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Cytotoxic T Lymphocyte–based Control of Simian Immunodeficiency Virus Replication in a Preclinical AIDS Vaccine Trial

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Abstract

Recently, encouraging AIDS vaccine trials in macaques have implicated cytotoxic T lymphocytes (CTLs) in the control of the simian human immunodeficiency virus SHIV89.6P that induces acute $CD4^+$ T cell depletion. However, none of these vaccine regimens have been successful in the containment of replication of the pathogenic simian immunodeficiency viruses (SIVs) that induce chronic disease progression. Indeed, it has remained unclear if vaccine-induced CTL can control SIV replication. Here, we show evidence suggesting that vaccine-induced CTLs control SIVmac239 replication in rhesus macaques. Eight macaques vaccinated with DNA-prime/Gag-expressing Sendai virus vector boost were challenged intravenously with SIVmac239. Five of the vaccinees controlled viral replication and had undetectable plasma viremia after 5 wk of infection. CTLs from all of these five macaques rapidly selected for escape mutations in Gag, indicating that vaccine-induced CTLs successfully contained replication of the challenge virus. Interestingly, analysis of the escape variant selected in three vaccinees that share a major histocompatibility complex class I haplotype revealed that the escape variant virus was at a replicative disadvantage compared with SIVmac239. These findings suggested that the vaccine-induced CTLs had "crippled" the challenge virus. Our results indicate that vaccine induction of highly effective CTLs can result in the containment of replication of a highly pathogenic immunodeficiency virus.

Key words: $CD8^+$ T lymphocytes • selection • MHC • SIV • Sendai virus

Introduction

Virus-specific $CD8^+$ CTL responses are critical for the control of immunodeficiency virus infections. The importance of CTLs in the control has been indicated by several clinical correlations in HIV-1–infected humans (1–3) and $CD8^+$ T cell depletion experiments in macaque AIDS

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Abbreviations used in this paper: aa, amino acid(s); B-LCL, B lymphoblastoid cell line; DGGE, denaturing gradient gel electrophoresis; L, leucine; nt, nucleotide; RSCA, reference strand–mediated conformation analysis; S, serine; SeV, Sendai virus; SHIV, simian HIV; SIV, simian immunodeficiency virus; VSV-G, vesicular stomatitis virus G; Vv, vaccinia virus.

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models (4–6). Therefore, recent vaccine approaches have focused on eliciting CTL responses (7, 8). However, HIV-1–infected individuals often have high plasma virus concentrations despite the presence of high frequencies of CTLs (9) and it has remained unclear if HIV-1 replication can be contained by vaccine-elicited CTL responses.

DNA vaccines, recombinant viral vector–based vaccines, and their combinations are promising delivery methods for AIDS vaccine because of their potential for inducing CTL responses. Recently, encouraging trials of these vaccines in macaques have implicated vaccine-induced CTLs in the control of the simian HIV (SHIV)89.6P that induces acute $CD4^+$ T cell depletion (10–14). However, most of these vaccine regimens used Env as an immunogen and it is likely that Env-specific antibodies played a role in control of this chimeric virus. Additionally, it has been suggested that SHIV89.6P may not be an appropriate challenge virus (15) and none of these vaccine regimens have been successful in the containment of the more realistic challenge of the pathogenic simian immunodeficiency viruses (SIVs) smE660, mac251, or mac239 (16–19). Thus, it is quite important to know if vaccine induction of CTL responses can lead to the containment of replication of these SIVs that induce chronic disease progression.

We previously developed a DNA-prime/Gag-expressing Sendai virus (SeV) vector boost vaccine system and showed its potential for efficiently inducing Gag-specific cellular immune responses (13, 20). In the preclinical trial, all the vaccinated macaques controlled viremia and were protected from acute AIDS progression after SHIV challenge (13, 21). In this study, we examined if CTL induction by our vaccine system can result in the containment of SIVmac239 replication.

Materials and Methods

Animals. Male rhesus macaques (*Macaca mulatta*) originally from southeastern Asia (Myanmar) were maintained in accordance with the Guideline for Laboratory Animals of National Institute of Infectious Diseases. These macaques were tested negative for SeV, SIV, and simian retrovirus type D before use. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia.

