half a kilometer of sediments accumulated on the sea floor. Due to the weight of Hawaii, the sea floor is depressed under the islands, and thus the oceanic sediment layer is at a depth of 9 to 10 km from the earth's surface at Kaoiki. Geodetic and seismologic evidence shows that near horizontal slip occurs at the depth of these sediments, allowing the southeast flanks of the volcanoes Kilauea and Mauna Loa to move away from the volcanoes and the rifts toward the southeast (*7, 13*) [A. S. Furumoto and R. L. Kovach, *Phys. Earth Planet. Inter.* **18**, 197 (1979); M. Ando, *J. Geophys. Res.* **84**, 7616 (1979)].
17. "Seismic quiescence" is a decrease of the rate of earthquake occurrence within the volume in question. The detection of quiescence presumes that a nearly constant background rate can be defined in the same volume. In two mainshocks in Hawaii, where detailed data were available, only parts of the source volume showed quiescence, whereas major asperities produced microearthquakes at constant rates up to the mainshock (*18, 19*).
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21. The *Volcano Letters* for seven earthquakes that might have been located in the Kaoiki area, according to the list given by Coffman and von Hake (*8*), showed that in all but one case, the writers called the earthquakes shallow (1 to 3 miles), saying they were part of ongoing eruptions and located on Kilauea or Mauna Loa. The single exception occurred on 25 May 1930. T. A. Jaggar [*Volcano Letters*, May 1930 (*8*)] did not attribute this event to either volcano. He even discussed and disproved the possibility that it might have been located at the west coast of Hawaii. He finally concluded, "All of these facts suggest a deep movement somewhere under Kilauea and Mauna Loa." In the terminology of the *Volcano Letters* of that time "deep" means about 10 km. Thus I conclude that the hypothesis of regular repeat times of the Kaoiki earthquake has passed its first test. The shock expected in 1931 ± 1.5 actually occurred in 1930.4, 11.3 years before the earliest well located event (Table 1). Using this as a fifth known value, $T_r(K)$ is estimated to be 10.7 ± 1.2 years. With six data points the prediction yields a date of 1995.25 for the next event.
22. The deformation characteristics and failure behavior of rocks in the laboratory are known to depend on strain rate. Therefore it is possible that precursors in Hawaii, where strain rates are high, may differ from those in areas of low strain rate.
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25. Supported by NSF grant ERA-8417014, the Alexander von Humboldt Foundation, and the Seismologisches Zentralobservatorium Gräfenberg, Germany. I thank R. Y. Koyanagi, T. L. Wright, P. Basham, and R. Kind for comments on the manuscript.

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T-Lymphocyte Priming and Protection Against Friend Leukemia by Vaccinia-Retrovirus *env* Gene Recombinant

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The current prevalence of the acquired immune deficiency syndrome in humans has provoked renewed interest in methods of protective immunization against retrovirus-induced diseases. In this study, a vaccinia-retrovirus recombinant vector was constructed to study mechanisms of immune protection against Friend virus leukemia in mice. The envelope (*env*) gene from Friend murine leukemia virus (F-MuLV) was inserted into the genome of a vaccinia virus expression vector. Infected cells synthesized gp85, the glycosylated primary product of the *env* gene. Processing to gp70 and p15E, and cell surface localization, were similar to that occurring in cells infected with F-MuLV. Mice inoculated with live recombinant vaccinia virus had an envelope-specific T-cell proliferative response and, after challenge with Friend virus complex, developed neutralizing antibody and cytotoxic T cells (CTL) and were protected against leukemia. In contrast, unimmunized and control groups developed a delayed neutralizing antibody response, but no detectable CTL, and succumbed to leukemia. Genes of the major histocompatibility complex influenced protection induced by the vaccinia recombinant but not that induced by attenuated N-tropic Friend virus.

