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# Protection against Friend Retrovirus-Induced Leukemia by Recombinant Vaccinia Viruses Expressing the *gag* Gene

MASAAKI MIYAZAWA,†* JANE NISHIO, AND BRUCE CHESEBRO

*Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, Hamilton, Montana 59840*

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**High sequence variability in the envelope gene of human immunodeficiency virus has provoked interest in nonenvelope antigens as potential immunogens against retrovirus infection. However, the role of core protein antigens encoded by the *gag* gene in protective immunity against retroviruses is unclear. By using recombinant vaccinia viruses expressing the Friend murine leukemia helper virus (F-MuLV) *gag* gene, we could prime CD4$^+$ T-helper cells and protectively immunize susceptible strains of mice against Friend retrovirus infection. Recovery from leukemic splenomegaly developed more slowly after immunization with vaccinia virus-F-MuLV *gag* than with vaccinia virus-F-MuLV *env*; however, genetic nonresponders to the envelope protein could be partially protected with Gag vaccines. Class switching of F-MuLV-neutralizing antibodies from immunoglobulin M to immunoglobulin G after challenge with Friend virus complex was facilitated in mice immunized with the Gag antigen. Sequential deletion of the *gag* gene revealed that the major protective epitope was located on the N-terminal hydrophobic protein p15.**

One of the major potential problems in developing an effective vaccine against human immunodeficiency virus (HIV) infection is the high degree of sequence heterogeneity among HIV isolates. This variability is particularly high in the third variable domain of the external envelope protein, gp120, in which the principal target for virus-neutralizing antibodies is located (4, 19, 35, 52). In contrast, sequence variability is lower in the *gag* gene that encodes internal core structural proteins (4). Therefore, Gag antigens might induce immune responses widely cross-reactive against many HIV variants. Humoral and cellular immune responses to retroviral Gag proteins in humans and animals have been documented (4, 20, 24, 31, 34, 37, 49, 50). Since Gag antigens are expressed on the surfaces of retrovirus-infected cells (13, 21, 43, 51), anti-Gag immune responses might directly eliminate virus-infected cells. Furthermore, T-helper cells primed with Gag proteins might facilitate immune responses to the envelope antigens through the hapten carrier mechanism, as has been suggested for other viral infections (16, 28, 42). Gag-specific immunity has been shown to mediate rejection of retrovirus-induced tumor cells in a mouse system (37). However, a protective role of anti-Gag immune responses against challenge with live retrovirus infection, which would better simulate HIV infection of humans, has not been demonstrated.

Friend murine leukemia retrovirus complex (FV) is composed of a replication-competent Friend murine leukemia helper virus (F-MuLV) and an acutely transforming, replication-defective, spleen focus-forming virus (17, 23, 47). FV causes rapid development of erythroleukemia associated with severe immunosuppression when inoculated into newborn or adult mice of susceptible strains. Since some strains of adult mice with mature immune systems are fully susceptible to FV-induced leukemia, FV infection of mice has provided an ideal system to analyze the role of host immune responses in spontaneous and induced resistance against retrovirus infections (7, 25).

The F-MuLV *gag* gene codes for two different precursor proteins (13). The precursor to virion core structural proteins is Pr65$^{gag}$, which is myristylated on the N-terminal glycine and proteolytically cleaved into four proteins in the virion: p15, p12, p30, and p10, from the N terminus to the C terminus. The precursor to the glycosylated cell surface Gag polyproteins, gPr80$^{gag}$, is not incorporated into the virion but instead is transported from the rough endoplasmic reticulum to plasma membrane through the Golgi complex and is expressed on the surfaces of infected cells as gP95$^{gag}$ and gP85$^{gag}$ after additional processing of carbohydrate chains and proteolytic cleavage (13). The translation of Pr65$^{gag}$ starts at the usual initiation codon ATG at position 618, while that of gPr80$^{gag}$ starts at an upstream CTG codon at position 355 within a favorable initiation context (38). Because the CTG initiation codon is in the same reading frame as the ATG codon, gPr80$^{gag}$ contains the entire amino acid sequence of Pr65$^{gag}$. In the present study, we expressed the entire F-MuLV *gag* gene or its shorter fragments with or without the CTG initiation site in recombinant vaccinia viruses and tested induction of protective immunity against Friend retrovirus infection in susceptible strains of mice.

## MATERIALS AND METHODS

**Mice.** Female C57BL/10SnJ (B10) and B10.A/SgSnJ (B10.A) mice and male A.BY/SnJ and A/WySnJ mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. BALB/cByJ mice were originally purchased from the Jackson Laboratory and have been maintained at the Rocky Mountain Laboratories. (B10 × A.BY)$F_1$, (B10.A × A.BY)$F_1$, and (B10.A × A/WySn)$F_1$ mice were bred and maintained in the animal facilities at the Rocky Mountain Laboratories. Both male and female mice, 2 to 12 months old, were used; however, sex and age distributions for each experimental group were matched as much as possible. These $F_1$ hybrid mice have the combination of genotypes *Fv-1*$^{b/b}$ *Fv-2*$^{r/s}$ *Rfv-3*$^{r/s}$ so that they would develop leukemic

* Corresponding author.
† Present address: Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980, Japan.

splenomegaly and die of leukemia after FV inoculation but could still be protected against FV under certain conditions (7).

**Virus.** The original B-tropic strain of FV was propagated in BALB/c mice as previously described (9), and the spleens were harvested 9 days after inoculation. A 20% homogenate of the spleens was prepared, and small aliquots were stored frozen at −70°C. The stock used in this study had titers of $3.2 \times 10^5$ spleen focus-forming units (SFFU) per ml for spleen focus-forming virus in $(B10.A \times A)F_1$ mice and $3 \times 10^7$ fluorescent focus-forming units per ml for F-MuLV on live *Mus dunni* cells by a focal immunofluorescence assay (44).

**Construction of recombinant vaccinia viruses expressing the F-MuLV *gag* gene.** All vaccinia virus-F-MuLV *gag* recombinants were made by cloning into the unique *Bgl*II site of pSC11SB, a derivative of the pSC11 (5) containing an oligonucleotide linker with a *Bgl*II site [d(pCAGATCTG); New England BioLabs, Beverly, Mass.] inserted at the unique *Sma*I site. Plasmid p3-4 containing a *Cla*I-*Eco*RI fragment including the whole long terminal repeat and *gag* genes of an infectious molecular clone of F-MuLV, FB29, in pBR322 (45) was cleaved with *Pst*I and *Eco*RI, and a 2.1-kb *Pst*I (position 737)-*Eco*RI fragment containing the 3′ portion of the *gag* gene was inserted into the polylinker site of pUC19. A 175-bp *Pst*I-digested fragment containing the remaining 5′ portion of the Pr65$^{gag}$ gene was then inserted into the unique *Pst*I site, and the 2.3-kb insert including the entire Pr65$^{gag}$ gene was cut out with *Sph*I and *Eco*RI. DNA ends of the *Sph*I-*Eco*RI fragment were blunted with Klenow enzyme and ligated to an oligonucleotide linker with a *Bam*HI site [d(pCGGATCCG); New England BioLabs]. This fragment was then inserted into the unique *Bam*HI site of pUC19 to form p100-2 and was later excised with *Bam*HI and recloned into the *Bgl*II site of pSC11SB to form p109-9, which was used to generate recombinant vaccinia virus-Pr65$^{gag}$ (TTTT TCT). Plasmid p100-2 was also digested with *Bam*HI and *Bgl*II, and the resultant fragment of the *gag* gene from positions 561 to 1904, which contained the sequence coding for p15, p12, and the N-terminal two-thirds of p30, was also inserted to the *Bgl*II site of pSC11SB, forming p108-2.