Vaccination and Challenge. An *env*- and *nef*-deleted SHIV DNA, SIVGP1, was constructed from an infectious $SHIV_{MD14YH}$ clone DNA as described previously (13, 22). The DNA is deleted with a gene fragment encoding Env surface protein (SU; nucleotide [nt] 6211 to nt 7726 in $HIV\text{-}1_{DH12}$; these sequence data are available from GenBank/EMBL/DDBJ under accession no. AF069140), the 3′ portion of the *env* gene (nt 8628 to nt 8764 in $HIV\text{-}1_{DH12}$), and the 5′ quarter of the *nef* gene (nt 9333 to nt 9481 in SIVmac239; GenBank/EMBL/DDBJ accession no. M33262). From SIVGP1 DNA, the 5′ long terminal repeat region was replaced with a CMV promoter with immediate early enhancer and the 3′ portion containing the remaining *nef* and the 3′ long terminal repeat was replaced with Simian virus 40 poly A to obtain CMV-SHIVdEN DNA. Therefore, the CMV-SHIVdEN DNA has SIV-derived *gag*, *pol*, *vif*, *vpx*, and partial *vpr* sequences and HIV-1–derived partial *vpr*, *tat*, *rev*, and partial env (nt 7726 to nt 8628 containing the second exon of *tat*, the second exon of *rev*, and RRE) sequences. At DNA vaccination, animals received 5 mg CMV-SHIVdEN DNA intramuscularly. We used two kinds of SeV vectors, a transmissible one (SeV-Gag) and an F-deleted nontransmissible one (F[−]SeV-Gag), for the boost. Recombinant SeV-Gag and F(−)SeV-Gag were constructed and recovered as described previously (20, 23, 24). 6 wk after the DNA prime, animals received 10^8 cell-infectious units of SeV-Gag or 6×10^9 cell-infectious units of F(−)SeV-Gag intranasally as a boost. Four macaques (V1, V2, V3, and V4) were vaccinated with DNA-prime/SeV-Gag-boost, and the other four (V5, V6, V7, and V8) were vaccinated with DNA-prime/F(−)SeV-Gag-boost. 13 wk after the boost, animals were challenged intravenously with 1,000 $TCID_{50}$ (50% tissue culture–infective dose) of SIVmac239 (25). An SIVmac239 molecular clone DNA, pBRmac239, was provided by T. Kodama (University of Pittsburg, Pittsburgh, PA) and R.C. Desrosiers (New England Primate Research Center, Southborough, MA), and the virus obtained from COS1 cells transfected with pBRmac239 was propagated on rhesus macaque PBMCs to prepare the SIVmac239 challenge stock.

Flow Cytometric Analysis of Virus-specific IFN-γ Induction. We measured virus-specific T cell levels by flow cytometric analysis of IFN-γ induction after specific stimulation as described previously (13). In brief, PBMCs were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs; reference 26) infected with a vaccinia virus (Vv) vector (27) for nonspecific Vv control stimulation and B-LCLs infected with a Vv vector expressing SIVmac239 Gag for Gag-specific Vv Gag stimulation, respectively. Intracellular IFN-γ staining was performed by using CytofixCytoperm kit (Becton Dickinson) according to the manufacturer's instructions. FITC-conjugated anti–human CD4, peridinin chlorophyll protein–conjugated anti–human CD8, allophycocyanin-conjugated anti–human CD3, and anti–human PE-conjugated IFN-γ antibodies (Becton Dickinson) were used. Gag-specific T cell levels were calculated by subtracting the IFN-γ^+ T cell frequencies after nonspecific Vv control stimulation from those after Gag-specific Vv Gag stimulation. Alternatively, for measurement of SIV-specific T cell levels, lymphocytes were cocultured with B-LCLs infected with a vesicular stomatitis virus G (VSV-G)–pseudotyped murine leukemia virus for nonspecific stimulation and B-LCLs infected with a VSV-G–pseudotyped SIVGP1 for SIV-specific stimulation, respectively. In the case of examining peptide-specific T cell levels, B-LCLs were pulsed with each peptide (at a final concentration of 1 μM) or peptide mixture (final concentration of each peptide was 1–10 μM) for peptide-specific stimulation or incubated without peptide for nonspecific stimulation. The peptides, including a panel of 117 overlapping peptides (15–17 amino acids [aa] in length and overlapping by 10 to 12 aa) spanning the entire SIVmac239 Gag sequence, were purchased from Sigma Genosys Japan. Specific T cell levels <100 cells per million PBMCs were considered negative, those between 100 and 200 borderline, and those >200 positive. Gag-specific T cells were undetectable before the vaccination in all of the vaccinees and before the challenge in all of the naive controls.