INTEREST IN THE PRODUCTION OF VACcines against retroviruses has been sparked by the discovery that members of this family cause human leukemia and acquired immune deficiency syndrome (AIDS). Protective immunization against retroviruses has been achieved in some systems by using live or killed virus (*1*) or viral envelope gene (*env*)-encoded glycoprotein (*2*). In other systems, however, immunization with killed virus or viral envelope proteins induced immunosuppression and enhanced disease (*3*). Since antigen presentation by vaccinia virus recombinants mimics that of natural infections, we were interested in determining whether protection against retroviruses could be achieved by the use of a recombinant vaccinia virus carrying a retroviral *env* gene. Previously, protective immunity was demonstrated with vaccinia virus recombinants that expressed genes from members of other virus groups including influenza, herpes simplex, hepatitis B, rabies, vesicular stomatitis viruses, and respiratory syncytial virus (*4*).

The Friend virus (FV) complex system is a particularly good retrovirus model for studying the potential for immune protection since it causes erythroleukemia even after infection of immunocompetent adult animals. Furthermore, the genetics, cell biology, immunology, and molecular biology of FV have been extensively studied (*5*). The disease, characterized by hepatosplenomegaly and polycythemia or anemia, occurs 1 to 3 weeks after inoculation and usually results in death within 1 to 3 months. The virus complex consists of a replication-defective spleen focus-forming virus (SFFV) and a replication-competent helper virus, referred to as Friend murine leukemia virus (F-MuLV). By itself, F-MuLV can induce leukemias with long latencies when inoculated into newborn mice; however, the rapid leukemia observed in adult mice inoculated with FV is believed to be induced by the defective SFFV component of the complex. The *env* gene of F-MuLV encodes a glycosylated protein, gp85, which is subsequently cleaved into the closely associated proteins gp70 and p15E (*5*). The SFFV is defective in its *env* gene, and so it utilizes the envelope protein of the helper F-MuLV. Thus, both F-MuLV and SFFV virions display the same envelope protein, and monoclonal antibodies directed to determinants on this polypeptide can neutralize infectious virus (*6*). gp70 is also expressed on the surface of infected cells where it can be recognized by cytotoxic antibodies or cytotoxic T lymphocytes (CTL) (*7*). Hunsmann *et al.* (*2*) showed that repeated immunization of mice with purified envelope protein resulted in protection against challenge with FV. Therefore, we chose to make a recombinant vaccinia virus capable of expressing the gp85 product of the F-MuLV *env* gene. The entire *env* gene, including a 60-nucleotide leader at the 5′ end and approximately 150 nucleotides at the 3′ end, was inserted into the Bam HI site of the vector pGS20 (*8, 9*). In the construct, the initiation codon of the *env* gene was the first ATG codon after the vaccinia virus P7.5 promoter. The resulting recombinant plasmid pPE2 was used as a vector to transfer the *env* gene into vaccinia virus by homologous recombination (*8*). Confirmation of the predicted location and structure of the *env* gene within the vaccinia virus genome was obtained by electropho-

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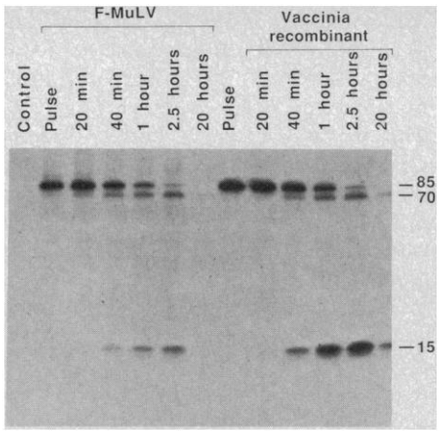