To remove the TTTTTCT sequence from the 5′ flanking sequence of the *gag* gene, p3-4 was cleaved with *Afl*II, *Cla*I, and *Bam*HI, and a pair of synthetic oligonucleotides

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5'-GATCCAACATGGGCCAGACTGTTACCACCCCC-3'
       3'-GTTGTACCCGGTCTGACAATGGTGGGGGAATT-5'
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that contained the 5′ portion of the *gag* sequence including the ATG initiation codon with a *Bam*HI and an *Afl*II site on the 5′ and 3′ ends, respectively, were annealed and inserted to form p17-3. This sequence (TTTTTNT) is known as a signal for the termination of transcription of the early class of vaccinia virus genes and thus might possibly affect the expression of the inserted genes in recombinant vaccinia viruses (14, 53). The *Eco*RI site was then replaced with a *Bam*HI site by inserting the oligonucleotide linker after blunting the DNA ends, forming p3-1. The resulting *gag* gene insert was excised from p3-1 with *Bam*HI and recloned into the *Bgl*II site of the pSC11SB to form p20-6, which was used to generate vaccinia virus-Pr65$^{gag}$.

To include the upstream CTG initiation codon for gPr80$^{gag}$ into the insert, p3-4 was cleaved with *Eco*RI and *Kpn*I, DNA ends were blunted with Klenow enzyme, and the *Bam*HI linker was ligated to the 2.8-kb fragment harboring the entire *gag* gene. This fragment was then cleaved with *Bam*HI and inserted into the *Bgl*II site of the pSC11SB, forming p9-28B, which was used to generate vaccinia virus-gPr80/Pr65$^{gag}$. The same *Bam*HI fragment was also inserted into the unique *Bam*HI site of pUC19 to form p8-22. This plasmid was digested with *Bam*HI and *Bgl*II, and a fragment of the *gag* gene from bases 310 to 1904 was inserted at the *Bgl*II site into pSC11SB. The resultant plasmid was used to generate vaccinia virus-p15/p12/p30$^{gag}$. To delete an additional portion of the *gag* gene, p8-22 was digested with *Stu*I and the *Bgl*II linker was inserted. A *gag* gene insert from positions 310 to 1045 was excised with *Bam*HI and *Bgl*II and cloned into pSC11SB to generate vaccinia virus-p15$^{gag}$. The structures of the *gag* genes that were reconstructed from separate fragments were confirmed by restriction enzyme digestion and DNA sequencing around the sites of ligation. All resultant recombinant vaccinia viruses were plaque purified three times.

A recombinant vaccinia virus (VSG-50$^{gag}$) expressing the *Pst*I (737)-*Eco*RI fragment of the F-MuLV *gag* gene was kindly constructed and provided by P. L. Earl and B. Moss, National Institute of Allergy and Infectious Diseases. Recombinant vaccinia viruses expressing the F-MuLV *env* gene (15) or influenza virus hemagglutinin (HA) gene (46) have been described previously.

**Immunofluorescence of the cells infected with a recombinant vaccinia virus.** The subconfluent layer of CV-1 cells seeded on a glass coverslip was infected with a low titer of a recombinant vaccinia virus overnight so that each infectious plaque was isolated. Cells were either fixed with methanol at room temperature for 15 min, washed with phosphate-buffered saline (PBS), and blocked with 10% skim milk at room temperature for 1 h before incubation with a primary antibody (fixed) or used without fixation (live). Infected cells were then incubated at 4°C overnight with hybridoma culture supernatant containing one of the following monoclonal antibodies (MAb): 34 (anti-p15$^{gag}$), 548 (anti-p12$^{gag}$), R18-7 (anti-p30$^{gag}$), and 48 (anti-gp70$^{env}$) (6, 8). To prevent damage of live cells during the incubation, hybridoma culture supernatant was supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). Cells were then washed and incubated with a 1/150 dilution of fluorescein isothiocyanate-conjugated immunoglobulin G (IgG) fraction of goat anti-rat or anti-mouse immunoglobulins (Cappel, Organon Teknika Corp., West Chester, Pa.) at room temperature for 1 h in the dark. Washing was done with phosphate-buffered balanced salt solution (9) containing 2% fetal bovine serum for live cells or with PBS containing 0.02% Tween 20 for fixed cells.

**Western blotting (immunoblotting).** Subconfluent cultures of CV-1 cells ($\sim 5 \times 10^6$ to $10 \times 10^6$) in 100-mm tissue culture dishes were infected with a recombinant vaccinia virus at a multiplicity of infection of 0.2 and incubated for 2 days. Cells scraped from one dish were centrifuged and dissolved directly into ~400 μl of sodium dodecyl sulfate (SDS) sample buffer (18, 31). Four to 10 μl of an infected cell sample was loaded on a well of an SDS–10% polyacrylamide gel, and proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore Corporation, Bedford, Mass.) after electrophoresis. After blocking with 10% skim milk at 4°C overnight, blotted membranes were incubated with hybridoma culture supernatant containing anti-p30$^{gag}$ MAb R18-7 (6) at 4°C overnight. Immunodetection of Gag proteins was done by a modified enzyme-labeled antibody technique as described previously (31).

**Immunization and protection experiments.** Mice were immunized by inoculating with $10^7$ PFU of a recombinant

vaccinia virus via tail scratch as described previously (15). Four weeks later, mice were challenged with FV complex by intravenous inoculation of 0.5 ml of a dilution via the tail vein. Some mice were challenged without prior immunization as controls for leukemogenicity of the virus stock. Mice were palpated under ether anesthesia for leukemic splenomegaly at a regular interval of 20 days. Only the mice having abnormally enlarged spleens (>0.4 g) were designated as leukemic (9). Mice dying with massive splenomegaly were designated as succumbing to leukemic death, and the incidence of leukemia at each time point was calculated by adding numbers of mice having leukemic splenomegaly and mice that had died of leukemia.

**Proliferative T-cell responses.** The assay for proliferative T-cell responses has been described previously (29). Nylon wool-passed T cells were prepared from the spleens and inguinal lymph nodes of (B10.A × A.BY)$F_1$ ($H$-$2^{a/b}$) mice 3 to 4 weeks after immunization with vaccinia virus-F-MuLV *env*, vaccinia virus-F-MuLV gPr80/Pr65$^{gag}$, vaccinia virus-F-MuLV Pr65$^{gag}$, or vaccinia virus-influenza virus HA as described above. Peritoneal exudate cells (PEC) from (B10 × A.BY)$F_1$ ($H$-$2^{b/b}$) and (B10.A × A/WySn)$F_1$ ($H$-$2^{a/a}$) mice were pulsed with purified F-MuLV virions as described previously (29) and were used as antigen-presenting cells. Friend erythroleukemia cell lines Y57-2C ($H$-$2^{b/b}$) and AA41 ($H$-$2^{a/a}$) (3, 11) were also used as stimulator cells after irradiation for 9,000 R in a cesium irradiator. T cells ($4 \times 10^5$ to $5 \times 10^5$) were mixed with $1 \times 10^4$ to $3 \times 10^5$ stimulator cells and cultured for 3 days before being labeled with [$^3$H]thymidine for 16 h. Stimulator cell dose-response curves were determined in each experiment, and the magnitude of proliferative responses is shown by mean Δ counts per minute (average incorporation of [$^3$H] thymidine after stimulation with antigen-pulsed PEC or leukemia cells minus average incorporation of [$^3$H]thymidine after stimulation with unpulsed PEC or without leukemia cells) + standard deviation as described previously (29).