Quantitation of Plasma Viral Loads. Plasma RNA was extracted using High Pure Viral RNA kit (Roche Diagnostics). For quantitation of plasma SIV RNA levels, serial fivefold dilutions of RNA samples were amplified in quadruplicate by RT and nested PCR using SIV *gag*-specific primers to determine the end point as described previously (22). For preparing the RNA standard, we first set up the method for quantitation of SHIV RNA copy

number by using HIV-1 *vpu*-specific primers and an HIV-1 standard quantitated by Amplicor HIV-1 Monitor (Roche Diagnostics). By using this method, we prepared an SHIV standard for the present assay. The lower limit of detection in this assay is $\sim 4 \times 10^2$ copies/ml. The plasma viral loads at several time points were confirmed by real time PCR (28).

Sequencing. Plasma RNA was extracted using High Pure Viral RNA kit or RNA extraction system in Amplicor HIV-1 Monitor. The fragment spanning from nt 1231 to nt 2958 in SIVmac239 containing all of the *gag* region was amplified from plasma RNA by nested RT-PCR. In case of the plasma with low viral loads (<2,000 copies/ml), 8–16 tubes of nested RT-PCR amplifications were performed for each plasma to avoid obtaining only unrepresentative clones. The PCR products were sequenced using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems). Alternatively, the PCR products were subcloned into a plasmid DNA by using the TOPO cloning system (Invitrogen) and sequenced.

Isolation of Mamu-A/B cDNA Clones. Total cellular RNA was used to synthesize oligo(dT)-primed cDNA with reverse transcriptase (Superscript II; Invitrogen). Full-length cDNAs of *Mamu-A* and *Mamu-B* were amplified by PCR with locus-specific primer pairs (*Mamu-A* forward: 5′-ATGGCGCCCCGAACCCTCCTCCTG-3′, *Mamu-A* reverse: 5′-TCACACTTTACAAGCCGTGAGAGA-3′; *Mamu-B* forward: 5′-ATGGCGCCCCGAACCCTCCTCCTG-3′, *Mamu-B* reverse: 5′-TCAAGCCGTGAGAGACACATC-3′) and cloned in pGEM-T Easy vector (Promega). The integrity of the clones was verified by reference strand–mediated conformation analysis (RSCA; 29) as the following and then sequenced.

Determination of Mamu MHC-I Haplotype. Locus-specific RT-PCR products were subjected to second round PCR to obtain 725-bp-long DNA fragments encoding Mamu-A/B extracellular domains using *Mamu-A/B* universal forward (5A: 5′-ATGGCGCCCCGAACCCTC-3′) and reverse (4R: 5′-CCAGGTCAGTGTGATCTCCG-3′) primers. The product was analyzed by RSCA conformation analysis essentially as described previously (31). In brief, the second round PCR products and "a reference strand," a fragment derived from the same PCR condition except for using 5′ Cy5-labeled forward primer and a certain cloned DNA template (its sequence is available upon request), were mixed together in a reaction tube, heat denatured, and then cooled down to form heteroduplex DNA. The mobility of heteroduplex DNA molecules in 6% nondenaturing Long Ranger gel (BioWhittaker Molecular Applications) was measured by ALF express II automated sequencing apparatus (Amersham Biosciences). Fluorescence electropherograms showed multiple peak patterns corresponding to multiple, different kinds of sequences expressed in individual macaques. The identity of each peak was determined by comparison of its mobility with those of heteroduplexes derived from parallel PCR using *Mamu-A/B* cDNA clones as templates. Alleles that were shared by a breeder macaque and subset of his sons were thought to be transmitted together and assigned to a single haplotype. The number of expressed alleles on one MHC-I haplotype ranged from one *A* and two *B* alleles to no less than three *A* and five *B* alleles.