Fig. 1. Immunoprecipitation of F-MuLV envelope proteins from cells infected with F-MuLV or recombinant vaccinia virus and uninfected control. Monolayers of mouse fibroblast SC-1 cells chronically infected with F-MuLV and CV-1 cells acutely infected with the vaccinia virus recombinant were incubated at 37°C for 1 hour in methionine-free medium containing 0.5% fetal bovine serum and then for 1 hour in methionine-free medium without serum. Cells were then pulse-labeled with 100 μCi of [^{35}S]methionine (1200 Ci/mmol) for 20 minutes. One 25-cm^2 flask of each was harvested at this time. All other flasks were washed three times with medium containing 2 m*M* (unlabeled) methionine and incubated in the same medium for 20 minutes, 40 minutes, 1 hour, 2.5 hours, or overnight. To prepare cytoplasmic extracts we disrupted cells in 0.5% NP 40 detergent and removed cell debris by centrifugation for 1 minute in a microcentrifuge. Proteins were precipitated with rabbit antiserum to gp85 and resolved by SDS–polyacrylamide gel electrophoresis. The molecular weights (×10^3) of the labeled proteins are shown to the right.

retic and hybridization analysis of DNA isolated from purified virions and cleaved with various restriction enzymes.

Pulse-chase analysis of cells metabolically labeled with [^{35}S]methionine indicated that gp85 was expressed and processed in cells infected with the recombinant vaccinia virus (Fig. 1). Rabbit antisera to gp85 reacted with both the gp85 precursor and the gp70 and p15E products. During the 20-minute pulse-labeling period, only gp85 was labeled; proteolytic cleavage to gp70 and p15E occurred during the chase period in cells infected with either virus. Both gp85 and gp70 produced by cells infected with recombinant vaccinia virus were glycosylated, since they comigrated with glycosylated polypeptides from F-MuLV–infected cells. Thus, analysis of the immunoprecipitated proteins suggested comparable envelope protein synthesis, glycosylation, and processing by F-MuLV and recombinant vaccinia virus. In addition, these data showed that glycosylation and processing of the F-MuLV envelope protein occurred in the absence of other retroviral functions. A similar conclusion was derived from studies with a mutant MuLV that synthesized *env*, but no detectable *gag*- or *pol*-encoded products (*10*).

The maturation process, including formation of complex oligosaccharides, is necessary for efficient transport of MuLV envelope proteins to the plasma membrane (*11*). Using indirect membrane immunofluorescence, we observed gp70 on the surface of cells infected with either F-MuLV or recombinant vaccinia virus (Fig. 2). Thus, transport of the F-MuLV envelope protein to the plasma membrane appeared to be normal in cells infected with vaccinia recombinant virus.

Next, we wished to determine whether immunization with the recombinant virus could protect mice against subsequent challenge with FV. Because of the strong influence of the major histocompatibility complex (H-2) genes on spontaneous recovery from FV (*12*), we tested two strains of congenic F_1 mice differing in H-2 genes and susceptibility to FV. Mice were infected with recombinant vaccinia virus encoding the F-MuLV *env* gene or encoding the influenza hemagglutinin (HA) gene as a control. Additional mice were immunized with live N-tropic FV (FV-N), which spreads inefficiently and does not cause leukemia in these mouse strains at the low doses used (*13*), or with formalin-fixed F-MuLV in Freund's complete adjuvant. Mice were challenged with B-tropic FV (FV-B) 26 days after a single immunization and were examined at intervals for splenomegaly by palpation under ether anesthesia (*12*). A challenge dose of FV, lower by a factor of 10, was used for H-2(a/a) mice because of their increased susceptibility to FV.

In initial experiments with live vaccinia–F-MuLV *env* recombinant virus inoculated intraperitoneally, female mice were better protected than males. However, when vaccinia–F-MuLV *env* was inoculated via a tail skin scratch, almost complete protection was observed in both male and female mice of the H-2(a/b) genotype (Table 1). The H-2(a/a) mice were not significantly protected by this recombinant vaccinia. Similarly, formalin-fixed F-MuLV in Freund's adjuvant protected all H-2(a/b) mice, but showed only partial protection of H-2(a/a) mice. In contrast, live FV-N protected all but one individual of both mouse strains tested.

