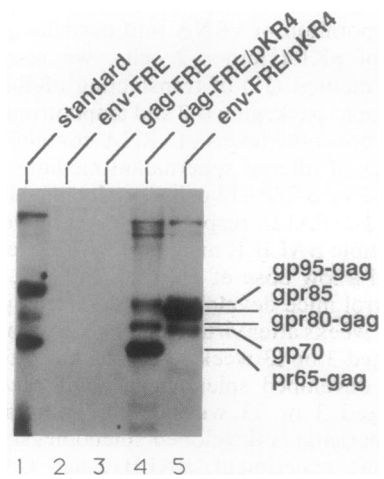

FIG. 2. Cytoplasmic expression of FMuLV *gag* and *env* gene products in pKR4-transfected cells. Metabolically labeled proteins were immunoprecipitated with anti-Rauscher MuLV gp70^{env} (lanes 2 and 4) or anti-Rauscher MuLV pr65^{gag} (lanes 3 and 5) from normal FRE cells (lanes 2 and 3) or a clone of pKR4-transfected FRE cells. Lane 1 shows molecular weight markers.

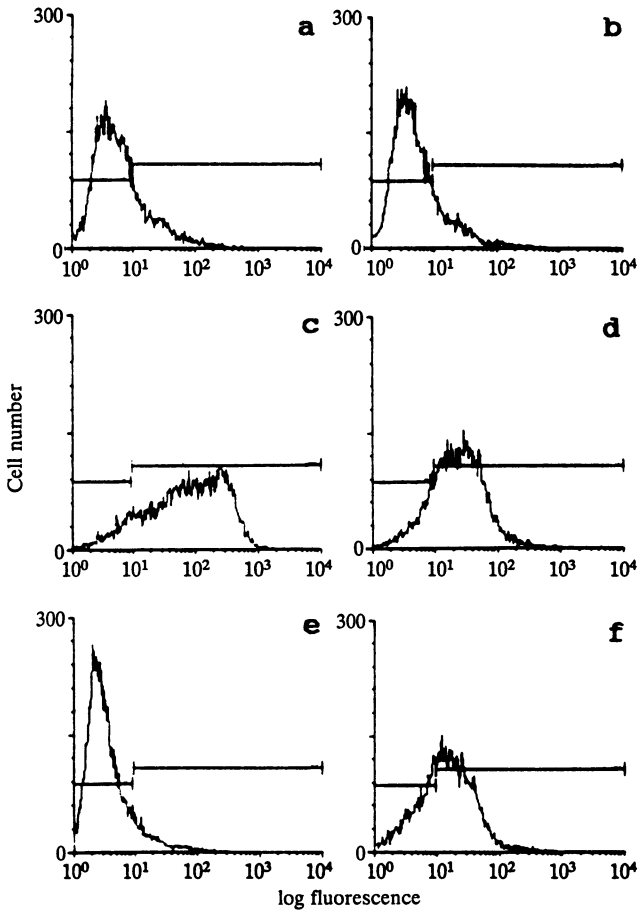

FIG. 3. Cell-surface expression of FMuLV *gag* and *env* gene products by pKR4-transfected cells. FACS analysis of normal FRE cells (*a* and *b*), F_{201}NIH (*c* and *d*), or a clone of pKR4-transfected FRE (*e* and *f*), using monoclonal goat anti-FMuLV gp70 (*a*, *c*, and *e*) or anti-FMuLV $p15^{gag}$ (*b*, *d*, and *f*) antibodies.

cells infected with unmodified FMuLV produce macroscopic plaques when cocultivated with rat XC cells (21). However, as predicted from the absence of cell-surface expression of the $gp70^{env}$ product of ΔFMuLV, SC-1 cells infected with the modified virus produced no XC plaques (data not shown).

Protection Against Challenge with FV. Because of the low level of incorporation of vRNA into particles produced by transfection of pKR4 into ψ-2 cells, we resorted to the "ping-pong" method (19) of transfecting pKR4 into cocultures of ecotropic-packaging ψ-2 and amphotropic-packaging PA12 cells to boost the levels of vRNA-containing particles. Two i.p. doses of filtered supernatant medium from pKR4-transfected ψ-2 or ψ-2/PA12 cells, producing ΔFMuLV(ψ-2) or ΔFMuLV(ψ-2/PA12), respectively, were given to groups of FV-susceptible BALB.K mice, and 3 or 13 weeks later the mice received a low dose of FV i.v. (Table 1). All 8 unimmunized control mice developed pronounced splenomegaly and died 6–11 weeks after virus challenge. By contrast, 11/16 mice challenged 3 or 13 weeks after immunization with ψ-2 supernatants developed splenomegaly and died, and 0/16 mice challenged 3 or 13 weeks after immunization with ψ-2/PA12 supernatants developed splenomegaly.