Typing of MHC-II (Mamu-DRB and Mamu-DQA). MHC-II alleles and haplotype compositions of macaques were analyzed by sequencing of cloned cDNA and denaturing gradient gel electrophoresis (DGGE; reference 30). Total RNA was extracted from B-LCLs and cDNA was generated by using SuperScript II reverse transcriptase. The entire DRB cDNA and the DQA exon 2 fragments were amplified by PCR using the following primer sets designed to hybridize with the conserved monomorphic regions: 5′-CGCGAATTCTCAGCTCAGGAGTCC-3′ and 5′-GCGGGATCCATGGTGTGTCTG-3′ for DRB, and 5′-CGCGAATTCGGTAGCAGCGGTAGAGT-3′ and 5′-GCGGGATCCGTGTAAACTTGTACCAGTT-3′ for DQA. The PCR products were subcloned into pUC19 and sequenced. When more than four clones with an identical sequence were obtained for an allele, the allele was considered to be expressed in the animal (see Table I).

The number of DRB alleles expressed in macaques and their haplotype relationships were analyzed by comparing the patterns of DGGE and by cloning and sequencing of the DNA extracted from each band in the gel. For DGGE analyses, the DRB exon 2 fragment was amplified by PCR using the forward (5′-CACTGGCTTTGGCTGGGGACAC-3′) and the GC-clamped reverse (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCGCCCCCGCCCGCAGGATACACAGTCACCTTAG-3′) primers. DGGE was performed in 6% polyacrylamide gel containing a gradient of 36–50% of the denaturant mixture (7 M urea and 40% formamide) at 100 V at 60°C for 2.5 h in a DCode system (Bio-Rad Laboratories). DNA eluted from each separate band was subcloned into a plasmid by using the TOPO cloning system and sequenced.

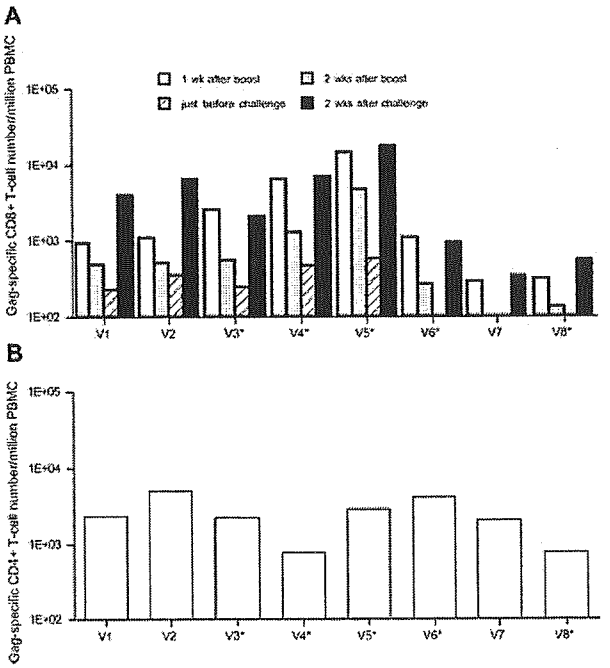


Figure 1. Gag-specific T cell frequencies in vaccinated macaques. Macaques V1, V2, V3, and V4 were boosted with a replication-competent SeV-Gag, whereas macaques V5, V6, V7, and V8 were boosted with a replication-defective F(−)SeV-Gag. *, macaques that controlled SIV replication after challenge. (A) Gag-specific $CD8^+$ T cell frequencies per million PBMCs. The frequencies at week 7 after vaccination (1 wk after boost), at week 8 after vaccination (2 wk after boost), at week 19 after vaccination (just before challenge), and at week 2 after challenge (2 wk after challenge) are shown. (B) Gag-specific $CD4^+$ T cell frequencies per million PBMCs at week 7 after vaccination (1 wk after boost). The frequencies were calculated by subtracting the IFN-γ^+ T cell frequencies after nonspecific Vv control stimulation from those after Gag-specific Vv Gag stimulation. The background IFN-γ^+ T cell frequencies after nonspecific stimulation were $<2.0 \times 10^2$.

Results

Gag-specific T Cell Induction after SeV-Gag-Boost. Our extremely simple vaccine protocol consisted of a single prime with DNA followed by a single boost with a recombinant SeV vector expressing SIVmac239 Gag 6 wk after the prime. Eight rhesus macaques (V1, V2, V3, V4, V5, V6, V7, and V8) were vaccinated with the prime/boost, and four naive controls (N1, N2, N3, and N4) received no vaccination before an intravenous SIVmac239 challenge.