To study the possible role of different immune mechanisms for protection, we tested immunized H-2(a/b) mice for various FV-specific immune parameters before challenging them with FV-B. Mice immunized with live FV-N had FV-neutralizing antibody in their sera. Their spleen cells were positive for FV-specific CTL, and these T lymphocytes were capable of proliferating after exposure to FV leukemia cells (Table 2). In contrast, mice immunized with vaccinia–F-MuLV *env* were negative for CTL and mostly negative for neutralizing antibody, but were positive for FV-specific pro-

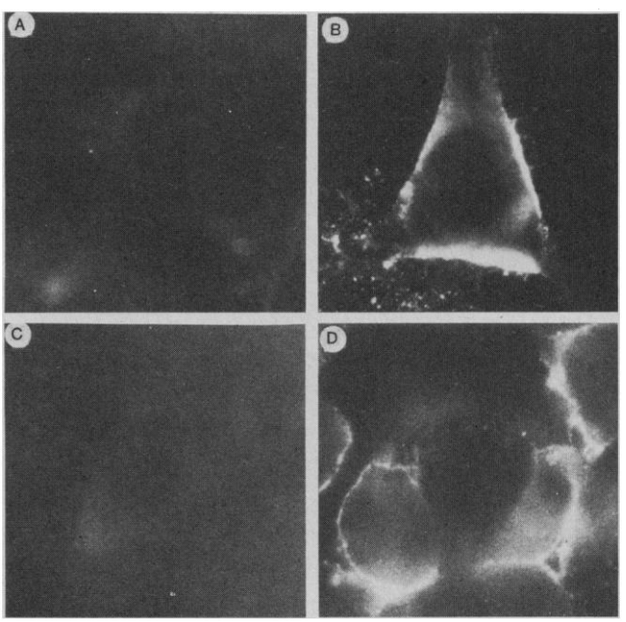

Fig. 2. Cell surface immunofluorescence of cells infected with F-MuLV or recombinant vaccinia virus. Cells were grown on eight-chamber Lab-Tek tissue culture slides. Vaccinia virus infections were done at a multiplicity of infection of 10. Cells were infected for 2 hours, overlayed with media, and incubated overnight. They were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 10 minutes, washed with PBS containing 0.5% bovine serum albumin (BSA), incubated with goat antibody to gp70 for 30 minutes on a rocking platform, washed three times with PBS containing 0.5% BSA, and incubated with fluorescein isothiocyanate (FITC)–conjugated antiserum to goat immunoglobulin G (Boehringer Mannheim Biochemicals) for 30 minutes. (A) SC-1 mouse fibroblasts; (B) SC-1 chronically infected with F-MuLV; (C) CV-1 cells infected with wild-type vaccinia virus; (D) CV-1 cells infected with vaccinia–F-MuLV recombinant. Similar results were obtained from live infected cells with the F-MuLV envelope–specific monoclonal antibody 48 (*6*) and FITC-conjugated goat antiserum to immunoglobulin G.

liferating T lymphocytes (Table 2). These results suggested that protection after immunization with the vaccinia recombinant virus did not work solely by antibody-mediated neutralization of the challenge FV-B inoculum.

This interpretation was confirmed by analysis of splenomegaly and FV replication in mice at several times early after FV challenge. Whereas none of the mice immunized with FV-N showed splenomegaly at any time, 45% of mice given vaccinia–F-MuLV *env* had splenomegaly 12 days after FV-B challenge (Fig. 3A). Most of these mice subsequently recovered. Testing of washed, dissociated spleen cells for release of infectious virus by infectious center assay indicated that all four groups tested had an increase in detectable virus by day 5 (Fig. 3B). Unimmunized and control groups, however, yielded 100- to 1000-fold more infectious centers than vaccinated groups at all times from day 5 after challenge. Nevertheless, even as late as day 35, some individuals in all groups still had detectable FV release from spleen cells. The observed recovery from leukemic splenomegaly correlated well with the appearance of both FV-specific CTL and neutralizing antibodies in mice immunized with vaccina–F-MuLV *env* (Fig. 3, C and D). Neutralizing antibody titers were high by day 10 in both protected groups. This was 7 days before antibody was found in unprotected groups, which suggested that the early antibody in immunized protected mice was a secondary immune response to the challenge virus. By days 24 to 35 in both unprotected groups neutralizing antibody titers rose to higher levels than were seen in protected groups. This result demonstrated the inability of neutralizing antibody alone to eliminate leukemic splenomegaly at this stage of disease.