In a separate experiment, BALB/c and BALB.B mice were challenged i.v. with a range of doses of FV 3 weeks after the second immunization with ΔFMuLV(ψ-2/PA12) (Table 2). BALB.B mice (*H-2*^{b}) are markedly less susceptible to the pathologic effects of FV than are BALB/c mice (*H-2*^{d}) (15) and at lower virus doses sometimes recover spontaneously from the initial splenomegalic response to the virus due to their stronger and more rapid antiviral immune responses. For this reason, the challenge doses of virus given to BALB.B mice were higher than those given to BALB/c mice. Although the numbers of mice in each group were small, the results suggest that the immunization with ΔFMuLV conferred a detectable level of protection against disease and mortality at the lower virus challenge doses studied.

Since some relatively large fraction of virions in these experiments is presumed to contain no vRNA, it was important to examine the capacity of such empty particles alone to generate resistance to FV. Three biweekly injections of culture fluids from untransfected ψ-2 cells, which contain high levels of RNA-free virions, were given to 10 mice each of the BALB/c and BALB.K strains and to 12 BALB.B mice. After challenge with a low dose of FV (25 SFFU for BALB/c and BALB.K, 125 SFFU for BALB.B), the incidences (80–100% in BALB/c and BALB.K, 50–67% in BALB.B) and rates of occurrence of disease did not differ between immunized mice and unimmunized controls of each strain.

Immune Responses to ΔFMuLV. The $FMuLV^{env}$-specific antibody responses to immunization with ΔFMuLV(ψ-2/PA12) were assayed using peroxidase-conjugated anti-mouse IgM or IgG antibodies in an ELISA system. BALB.B and BALB/c mice were bled and immunized on day 0, days 0 and 14, or days 0, 14 and 28. Analysis of serum samples taken on days 0, 7, 21, 35, and 42 indicated that mice in all groups of both strains showed similar IgM responses that peaked on day 21 and declined thereafter (data not shown). IgG responses, however, increased with increasing numbers of immunizations and were more rapid in BALB.B than in BALB/c mice (Fig. 5).

To examine the FV-specific CTL response of mice immunized with ΔFMuLV(ψ-2/PA12), BALB.B mice were given the virus on days 0 and 14, days 14 and 28, day 28 only, or not at all. On day 38 spleen cells from these mice were restimulated in culture with irradiated spleen cells from FV-infected BALB.B mice. CTL activity for Df45 targets was significant with spleen cells from mice immunized on days 14 and 28, but very little activity was detected with spleen cells from mice of the other groups (Table 3).

Persistence of Infection with ΔFMuLV. Mice that had received either FMuLV-containing F_{201}NIH supernatants or ΔFMuLV(ψ-2) on day 0 or days 0 and 14 were killed at various times after the last injection, and DNA was extracted from their livers. The levels of proviral DNA were estimated by PCR using primers spanning the site of the *env* gene deletion (18). As seen in Fig. 6, livers from BALB.K mice receiving a single injection of ΔFMuLV did not show a significant level of viral DNA on day 1 but did so on days 2, 7, and 14. In contrast, BALB.K mice receiving one injection of unmodified FMuLV showed proviral DNA on days 1 and 3, but this DNA was no longer detectable on days 7 and 21. Although no proviral DNA was detected from either a BALB.B or a BALB/c mouse receiving a single injection of ΔFMuLV and killed on day 42, a BALB.B mouse that received a second injection on day 14 showed a high level of viral DNA on day 42.