We measured virus-specific T cell levels in the vaccinated macaques by flow cytometric detection of antigen-specific IFN-γ induction. SIV- and Gag-specific T cell responses were examined in PBMCs at weeks 2 and 6 after the DNA vaccination, respectively, but no responses to either SIV or Gag were detectable in any of the vaccinated macaques (not depicted). After the SeV boost, however, we found induction of Gag-specific $CD8^+$ T cells in all of the vaccinees (Fig. 1 A). The levels differed among the macaques, with five (V1, V2, V3, V4, and V5) maintaining detectable levels of Gag-specific $CD8^+$ T cells until challenge. The SeV boost also induced Gag-specific $CD4^+$ T cells in all eight vaccinees (Fig. 1 B).

Control of SIVmac239 Replication in Five of Eight Vaccinees. These vaccinated macaques were challenged intravenously with 1,000 $TCID_{50}$ of SIVmac239 at week 19 after the DNA prime (13 wk after the SeV boost). The unvaccinated control macaques had high peak viremia (>10^7 SIV RNA copies/ml plasma) on day 10 after challenge and maintained relatively high plasma viral concentrations (10^4–10^6 SIV RNA copies/ml plasma; Fig. 2). Three of them showed gradual loss of percent CD4 in peripheral T lymphocytes. In contrast, five vaccinated macaques (V3, V4, V5, V6, and V8) controlled replication of this highly pathogenic challenge virus. In these macaques, plasma viremia became undetectable after week 5 and peripheral $CD4^+$ T cells were maintained. The other three vaccinees (V1, V2, and V7) failed to control virus replication and

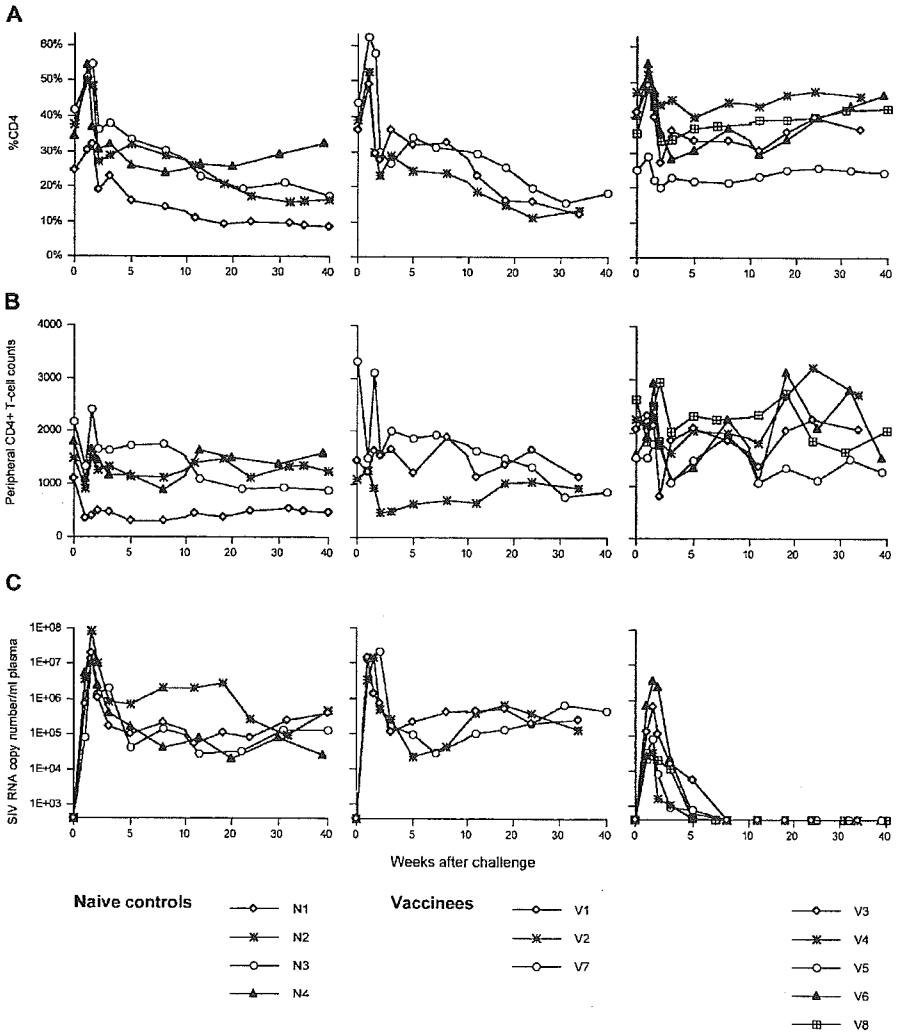
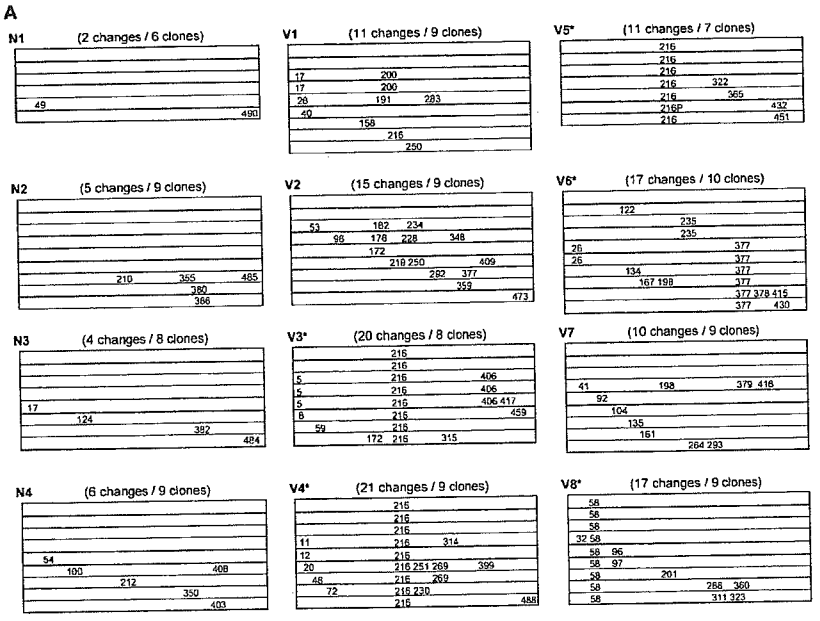