Live FV-N appeared to be a more potent immunogen than recombinant vaccinia, since it protected mice of both H-2 genotypes tested and also induced neutralizing antibodies and CTL prior to FV-B challenge. This difference in immunogenicity is probably caused by the ability of the "attenuated" FV-N to replicate in mice better than the vaccinia viruses, although expression of additional F-MuLV antigens may also be a factor. Comparison of FV-specific immune response parameters both before and after challenge in protected and unprotected groups suggested that vaccinia–F-MuLV *env* induced protection by priming T-lymphocyte populations to FV-specific envelope antigens, and not by completely blocking initial infection by the challenge virus. After subsequent challenge with FV, immunized mice rapidly generated both CTL and a secondary neutralizing antibody response, both of which might contribute to the protection observed. A similar anamnestic response occurred in chimpanzees that were protectively immunized with a recombinant vaccinia virus expressing hepatitis B surface

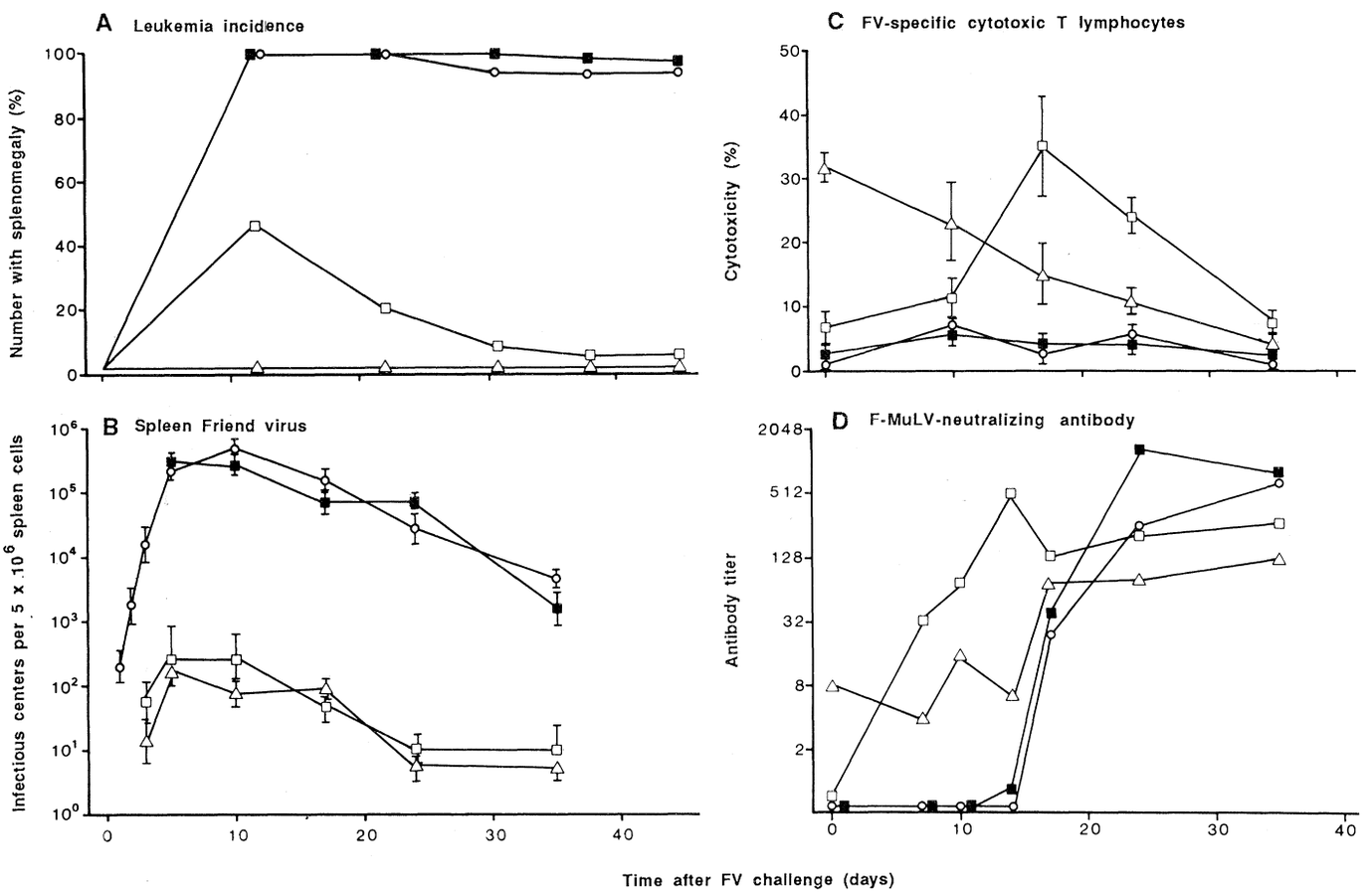

Fig. 3. (A) Incidence of leukemic splenomegaly detected by spleen palpation during ether anesthesia (43 to 57 mice per group); (B) spleen FV infectious centers; (C) FV-specific CTL; (D) F-MuLV–neutralizing antibodies. Data are shown for (B10.A × A.BY)F_1 mice, H-2(a/b), at various times before or after intravenous challenge with 2×10^4 SFFU of FV-B. Mice were immunized 26 days prior to challenge with live vaccinia–F-MuLV *env*, 1×10^7 plaque-forming units (PFU), via tail scratch (□), live vaccinia–influenza HA, 1×10^7 PFU, via tail scratch (■), or live FV-N complex, 5×10^3 SFFU intravenously (△), or they were not immunized (○). Infectious centers were detected by plating dilutions of dissociated washed live spleen cells from four to five mice per point onto NIH/3T3 cell monolayers; 4 days later we counted foci of infection using the FIA (*6*). Values shown are the geometric means ± SE. Methods