DISCUSSION

In an initial effort to produce an anti-FV vaccine, we have generated virions comprised of the genome of the helper virus component of FV, FMuLV, containing a short in-frame deletion in its *env* gene and packaged in proteins encoded by the immunologically crossreactive Moloney strain of MuLV or an amphotropic MuLV. Protection against FV disease by immunization with this replication-defective ΔFMuLV was observed at levels ranging from weak to relatively strong and long-lasting in mice of three *H-2*-congenic strains, BALB/c,

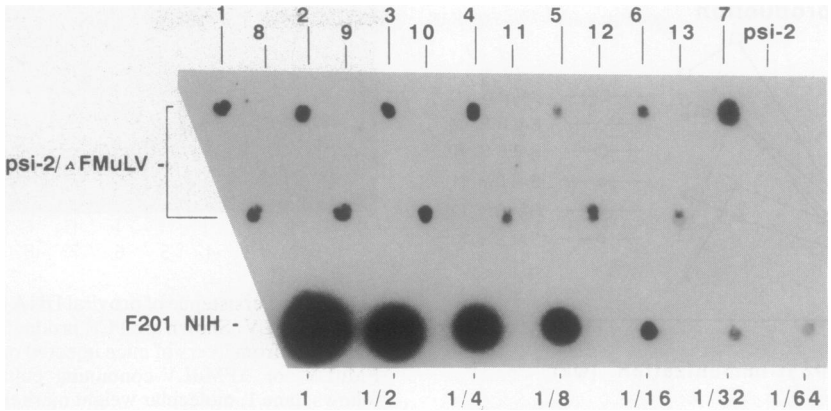

FIG. 4. vRNA in virions from pKR4-transfected ψ-2 cells. Shown are dot blots of RNA extracted from filtered culture fluids from clones of ψ-2 cells transfected with pKR4 (*Upper*) or from dilutions of culture fluids from F_{201}NIH cells (*Lower*).

BALB.B, and BALB.K (Tables 1 and 2). Tests of immunized BALB.B and BALB/c mice showed production of IgM and IgG anti-FV antibody responses (Fig. 5). Tests in immunized BALB.B mice demonstrated a strong anti-FV CTL response (Table 3). These preliminary experiments suggest that the approach is a valid one, although many aspects of the findings indicate a need for much further study and development in the system.

Among the simpler questions raised by these studies are those concerning quantitative aspects of the ΔFMuLV used. To date we have examined only fresh virion-containing culture fluids given i.p., without assaying for factors affecting the relative potencies of different batches. It will be necessary to standardize their content in vRNA-containing virions in order to optimize the values for the induction of resistance. The effects of different dosage levels and immunization schedules also remain to be optimized. It is possible that the varying levels of resistance obtained with the vaccine in different experiments with mice of different strains are due as much to factors related to these questions as to the different genetic constitutions of the mice. This seems particularly likely since the levels of protection conferred in mice of the three *H-2*-congenic strains did not correlate with the levels of innate resistance associated with their *H-2* types. Among these strains, naïve BALB.B mice show relatively strong resistance to FV, BALB.K show a moderate resistance, and BALB/c show little resistance, but the levels of protection against FV conferred by immunization did not parallel this series.

Another open question is whether there might be additional or alternative deletions from the FMuLV genome that would produce a marked improvement over the defective version studied here. The present 81-bp deletion in the p15E coding region of ΔFMuLV prevents the expression of gp70 on cell surfaces (Fig. 3) and presumably also on virion surfaces, thereby rendering any particles produced by infected cells noninfectious. However, the slightly (4%) truncated *env* gene is abundantly expressed in the cytoplasm where it is available for antigen processing and presentation to T cells in the form of oligopeptides loaded into major histocompatibility molecules (18). Candidates for alternative deletions in FMuLV include various portions of the *gag* and *pol* genes. For example, it should be possible to identify deletions in *gag* that would prevent the production of even noninfectious virus particles. However, since strong epitopes recognized by T-cell receptors in mice of different *H-2* haplotypes may occur within any viral protein, relatively short in-frame deletions that do not eliminate possible T-cell epitopes or prevent translation of the truncated gene are to be preferred.

Table 1. Protection against FV by immunization with ΔFMuLV in BALB.K mice

Group	Immunization*	Virus challenge	Splenomegalic mice/total mice,† no. 14	28	42	56	% final mortality
1	None	3‡	1/8	7/8	4/4	1/1	100
2	ψ-2	3	2/8	4/8	3/6	0/3	63
3	ψ-2/PA12	3	0/8	0/8	0/8	0/8	0
4	ψ-2	13	0/8	2/8	2/8	4/7	75
5	ψ-2/PA12	13	0/8	0/8	0/8	0/8	0

*Mice received 2-ml injections i.p. of filtered supernatant fluids from pKR4-transfected ψ-2 cell cultures or ψ-2/PA12 cocultures on days 0 and 14. Three or 13 weeks later, they were challenged with 30 SFFU of FV.
†On day after challenge.
‡No. of weeks between the second immunization and virus challenge.