Figure 2. Changes in peripheral $CD4^+$ T cell levels and plasma viral loads after SIVmac239 challenge. (A) Percents of $CD4^+$ T cells in peripheral blood. (B) $CD4^+$ T cell counts in peripheral blood. (C) Plasma viral loads (SIV RNA copy number/ml). The left panels show the naive controls (N1, N2, N3, and N4), the middle panels show the vaccinees that failed to control SIV replication (V1, V2, and V7), and the right panels show the vaccinees that controlled SIV replication (V3, V4, V5, V6, and V8). The portion until week 10 after challenge is enlarged.



Animal	CTL escape mutant	Frequencies of the mutant		
		week 2	week 3	week 5
V3	mutation at the 216th aa	1/9	5/9	8/8
V4	mutation at the 216th aa	0/8	9/9	9/9
V5	mutation at the 216th aa	0/7	3/9	7/7
V6	mutation at the 377th aa	ND	1/10	6/10
V8	mutation at the 58th aa	ND	0/9	9/9

Figure 3. Mutations in SIV *gag*. (A) Schematic representation of the positions of aa changes in SIV Gag in each macaque after challenge. 6–10 clones of plasmids carrying the whole *gag* region amplified from plasma RNA at week 5 after challenge were obtained from each macaque and sequenced. Each lane represents the whole *gag* sequence derived from each clone and the positions of aa changes detected are indicated. Total number of aa changes and number of clones sequenced are shown in the parentheses. All the changes at aa 58 were glutamine to lysine, and all at aa 377 were isoleucine to threonine. All the changes at aa 216 other than the one indicated as 216P were L to S. The 216P represents L to P change at aa 216. (B) Frequencies of the CTL escape mutants at weeks 2, 3, and 5 in the vaccinees that controlled SIV replication. The ratio of the number of the clones with the escape mutation to the number of the sequenced clones is shown.

showed gradual loss of percent CD4 in peripheral T lymphocytes similar to the naive control animals. One of them (macaque V2) was killed at week 42 because of dyspnea, loss of body weight, and loss of peripheral $CD4^+$ T cells (4.4%, 97 cells/μl at week 42). Autopsy revealed that this animal developed AIDS with *Pneumocystis carinii* pneumonia.