for detecting FV-specific CTL were described previously (*7*). The effector-to-target ratio was 200 to 1. Values are means ± SE for four to five mice per point. Neutralizing antibodies were detected as described in Table 2. Values shown are geometric means of four to five mice per point. Standard error in all cases was less than or equal to one twofold dilution, and was omitted from the graph.

Table 1. Effect of H-2 genotype on immunization against challenge with Friend virus. Statistical significance (P) was calculated by the χ^2 test for a 2×2 table without the continuity correction, corrected for multiple comparisons with the Bonferroni correction.

Immunization*	Number leukemic/total†			
	H-2(a/b)		H-2(a/a)	
	Female	Male	Female	Male
None	25/25	24/26	19/20	19/20
FV-N	0/25‡	1/31‡	0/20‡	0/20‡
Fixed F-MuLV in CFA	0/8‡	0/12‡	6/8	4/10§
Vaccinia–F-MuLV *env*	1/28‡	2/29‡	13/15	10/15
Vaccinia–influenza HA	20/20	21/23	10/10	9/10

*Doses and routes of immunization were as follows: Live FV-N 5×10^3 SFFU, intravenously; formalin-fixed F-MuLV (44 μg) in Freund's complete adjuvant (CFA) injected in rear foot pads; live recombinant vaccinia viruses 1×10^7 plaque-forming units (PFU) in 1 μl was applied to a 3-mm needle scratch in the tail skin. †H-2 congenic FV-1(b/b) mice [(B10.A × A.BY)F_1, H-2(a/b) and (B10.A × A/WySn)F_1, H-2(a/a)] were challenged by intravenous inoculation with FV-B 26 days after a single immunization with various preparations. H-2(a/b) and H-2(a/a) mice were challenged with 2×10^4 or 2×10^3 SFFU, respectively. Data shown are number of mice with leukemic splenomegaly over total at 14 weeks after challenge and are the compiled results of three separate experiments. We examined mice under ether anesthesia by spleen palpation at approximately weekly intervals, starting 12 days after challenge. After challenge all mice survived for 8 weeks, and mice with splenomegaly died between 8 and 14 weeks. ‡$P < 0.00004$. §$P = 0.003$.

antigens and were then challenged with hepatitis B virus (*4*).