**Elimination of a T-cell subset with MAb and complement.** Nylon wool-passed T cells were incubated with culture supernatant of hybridoma GK1.5 (anti-L3T4) (12) or 2.43 (anti-Lyt-2) (41) at 4°C for 1 h, washed, and then incubated with a 1/8 dilution of rabbit complement (Organon Teknika Corp., Durham, Calif.) at 37°C for 30 min to eliminate $CD4^+$ or $CD8^+$ populations. The purity of each population after MAb plus complement treatment was confirmed by flow cytometry analysis with fluorescein isothiocyanate-conjugated anti-mouse Lyt-2, phycoerythrin-conjugated anti-mouse L3T4, and a Becton-Dickinson FACStar (Becton-Dickinson Immunocytometry System, Mountain View, Calif.). Culture medium for the hybridomas was used as a negative control (complement alone). T cells ($2.5 \times 10^5$ to $5 \times 10^5$) after antibody and complement treatment were cultured either with antigen-pulsed $H$-$2^{b/b}$ PEC in the case of envelope-specific proliferative responses or with irradiated Y57-2C cells in the case of Gag-specific proliferative responses.

**Assays for virus-neutralizing antibodies.** $H$-$2^{a/b}$ mice were immunized with vaccinia virus-Pr65$^{gag}$, vaccinia virus-gPr80/Pr65$^{gag}$, vaccinia virus-F-MuLV *env*, or the vaccinia virus-influenza virus HA as described above and challenged 4 weeks later with an intravenous inoculation of 1,500 SFFU of FV. Mice were bled of 100 μl from the retro-orbital sinus 3 weeks after immunization (7 days before challenge) and 10, 20, and 40 days after challenge. To prevent possible influence of bleeding on the course of leukemia or antiviral immune responses, each mouse was bled either only once or at the beginning and the end of the experiment. The method for determining F-MuLV-neutralizing IgG and IgM titers has been described previously (15, 22, 32, 39). In brief, a sample of 15 μl of various serum dilutions, 15 μl of diluted live F-MuLV to have 30 to 50 infectious foci per well, and 10 μl of a 1/8 dilution of normal guinea pig serum as a complement source were mixed in wells of 96-well tissue culture plates and incubated at 37°C for 1 h. Culture supernatant of *Mus dunni* cells chronically infected with FB29 (45) was used as the source of F-MuLV. A part of each serum was mixed with an equal volume of 0.2 M 2-mercaptoethanol and incubated at 37°C for 30 min before dilution with 0.01 M 2-mercaptoethanol. These neutralization mixtures were then diluted by adding 120 μl of cold phosphate-buffered balanced salt solution containing 2% fetal bovine serum, and 50 μl of each mixture was inoculated onto a culture of NIH 3T3 cells in wells of 24-well tissue culture plates. The focal immunoassay with anti-F-MuLV gp70 MAb 48 (6) or 720 (39) was performed to count foci of infected cells after 4 days of infection. Neutralizing titer was defined by maximum dilution of samples giving >75% reduction of infected cell foci. The titer of IgG virus-neutralizing antibodies was determined by assaying 2-mercaptoethanol-treated sera, whereas IgG plus IgM neutralizing antibody titers were determined by assaying untreated sera.

**Statistical analyses.** $\chi^2$ test for a $2 \times 2$ table was used to compare incidences of leukemia. Means of antibody titers were compared by Student's $t$ test.

## RESULTS

**Characterization of the F-MuLV *gag* gene products expressed in vaccinia virus recombinants.** We inserted the entire F-MuLV *gag* gene from an infectious molecular clone, FB29 (45), with or without the fragment that contained the CTG initiation site for gPr80$^{gag}$, into the vaccinia virus expression vector pSC11 and produced recombinant vaccinia viruses by standard homologous recombination techniques (26) (Fig. 1). In one construct, vaccinia virus-Pr65$^{gag}$, a segment of DNA including the ATG initiation codon was replaced with a pair of synthetic oligonucleotides to remove a poxvirus transcription terminating signal, TTTTTCT, found ~20 bp upstream from the ATG codon (Fig. 1).

Cells infected with resultant recombinant vaccinia viruses expressed Gag proteins of appropriate antigenicity and relative molecular masses as detected with specific MAb. When cells were infected with recombinant vaccinia viruses that lacked the upstream sequence containing the CTG initiation codon for gPr80$^{gag}$, Gag antigens were detected only by cytoplasmic immunofluorescence after methanol fixation (Fig. 2). In contrast, when cells were infected with vaccinia virus-gPr80/Pr65$^{gag}$ containing the CTG initiation codon for gPr80$^{gag}$, antigenicities of p15, p12, and p30 were detected both on the surfaces of live cells as well as in the cytoplasms of fixed cells (Fig. 2 and 3). By Western blotting, a band with a relative molecular mass of 65,000 was detected with anti-p30 MAb R18-7 from cells infected with either vaccinia virus-Pr65$^{gag}$ or vaccinia virus-gPr80/Pr65$^{gag}$ (Fig. 4). However, from the cells infected with vaccinia virus-gPr80/Pr65$^{gag}$ additional bands with relative molecular masses of ~80,000 to 95,000 were also detectable, indicating that the cell surface Gag polyproteins gP95 and gP85 and their precursor, gPr80, were expressed (Fig. 4). Although the *gag* gene insert in vaccinia virus-Pr65$^{gag}$ and vaccinia virus-gPr80/Pr65$^{gag}$ contained the portion of the *pol* gene coding for the protease, obvious proteolysis of Pr65$^{gag}$ polyprotein

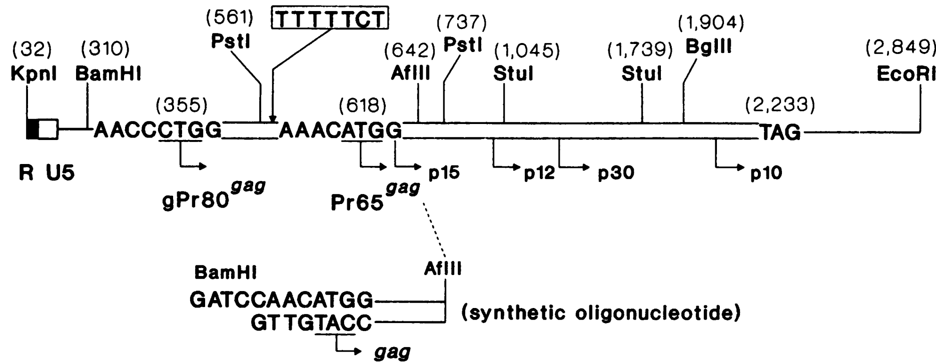


FIG. 1. A diagrammatic representation of the F-MuLV *gag* gene and synthetic oligonucleotides used for construction of the recombinant vaccinia viruses. Base numbering of the *gag* gene is according to the complete sequence of F-MuLV FB29 (36).