At week 2 after challenge, we detected anamnestic Gag-specific $CD8^+$ T cell responses in all of the vaccinated macaques, indicating efficient secondary responses during the acute phase of infection (Fig. 1 A). These levels varied from macaque to macaque. Macaque V5 showed the highest level of Gag-specific $CD8^+$ T cells and macaque V7 showed the lowest. No significant difference in the levels was observed between the macaques that controlled viral replication and those that did not. The magnitude of the total prechallenge Gag-specific $CD8^+$ T cell or $CD4^+$ T cell responses did not appear to correlate with the level of control. We examined plasma-neutralizing activities against SIVmac239 as described previously (31), but found no neutralizing activities in any of the controls or the vaccinees at weeks 5 or 12 after challenge (not depicted), indicating that neutralizing antibodies were not essential for the control of SIV replication observed in this experiment.

Rapid Selection of CTL Escape Variants in the Vaccinees That Controlled SIVmac239 Replication. To determine whether vaccine-induced Gag-specific T cell responses exerted a selective pressure on the virus, we sequenced the SIV *gag* region in the viral genomes obtained from plasma RNA at week 5 after challenge (Fig. 3 A). The numbers of aa changes per clone in the vaccinated macaques were significantly higher than those in the unvaccinated (mean: unvaccinated, 0.51; vaccinated, 1.75; $P = 0.0006$ by t test). This may reflect the immune pressure by vaccine-induced Gag-specific T cell responses. Interestingly, all of the macaques that controlled SIVmac239 replication (V3, V4, V5, V6, and V8), but not those unable to control the virus, showed consistent aa changes in Gag (Fig. 3 A). Among them, three macaques (V3, V4, and V5) had a common aa change, leucine (L) to serine (S) at the 216th aa in Gag. We then examined peptide-specific T cell responses after the SeV boost and found, in these three macaques but not in the other vaccinees, efficient expansion of $CD8^+$ T cells specific for an epitope ($Gag_{206-216}$; IINEEAADWDL) spanning from the 206th to the 216th aa in SIVmac239 Gag. Interestingly, these three macaques showed no or diminished recognition of the mutant peptide, IINEEAADWDS ($Gag_{206-216}$L216S; Fig. 4 A), indicating that this mutant likely represents an escape variant. Sequence analysis of vi-

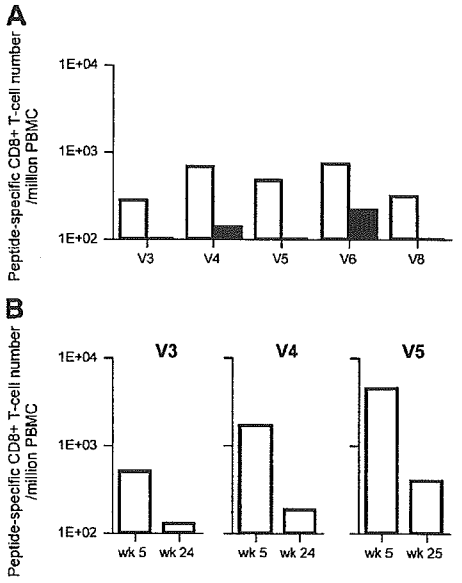
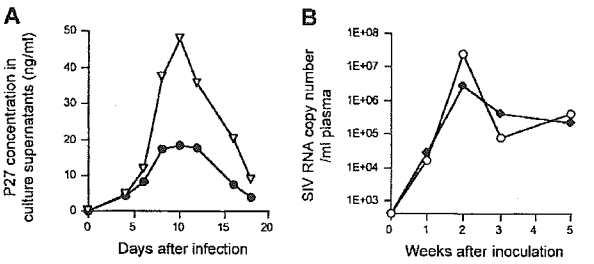


Figure 4. Peptide-specific T cell frequencies in the vaccinees that controlled SIV replications. (A) Comparison between the epitope peptide–specific and the variant peptide–specific $CD8^+$ T cell responses. PBMCs at week 10 after vaccination in macaque V3, at week 10 after vaccination in V4, at week 15 after vaccination in V5, at week 3 after challenge in V6, and at week 3 after challenge in V8 were used. The open bars indicate the levels of $CD8^+$ T cells specific for $Gag_{206-216}$ peptide in V3, V4, and V5, $Gag_{367-381}$ peptide in V6, and Gag_{50-65} peptide in V8, respectively. The solid bars indicate the levels of $CD8^+$ T cells specific for $Gag_{206-216}$L216S peptide in V3, V4, and V5, $Gag_{367-381}$I377T peptide in V6, and Gag_{50-65}Q58K peptide in V8, respectively. (B) $Gag_{206-216}$-specific $CD8^+$ T cell levels in macaques V3, V4, and V5 after challenge. The background IFN-γ^+ $CD8^+$ T cell frequencies after nonspecific stimulation were $<1.0 \times 10^2$.