In the present studies, vaccinia–F-MuLV *env* and formalin-killed F-MuLV were unable to protectively immunize H-2(a/a) mice. Previously mice of this same H-2(a/a) strain differed from congenic H-2(a/b) mice in that they had a poor recovery rate and failed to develop FV-specific proliferating T cells even after very low doses of FV-B (*14*). The mechanism of H-2 influence on this recovery is not known, but these results suggest that major histocompatibility genes may have a strong influence on ability to immunize against human retroviruses.

A surprising finding of the present studies was the observation of residual FV-infected spleen cells in mice protectively immunized by both FV-N and recombinant vaccinia. Such cells were also found in certain strains of mice after spontaneous recovery from FV leukemia (*15*). These cells appear to represent a type of "carrier" status common to mice that have recovered spontaneously or successfully resisted FV-B challenge. Since all these protected or recovered mice had evidence of FV-specific CTL and neutralizing antibodies, it is unclear how the residual infected cells escaped immune elimination. Whether this type of carrier status might occur in humans immunized against retroviruses is important to consider in future vaccine development and use.

In conclusion, we have demonstrated that vaccinia virus vectors can be used to properly express the *env* gene of a retrovirus and that such recombinants can protect experimental animals against retrovirus disease. The immunity induced by the recombinant vaccinia virus containing the F-MuLV *env* gene, however, was weaker and influenced more by host factors than that induced by live N-tropic F-MuLV. It will now be important to characterize the FV-specific proliferating T lymphocytes induced by vaccinia–F-MuLV recombinant virus and to determine which antibody or lymphocyte populations are capable of transferring protective immunity to unimmunized recipients. Such data may be useful in considering vaccine potential of recombinant vaccinia viruses that express AIDS virus recombinant antigens (*16*).

Table 2. Effect of immunization of H-2(a/b) mice before challenge with FV.

Immunization*	FV-specific immune parameters†		
	Neutralizing antibody‡ (% positive)	Cytotoxic T lymphocytes§ (% lysis)	T-cell proliferation (cpm)
None	0	2.2 ± 0.8	2150 ± 248
FV-N	58	31.2 ± 2.6	9189 ± 851
Vaccinia–F-MuLV *env*	16	6.7 ± 2.7	9180 ± 142
Vaccinia–influenza HA	0	2.7 ± 0.6	1755 ± 286

*Doses and routes of immunization: Live FV-N, 5×10^3 SFFU, intravenously; live vaccinia recombinant viruses, 1×10^7 PFU, via tail scratch. †Immune parameters were measured 25 days after immunization; values are ±SE. F-MuLV neutralization was assayed as described (*6*) with slight modifications. Briefly, 15 μl containing 1200 focus-forming units of F-MuLV 57 plus 15 μl of antiserum dilutions plus 10 μl of a 1/8 dilution of fresh frozen guinea pig serum (as a source of complement) were mixed and incubated for 1 hour at 37°C. Cold PBS (120 μl) was added, and 50 μl was used to infect 60-mm dishes previously seeded with NIH/3T3 cells in medium containing Polybrene at 8 μg/ml. We counted foci of virus infection 4 days later, using a focal immunofluorescence assay (FIA) with monoclonal antibody 48 (*6*). Titers were defined as the highest dilution giving greater than 75% neutralization. ‡Percent of mice with neutralizing titers greater than 2. Average titer of positive mice after FV-N was 32 and after vaccinia–F-MuLV *env* was 8. There were 15 to 20 mice in each group. §CTL and FV-specific proliferating T lymphocytes were assayed as described (*7, 14*). The effector-to-target ratio was 200 to 1 in the CTL assay. In the CTL assay there were 5 mice per group, and in the T-cell proliferation assay there were 15 to 20 mice per group.

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