was not observed by radioimmunoprecipitation and Western blotting even at 3 days after infection of CV-1 cells (data not shown). Truncation of the gPr80$^{gag}$ gene by removing restriction fragments from the 3′ end resulted in two additional recombinant vaccinia viruses. Vaccinia virus-p15$^{gag}$ expressed the entire p15 plus an N-terminal small fragment of p12 up to the *Stu*I site at position 1045, and vaccinia virus p15/p12/p30$^{gag}$ expressed the entire p15 and p12 plus the N-terminal two-thirds of p30 up to the *Bgl*II site at position 1904 (Fig. 3 and 4). The latter product retained the antigenicity of p30 detected by MAb R18-7 (Fig. 3 and 4). On the other hand, no antigenicities of p12 or p30 were detectable on the surfaces of live cells (Fig. 3) or in the cytoplasms of methanol-fixed cells when infected with vaccinia virus-p15$^{gag}$. Cells infected with vaccinia virus-p15$^{gag}$ were also negative for the expression of the antigenicity of p30 in Western blotting (data not shown).

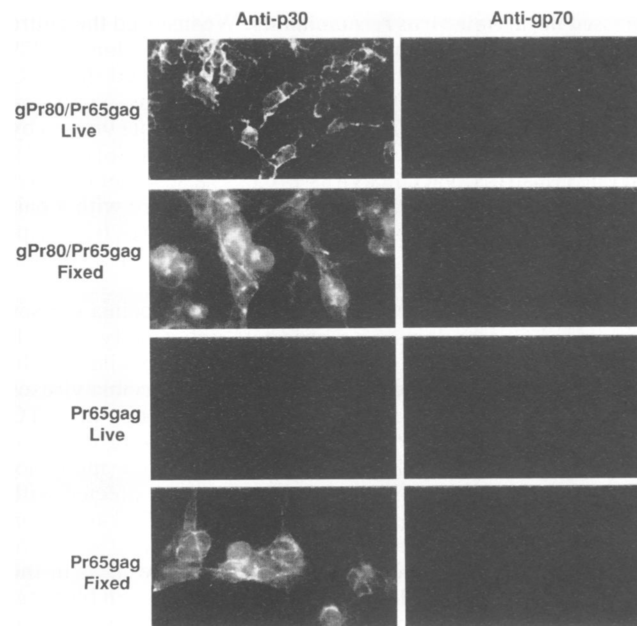


FIG. 2. Immunofluorescence of CV-1 cells infected with a recombinant vaccinia virus expressing the F-MuLV Pr65$^{gag}$ gene or the F-MuLV gPr80$^{gag}$ and Pr65$^{gag}$ genes. Infected CV-1 cells were incubated with hybridoma culture supernatant that contained either anti-p30$^{gag}$ MAb R18-7 or anti-gp70$^{env}$ MAb 48. Cells were either fixed with methanol before incubation with the first antibody or used alive without fixation. When anti-p12$^{gag}$ MAb 548 was used, results were the same as those shown here for anti-p30. The cells infected with vaccinia virus-Pr65$^{gag}$ containing the TTTTTCT sequence showed the same pattern of fluorescence as those infected with vaccinia virus-Pr65$^{gag}$ (data not shown).

**Priming of CD4$^+$ T-helper cells with vaccinia virus-F-MuLV *gag*.** Priming of T-helper cells with the Gag antigen in mice immunized with live recombinant vaccinia virus-Pr65$^{gag}$ or vaccinia virus-gPr80/Pr65$^{gag}$ was tested by antigen-specific T-cell proliferative responses in vitro. Recombinant vaccinia viruses expressing the F-MuLV *env* (15) or influenza virus HA gene (46) were used as positive and negative controls. T cells from the *H-2$^{a/b}$* mice primed with vaccinia virus-F-MuLV *env* showed an *H-2$^b$*-restricted proliferative response under the stimulation of PEC pulsed with inactivated F-MuLV particles (Fig. 5A). However, T cells from mice primed with vaccinia virus-Pr65$^{gag}$ or vaccinia virus-gPr80/Pr65$^{gag}$ showed no proliferative responses to stimulation by F-MuLV virions plus PEC in repeated experiments (Fig. 5A). In contrast, irradiated Friend erythroleukemia cells of both *H-2$^b$* and *H-2$^a$* haplotypes (Y57-2C and AA41) induced significant proliferative response of T cells from mice immunized with either vaccinia virus-F-MuLV *env* or vaccinia virus-Pr65$^{gag}$ (Fig. 5A). Therefore, in *H-2$^{a/b}$* mice immunized with recombinant vaccinia virus-Pr65$^{gag}$, proliferative T cells could recognize cell-associated Gag antigens on FV-induced leukemia cells, but these T cells seemed to be unable to recognize virion core Gag proteins in killed virus particles presented by PEC. These results suggested that the leukemia cell-associated Gag antigens contain T-cell epitopes that are absent from virion core Gag proteins or that virion-associated Gag antigens may not be properly processed by PEC to stimulate Gag-specific T-helper cells.

In both the envelope-specific and Gag-specific proliferative responses, cells proliferating under antigenic stimulation were mainly CD4$^+$ (Fig. 5B). Thus, CD4$^+$ T-helper cells were primed with Gag antigens by immunization with vaccinia virus-F-MuLV *gag*. The apparent lack of *H-2* restriction with leukemia cells as stimulators (Fig. 5A) was due to a small number of residual *H-2$^{a/b}$* antigen-presenting cells in responder T-cell fractions, because CD4$^+$ or CD8$^+$ T cells purified with a fluorescence-activated cell sorter did not show a proliferative response when cultured with the leukemia cells (data not shown).