Table 2. Protection against FV by immunization with ΔFMuLV in BALB.B and BALB/c mice

Virus dose, SFFU	No. of splenomegalic mice/total mice on day after virus challenge 14	28	42	56	70	% final mortality
			*BALB.B immunized**			
1000	0/5	2/5	2/5	2/5	1/4	40
333	0/5	4/5	4/5	2/3	0/1	80
100	0/5	1/5	1/5	1/5	0/4	20
33	0/5	0/5	0/5	0/5	0/5	0
			BALB.B control			
1000	1/3	2/3	3/3	—	—	100
333	2/3	3/3	2/2	1/1	—	100
100	0/3	1/3	2/2	1/2	0/1	67
33	0/3	0/3	3/3	2/3	0/1	67
			*BALB/c immunized**			
100	0/4	3/4	1/1	—	—	100
50	0/5	4/5	2/3	0/1	0/1	80
25	0/5	4/5	4/5	1/2	0/1	80
12	0/5	4/5	5/5	4/5	0/1	80
6	0/5	1/5	2/5	0/4	0/4	20
			BALB/c control			
100	0/3	3/3	1/1	—	—	100
50	0/3	3/3	2/2	—	—	100
25	0/3	3/3	2/2	1/1	—	100
12	0/3	3/3	2/2	—	—	100
6	0/3	3/3	2/2	—	—	100

*Mice received 2-ml injections i.p. of filtered culture fluids from pKR4-transfected ψ-2/PA12 cocultures on days 0 and 14. Three weeks later they were challenged with the indicated numbers of SFFU of Fv.

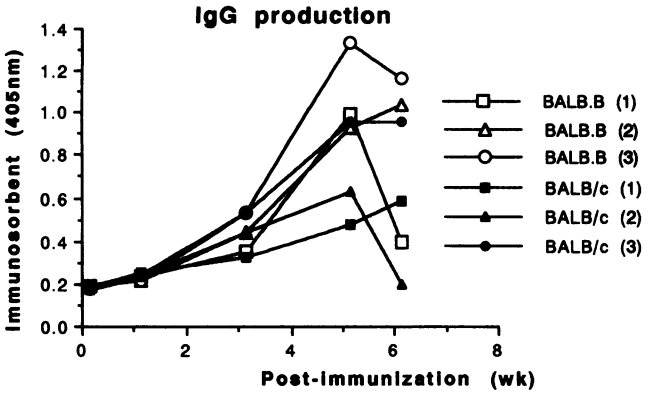

FIG. 5. Anti-FV antibody production by mice immunized with ΔFMuLV. ELISAs for anti-FMuLV^{env} IgG antibodies in sera from BALB.B and BALB/c mice immunized with ΔFMuLV on day 0, days 0 and 14, or days 0, 14, and 28 detected with antigen extracted from pKR1-transfected cells.

A more problematic question raised by these results concerns the persistence of the defective provirus in tissues of the vaccinated mice. In a small experiment we noted that the proviral form of the replication-competent parent FMuLV was no longer detected after 7 days in host liver DNA, whereas that of the ΔFMuLV derivative persisted for at least 3 weeks (Fig. 6). In an earlier unpublished study of the immunizing capacity of ψ-2-packaged helper-free SFFV virions, which do not cause disease in normal FV-susceptible hosts (22, 23), we found that injecting helper FMuLV up to 2 months after immunization rescued latent SFFV and led to full-blown FV disease. It is possible that cells infected with defective retroviruses—even those encoding strong viral antigens—are more resistant to immune elimination than those infected with their competent relatives. It might be argued that, since the present vaccine has so far shown no pathogenic properties, some level of proviral persistence might foster persistence of the anti-FV resistance obtained. However, it is not difficult to conceive of other potential problems that might ensue from proviral persistence, such as the initiation of a malignancy by proviral integration near an oncogene in the DNA of a particular host cell.

The possibility that elements of the approach presented here might be used to develop a vaccine protective against human retroviruses of the human immunodeficiency virus and human T-lymphotropic virus families remains to be explored.

Table 3. CTL response of BALB.B mice immunized with ΔFMuLV

Immunization			% ^{51}Cr release at effector:target ratio		
Day 0	Day 14	Day 28	25:1	50:1	100:1
+	+	−	4	6	14
−	+	+	40	48	67
−	−	+	5	6	18
−	−	−	2	4	9

BALB.B mice received 2-ml injections of filtered supernatant fluid from pKR4-transfected ψ-2/PA12 cocultures on the indicated days. On day 46 their spleen cells were stimulated in culture with irradiated (50 Gy) spleen cells from BALB.B mice infected with FV 14 days earlier and used 6 days later as effector cells with ^{51}Cr-labeled Df45 target cells at the indicated effector:target ratios.