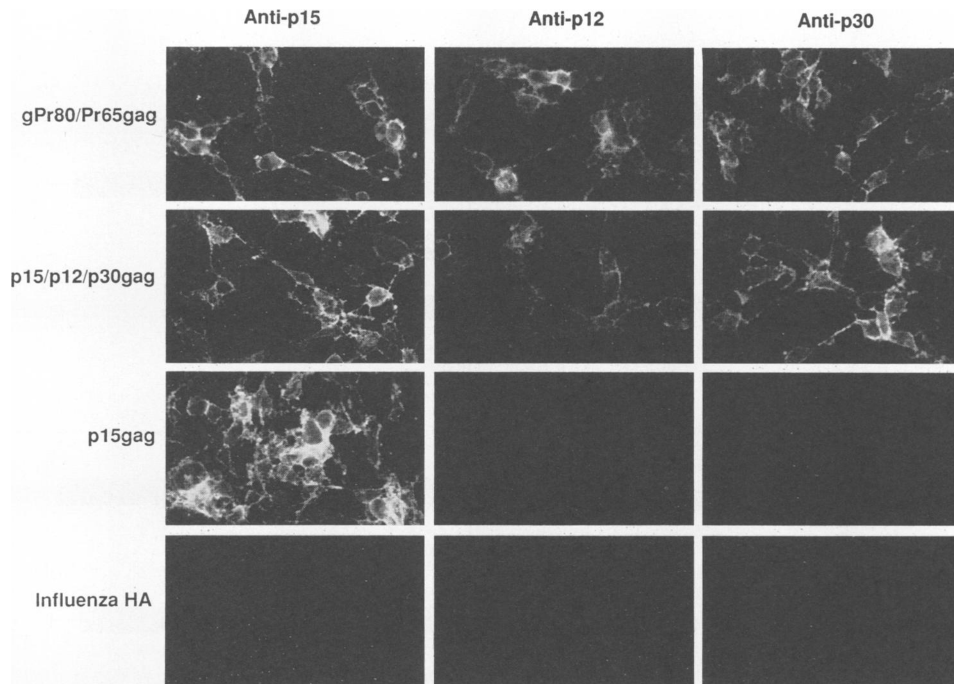


FIG. 3. Indirect immunofluorescence of CV-1 cells infected with a recombinant vaccinia virus expressing the whole or truncated F-MuLV *gag* gene. Live cells on a coverslip were incubated with one of the following MAb: 34 (anti-p15), 548 (anti-p12), R18-7 (anti-p30), and 48 (anti-gp70). Vaccinia virus recombinants tested were vaccinia virus-gPr80/Pr65$^{gag}$, vaccinia virus-p15/p12/p30$^{gag}$, vaccinia virus-p15$^{gag}$, and vaccinia virus-influenza virus HA. None of the cells infected with the four recombinant vaccinia viruses was positive for surface immunofluorescence with gp70-specific MAb 48 as a negative control (data not shown). Immunofluorescence of the methanol-fixed cells gave the same results.

**Induction of protective immunity against FV infection with recombinant vaccinia virus-*gag*.** We next tested possible induction of protective immunity against FV infection with recombinant vaccinia viruses expressing the whole or truncated F-MuLV *gag* gene in susceptible (B10.A × A.BY)$F_1$ ($H$-$2^{a/b}$) mice. When inoculated with 1,500 SFFU of FV without prior immunization, ~100% of mice of this strain developed leukemic splenomegaly within 3 weeks and >60% died of leukemia by 100 days after inoculation (7, 9, 30a). When challenged with the high dose of FV after prior

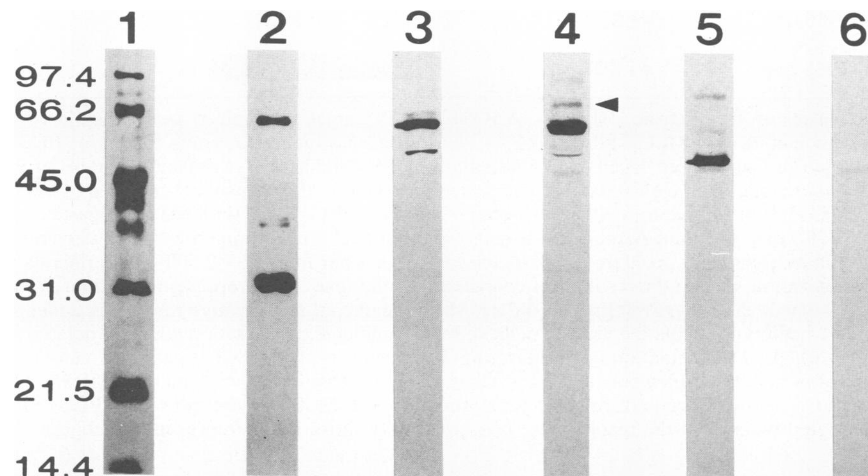


FIG. 4. Detection of Gag proteins by Western blotting of whole cell extracts with anti-p30 MAb R18-7. Lanes: 1, biotinylated molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.) (relative molecular mass, $10^3$); 2, F-MuLV particles purified by Percoll density gradient centrifugation as described previously (29, 31); 3, CV-1 cells infected with vaccinia virus-Pr65$^{gag}$; 4, CV-1 cells infected with vaccinia virus-gPr80/Pr65$^{gag}$; 5, CV-1 cells infected with vaccinia virus-p15/p12/p30$^{gag}$; 6, CV-1 cells infected with vaccinia virus-influenza virus HA. Note the strong reaction of the anti-p30 MAb with p30 and Pr65$^{gag}$ in lane 2. The arrowhead in lane 4 probably indicates gPr80$^{gag}$, and the faint band above this is probably gP95$^{gag}$ or its proteolytic cleavage product, gP85$^{gag}$. CV-1 cells infected with vaccinia virus-p15$^{gag}$ did not react with the anti-p30 MAb in the Western blotting experiments.

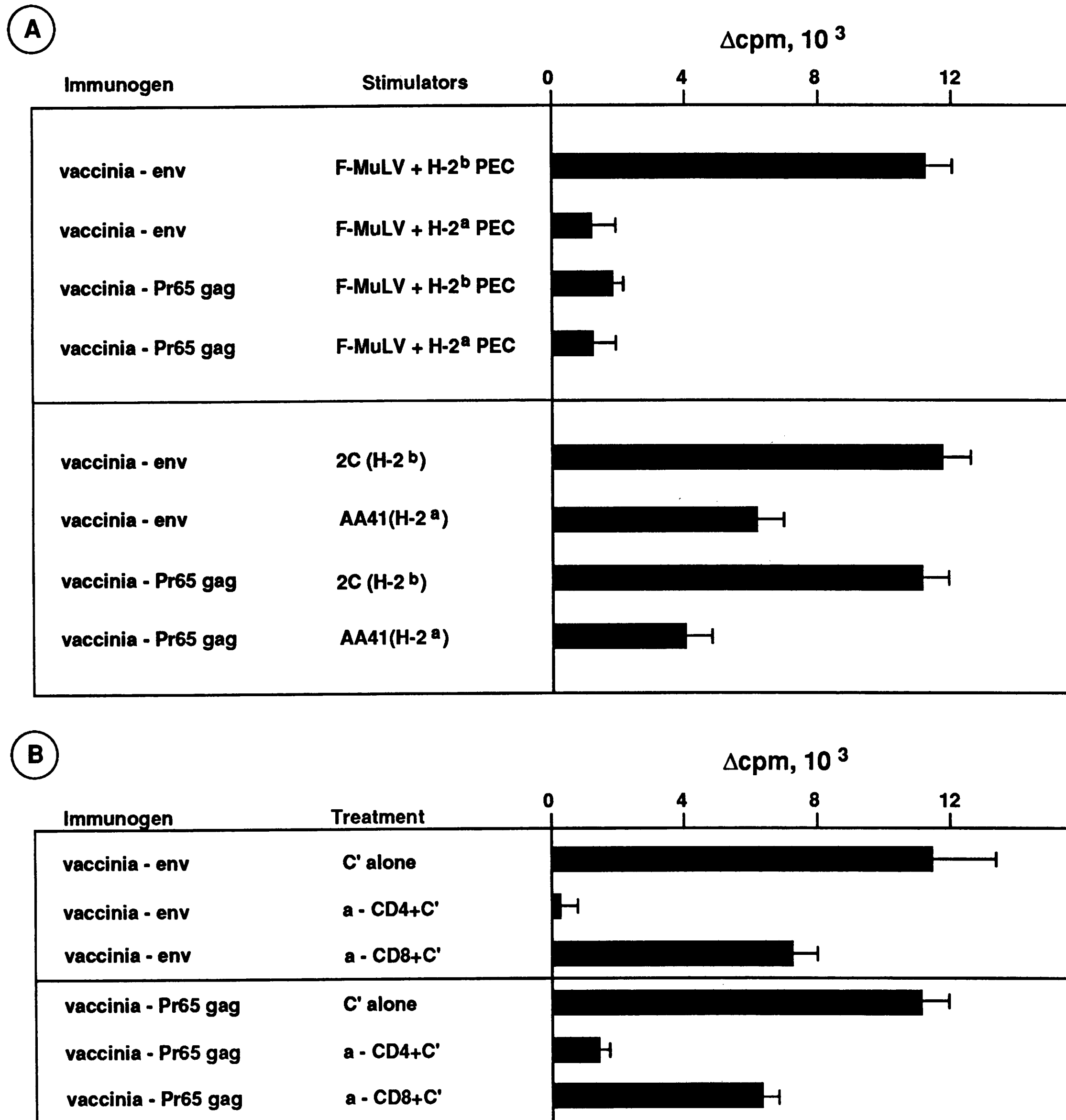


FIG. 5. Proliferative responses of T cells from (B10.A × A.BY)$F_1$ ($H$-$2^{a/b}$) mice immunized with vaccinia virus-F-MuLV *env*, vaccinia virus-F-MuLV $Pr65^{gag}$, or vaccinia virus-F-MuLV gPr80/$Pr65^{gag}$. (A) Representative proliferative responses (mean Δ counts per minute + standard deviation) of envelope- or Gag-primed T cells under stimulation of FV-induced erythroleukemia cells or PEC pulsed with F-MuLV virions. For pulsing, $10^7$ PEC were incubated with 100 μg of inactivated virions in 1 ml of phosphate-buffered balanced salt solution containing 10% fetal bovine serum at 37°C for 1 h. Stimulator cells used were $H$-$2^b$ mouse PEC pulsed with F-MuLV particles, $H$-$2^a$ mouse PEC pulsed with F-MuLV particles, Y57-2C cells (2C), and AA41 cells. T cells from the $H$-$2^{a/b}$ mice immunized with vaccinia virus-influenza virus HA as negative controls did not show significant proliferative responses (Δ counts per minute, <2,500). Experiments were repeated four times from 20 to 30 days after immunization, and all the results were consistent with those in this representative figure. T cells from the $H$-$2^{a/b}$ mice that had been immunized with vaccinia virus-gPr80/$Pr65^{gag}$ did not show significant proliferative responses under stimulation with F-MuLV virions plus PEC of either *H-2* haplotype, while the same T cells showed significant proliferation when stimulated with the irradiated Friend leukemia cells (data not shown). (B) Determination of proliferating T-cell subsets. Nylon wool-passed T cells from immunized mice were treated with anti-CD4 MAb plus rabbit complement (a–CD4 + C′), anti-CD8 MAb plus complement (a–CD8 + C′), or complement alone. After washing, T-cell proliferative assays were performed under stimulation of F-MuLV virions plus $H$-$2^b$ PEC or irradiated Friend leukemia cells. Experiments were repeated twice, and the results were consistent with those in this representative figure.

immunization, almost all of the $H$-$2^{a/b}$ mice immunized with the vaccinia virus-F-MuLV *env* were protected against FV infection, while the vaccinia virus-influenza virus HA did not protect (Fig. 6A and B). Mice immunized with recombinant vaccinia viruses expressing $gPr80^{gag}$ and/or $Pr65^{gag}$ of F-MuLV recovered slowly, but steadily, from initial development of splenomegaly (Fig. 6A). Significant protection was observed with vaccinia virus-$Pr65^{gag}$ (TTTTTCT) that contained the TTTTTCT sequence within the *gag* gene insert. Expression of the cell surface Gag polyprotein, $gPr80^{gag}$, in addition to $Pr65^{gag}$ did not change the incidence or kinetics of recovery (Fig. 6A). None of the mice that had

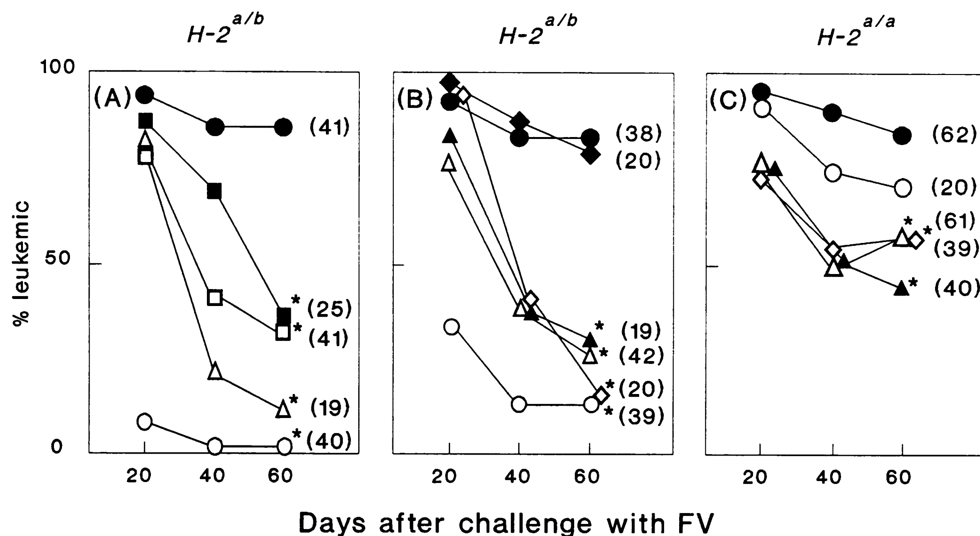


FIG. 6. Induction of protective immunity against FV infection in (B10.A × A.BY)F$_1$ ($H\text{-}2^{a/b}$) and (B10.A × A/WySn)F$_1$ ($H\text{-}2^{a/a}$) mice. Mice were immunized via tail scratch with $10^7$ PFU of a live recombinant vaccinia virus expressing one of the following F-MuLV genes: Pr65$^{gag}$ (■), Pr65$^{gag}$ containing the TTTTTCT sequence (□), gPr80$^{gag}$ and Pr65$^{gag}$ (△); p15, p12, and a part of p30$^{gag}$ (◇); and p15$^{gag}$ (▲). Recombinant vaccinia virus (VSG-50$^{gag}$) containing the *Pst*I (position 737)-*Eco*RI fragment of the *gag* gene (Fig. 1) was also tested (results in panel B [◆]). Vaccinia viruses expressing the F-MuLV *env* (○) or influenza virus HA (●) were used as controls. Four weeks after immunization, mice were challenged by intravenous inoculation of FV complex (1,500 SFFU for $H\text{-}2^{a/b}$ mice and 150 SFFU for $H\text{-}2^{a/a}$ mice) and monitored for the presence of leukemic splenomegaly or death. The total number of mice in each group is shown in parenthesis. Panels A and B show results for separate experiments with the same mouse strain, while panels B and C show results for experiments done at the same time. Asterisks indicate results significantly lower than the incidence of leukemia in mice immunized with vaccinia virus-influenza virus HA ($P < 0.01$ by $\chi^2$ analysis).

recovered from splenomegaly by 60 days after challenge showed relapse of leukemia during the follow-up period until 105 days after challenge.