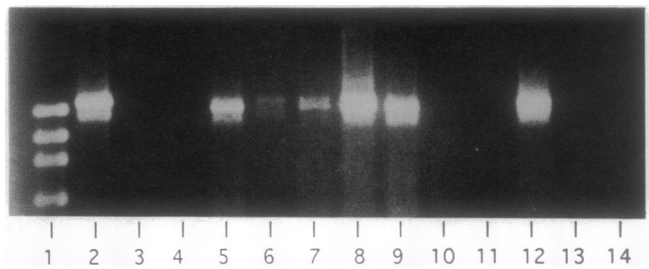

FIG. 6. Persistence of proviral DNA in tissues of mice immunized with ΔFMuLV. Shown are PCR products using ΔFMuLV^{env} primers with DNA from livers of mice injected on day 0 or days 0 and 14 with FMuLV- or ΔFMuLV-containing culture fluids. DNAs were as follows: lane 1, molecular weight markers; lane 2, plasmid 1a1c; lane 3, BALB.K normal control mice; lanes 4–7, BALB.K mice 1, 2, 7, and 14 days, respectively, after receiving ΔFMuLV once; lanes 8–11, BALB.K mice 1, 3, 7, and 21 days, respectively, after receiving FMuLV once; lane 12, BALB.B mice 28 days after receiving ΔFMuLV twice; lanes 13 and 14, BALB.B and BALB/c mice, respectively, 42 days after receiving ΔFMuLV once.

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1. Friend, C. (1957) *J. Exp. Med.* **105**, 307–318.
2. Ruscetti, S. & Wolff, L. (1984) *Curr. Top. Microbiol. Immunol.* **112**, 21–44.
3. Johnson, C. S., Thurlow, S. M. & Furmanski, P. (1986) *Cancer Res.* **46**, 183–189.
4. Lilly, F. (1967) *Science* **155**, 461–462.
5. Earl, P. L., Moss, B., Morrison, R. P., Wehrly, K., Nishio, J. & Chesebro, B. (1986) *Science* **234**, 728–731.
6. Miyazawa, M., Nishio, J. & Chesebro, B. (1992) *J. Virol.* **66**, 4497–4507.
7. Chesebro, B. & Wehrly, K. (1976) *J. Exp. Med.* **143**, 73–84.
8. Scolnick, E. M., Williams, D., Maryak, J., Vass, W., Goldberg, R. J. & Parks, W. P. (1976) *J. Virol.* **20**, 570–582.
9. Troxler, D. H., Boyars, J., Parks, W. P. & Scolnick, E. M. (1977) *Virology* **76**, 606–615.
10. Holt, C. A., Osorio, K. & Lilly, F. (1986) *J. Exp. Med.* **164**, 211–226.
11. Hartley, J. W. & Rowe, W. P. (1975) *Virology* **65**, 128–134.
12. Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33**, 153–159.
13. Miller, A. D. & Buttimore, C. (1986) *Mol. Cell. Biol.* **6**, 2895–2902.
14. Troxler, D. H., Parks, W. P., Vass, W. C. & Scolnick, E. M. (1977) *Virology* **76**, 602–615.
15. Polsky, D. & Lilly, F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9243–9247.
16. Axelrad, A. A. & Steeves, R. A. (1964) *Virology* **24**, 513–518.
17. Chesebro, B., Wehrle, K., Cloyd, M., Portis, J., Collins, J. & Nishio, J. (1981) *Virology* **112**, 131–144.
18. Ruan, K.-S. & Lilly, F. (1991) *Virology* **181**, 91–100.
19. Kozak, S. L. & Kabat, D. (1990) *J. Virol.* **64**, 3500–3508.
20. Graham, F. L. & van der Erb, A. J. (1973) *Virology* **52**, 456–467.
21. Rowe, W. P., Pugh, W. E. & Hartley, J. W. (1970) *Virology* **42**, 1136–1139.
22. Wolff, L. & Ruscetti, S. (1985) *Science* **228**, 1549–1552.
23. Bestwick, R. K., Hankins, W. D. & Kabat, D. (1985) *J. Virol.* **56**, 660–664.