In order to test which portions of the *gag* gene contained the immunoprotective epitopes, mice were also immunized with vaccinia viruses expressing a truncated form of the *gag* gene (Fig. 1 to 3). Interestingly, both constructs with deletions of the *gag* gene from the 3′ end of the gPr80$^{gag}$ sequence (vaccinia virus-p15/p12/p30$^{gag}$ and vaccinia virus-p15$^{gag}$) gave protection against the high dose of FV equal to that induced by vaccinia virus-gPr80/Pr65$^{gag}$ in $H\text{-}2^{a/b}$ mice (Fig. 6B). Similarly, a recombinant vaccinia virus expressing the *Pst*I (position 561)-*Bgl*II (position 1904) fragment of the *gag* gene could induce protection against FV challenge in the $H\text{-}2^{a/b}$ mice, which was not different in the frequency or kinetics from the protection induced with the vaccinia virus-Pr65$^{gag}$ (TTTTTCT) (data not shown). In contrast, a recombinant vaccinia virus (VSG-50$^{gag}$) that contained the *Pst*I (position 737)-*Eco*RI fragment of the *gag* gene and thus lacked expression of p15 did not induce protective immunity against FV (Fig. 6B). The cells infected with this construct expressed a partial Gag protein with a relative molecular mass of ~42,000, which was immunoprecipitable with the anti-p30 MAb and appeared to be initiated at an in-frame ATG codon in the middle of the p12 sequence (28a). Thus, it seemed likely that the p15 Gag protein, or possibly the N-terminal small fragment of p12 included in the shortest insert, contained protective epitopes.

**Class switching of virus-neutralizing antibodies from IgM to IgG in $H\text{-}2^{a/b}$ mice after immunization with vaccinia virus-*gag* and challenge with FV.** We next tested the induction of F-MuLV-neutralizing antibodies in immunized and challenged $H\text{-}2^{a/b}$ mice. Before challenge with FV, virus-neutralizing antibodies were not detectable in any sera from immunized mice. In nonimmunized mice or mice immunized with vaccinia virus-influenza virus HA, IgM neutralizing antibodies appeared at 20 days after FV challenge (Fig. 7) because of the presence of the *Rfv-3$^{r/s}$* genotype (7, 15). In contrast, immunization with vaccinia virus-F-MuLV *env* induced the production of virus-neutralizing IgG antibodies promptly after challenge, as has been demonstrated previously (15). Surprisingly, mice immunized with vaccinia virus-F-MuLV *gag* recombinant vaccines also produced virus-neutralizing IgG antibodies after challenge. Virus-neutralizing IgG antibody titers after challenge were significantly higher than IgM titers in mice immunized with the Gag antigen (Fig. 7). However, induction of virus-neutralizing IgG antibodies was slower in mice that were immunized with vaccinia virus-F-MuLV *gag* (Fig. 7) than in animals immunized with vaccinia virus-F-MuLV *env* (15). This correlated with the slower kinetics of recovery from FV-induced splenomegaly in mice immunized with vaccinia virus-F-MuLV *gag* (Fig. 6).

**Protection of envelope-nonresponder $H\text{-}2^{a/a}$ mice against FV infection with recombinant vaccinia virus-*gag*.** In contrast to $H\text{-}2^{a/b}$ mice, $H\text{-}2^{a/a}$ mice are not protected against FV by immunization with the recombinant vaccinia virus expressing the F-MuLV *env* gene (15, 32), because they are genetic nonresponders to the envelope antigen (29). $H\text{-}2^{a/a}$ mice are also genetically more susceptible to FV infection than are $H\text{-}2^{a/b}$ mice (7, 30). Therefore, we next immunized the envelope-nonresponder (B10.A × A)F$_1$ ($H\text{-}2^{a/a}$) mice with vaccinia virus-F-MuLV *gag* recombinant vaccines and tested possible protection against FV infection. Because of the increased susceptibility of $H\text{-}2^{a/a}$ mice, a challenge dose of 150 SFFU was used in the protection experiments. About half of the $H\text{-}2^{a/a}$ mice immunized with vaccinia virus-F-MuLV *gag* recovered from the initial development of splenomegaly by 60 days after challenge (Fig. 6C). Again, all

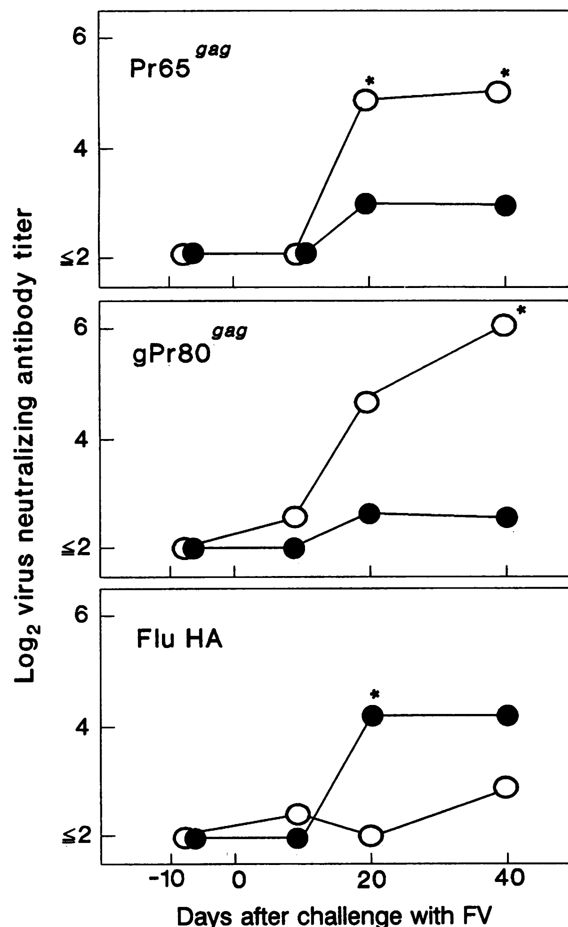


FIG. 7. Titers of IgG (○) and IgM (●) virus-neutralizing antibodies in $H\text{-}2^{a/b}$ mice immunized with a recombinant vaccinia virus and challenged with FV. Data shown here are geometric mean titers in experiments with 5 (day −7) to 21 (day 42) mice per group, and standard errors of the means were ≤1.44. The mean titer of IgG virus-neutralizing antibody was $2^{3.8}$ from mice immunized with vaccinia virus-F-MuLV *env* at 10 days after FV challenge. Asterisks indicate titers significantly higher than the titer of the other isotype ($P < 0.01$ by Student's $t$ test.)

three recombinant vaccinia viruses expressing the intact or truncated form of Gag precursor proteins were equally protective. However, vaccinia virus-F-MuLV *env* was not significantly protective in this nonresponder mouse strain. Thus, in this nonresponder mouse strain, Gag proteins can serve as an alternative to the envelope protein for the induction of partial protective immunity against retrovirus infection.

## DISCUSSION

Both humoral and cellular immune responses against retroviral Gag proteins have been described for humans and animals (4, 20, 24, 31, 34, 37, 48–50). Gag-specific cytotoxic T lymphocytes (CTL) and antibody responses in HIV-infected humans have been demonstrated (34, 49, 50), and the presence of $p24^{gag}$-specific antibodies has been correlated with better prognosis (50). In mice, polyclonal CTL or CTL lines generated by immunization with killed FV-induced tumor cells and in vitro restimulation by mixed lymphocyte-tumor cell culture recognized both *gag* and *env* gene products of F-MuLV on infected or transfected target cells (20, 24). In addition, recognition of both *env*- and *gag*-encoded antigens by CTL generated against retrovirus-induced tumor cells after mixed lymphocyte-tumor cell culture has been shown in the Gross murine leukemia virus system (37). These results in human and murine systems suggested that Gag-specific immune responses might play a role in the elimination of retrovirus-infected cells in vivo. However, a potential role of Gag-specific immune responses against challenge with infectious retroviruses has not been tested. Moreover, antigenic specificities of polyclonal CTL and CTL lines may be skewed because of immunization with killed tumor cells and mixed lymphocyte-tumor cell culture. In fact, our previous experiments with mice recovering from FV-induced splenomegaly revealed that primary CTL in recovering spleens recognized the *env*-encoded gp70 and that leukemia cell clones lacking expression of gp70 were not recognized well by the primary CTL (10). In the present study, $CD4^+$ T-helper cells from Gag-primed $H\text{-}2^{a/b}$ mice proliferated only after stimulation with irradiated Friend leukemia cells but not after stimulation with PEC pulsed with F-MuLV virions (Fig. 5A), while envelope-specific T-helper cells were stimulated well with PEC plus virions (30 and Fig. 5A). These results suggested that immunization of mice with killed leukemia cells and in vitro restimulation by mixed lymphocyte-tumor cell culture might preferentially induce Gag-specific cellular immune responses.

The present study has shown directly that recombinant vaccinia viruses expressing the *gag* gene of F-MuLV can induce protective immunity against challenge with live FV complex. After inoculation of FV, all mice developed initial splenomegaly regardless of the antigen used for prior immunization (15 and Fig. 6). However, mice immunized with recombinant vaccinia virus-*gag* recovered steadily, and more than 70% of the immunized $H\text{-}2^{a/b}$ mice and nearly half of the $H\text{-}2^{a/a}$ mice had eliminated leukemia cells by 60 days after challenge. The actual effector mechanisms involved in the protection against FV infection in mice immunized with vaccinia virus-*gag* is currently unknown. However, priming of $CD4^+$ T-helper cells with the *gag* gene products and class switching of virus-neutralizing antibodies from IgM to IgG after challenge with live FV complex in Gag-immune mice suggested cooperation between Gag-specific T-helper cells and envelope-specific effector mechanisms. Since Gag proteins are located inside the virion and anti-Gag monoclonal or polyclonal antibodies do not neutralize F-MuLV infectivity (6, 13), F-MuLV neutralization by sera from the mice immunized with the Gag antigen and challenged with FV was probably due to the presence of antienvelope antibodies. Therefore, it is possible that immunoglobulin class switching of envelope-reactive B cells was helped by Gag-specific T cells in mice immunized with vaccinia virus-F-MuLV *gag*. Similar cooperation between viral core antigen-specific T-helper cells and envelope-specific immune effector mechanisms by the hapten carrier phenomenon has been demonstrated in a few other systems (16, 28, 42). Alternatively, although unlikely, it is also possible that antibodies to N-terminal Gag protein p15 might neutralize F-MuLV infectivity, as has been suggested for the p17 Gag protein of HIV (33, 40).

Sequential deletion of the F-MuLV *gag* gene has revealed that the major protective epitope resides in the N-terminal p15 protein. The hydrophobic p15 Gag protein of F-MuLV has never been tested separately for its immunogenicity; however, a mixture of p10, p12, and p15 core proteins plus

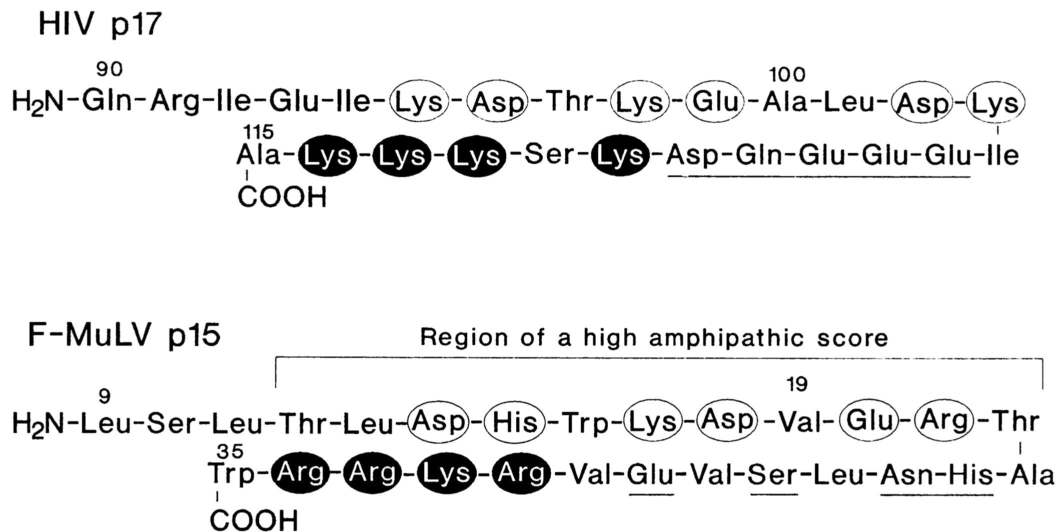


FIG. 8. Structural similarities between the antigenic peptide of HIV p17 and the putative immunogenic portion of F-MuLV p15. Both peptides have a strongly amphipathic region that contains three pairs of acidic and basic amino acids (circled). This amphipathic portion is followed by a stretch of four to five hydrophilic amino acids (underlined) and four basic amino acids (shaded circles) in both proteins.

ssp15E envelope protein from purified F-MuLV particles, along with a strong adjuvant, induced significant protective immunity against FV in $H\text{-}2^{a/b}$ mice (22). We have not yet determined the precise epitope(s) involved in immunization with vaccinia virus-p15$^{gag}$. However, it was shown recently that p17, the N-terminal HIV Gag protein analogous to F-MuLV p15, contains a peptide which is immunogenic (33, 40) and carries epitopes detected by antibodies (2), proliferating T cells (48), and CTL (1) from HIV-seropositive patients. Interestingly, computer analysis of the amino acid sequence of F-MuLV p15 by the previously described algorithm (27) revealed the presence of a potential T-helper cell antigenic site, at which this protein shares the predicted secondary structural features with HIV p17 (Fig. 8). Thus, it is possible that this region might be involved in the T-helper cell priming and immunoprotection that we have observed (Fig. 6). Future studies with peptide antigens and/or minigenes will be required to determine the precise epitopes and immune reactions which are utilized in this system.

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