ral genomes from the three macaques that made responses to this epitope at weeks 2 and 3 revealed that this CTL escape mutant became dominant around week 3 after challenge (Fig. 3 B). Thus, in these three macaques with high levels of $Gag_{206-216}$-specific $CD8^+$ T cells, the wild-type challenge virus disappeared quickly and only the CTL escape mutant was detectable in plasma at week 5. These three macaques had high levels of $Gag_{206-216}$-specific $CD8^+$ T cells 3 wk after challenge. However, these levels were considerably reduced in the chronic phase (Fig. 4 B).

We further examined epitope-specific $CD8^+$ T cell responses in the other two macaques that controlled viral replication. In macaque V6, a mutation leading to a change at the 377th aa (isoleucine to threonine) was observed at week 5 (Fig. 3 A). Analysis of peptide-specific responses revealed that this macaque had a high level of $CD8^+$ T cells specific for a 15-mer peptide corresponding to aa 367–381 in SIVmac239 Gag ($Gag_{367-381}$) at week 3 after challenge. Stimulation by the mutant $Gag_{367-381}$ peptide with the substitution ($Gag_{367-381}$I377T) induced IFN-γ^+ $CD8^+$ T cells, but their frequency was lower than that after stimulation by the wild-type $Gag_{367-381}$ peptide (Fig. 4 A). Additionally, viruses from macaque V8 had a mutation leading to a change at the 58th aa (glutamine to lysine; Fig. 3 A). In this animal, $CD8^+$ T cell responses specific for a 16-mer peptide corresponding to aa 50–65 in SIVmac239 Gag (Gag_{50-65}) were observed at week 3 after challenge. Stimulation by the mutant Gag_{50-65} peptide with the substitution (Gag_{50-65}Q58K) failed to induce IFN-γ^+ $CD8^+$ T cells (Fig. 4 A). Each of these mutants became dominant at approximately week 5 after challenge in the corresponding macaque (Fig. 3 B).

Among the 12 macaques used in the challenge experiment, 8 macaques (2 naive controls and 6 vaccinees) descended from a single male, macaque R90-120 (its sons: N2, V2, and V3; its grandsons: N3, V4, V5, V6, and V7; Table I). Analysis of MHC-I *Mamu-A* and *Mamu-B* alleles indicated that four macaques of the eight R90-120 descendants, N2, V3, V4, and V5, share an MHC-I haplotype (90–120-Ia) derived from macaque R90-120. Analysis of MHC-II also suggested that these macaques possibly share an MHC-II haplotype derived from macaque R90-120. Among these macaques possessing the 90–120-Ia haplotype, three (V3, V4, and V5) were vaccinees that controlled SIV replication with high levels of $Gag_{206-216}$-spe-



C

Animal	Sequencing	Frequencies of the L216S mutant			
		week 1	week 2	week 3	week 5
M1	direct	++	+	-	-
	clones	ND	3/10	0/10	ND
M2	direct	++	-	-	-
	clones	ND	0/10	0/10	ND

Figure 5. Comparison of replication efficiencies between the wild-type SIVmac239 and the escape variant SIVmac239G216S. (A) Replication kinetics of SIVmac239 (▽) and SIVmac239G216S (●) in macaque PBMCs. MT4 cells were transfected with pBRmac239 and pBRmac239G216S to obtain SIVmac239 and SIVmac239G216S, respectively. PBMCs were infected with the viruses at a multiplicity of infection of 0.0002 and concentrations of SIV Gag p27 in their culture supernatants were measured by ELISA (Beckman Coulter). A representative result from three independent experiments is shown. (B) Plasma viral loads (SIV RNA copy number/ml) in macaques M1 (○) and M2 (◆) after inoculation with both of the wild-type SIVmac239 molecular clone DNA and the mutant SIVmac239G216S molecular clone DNA. (C) Frequencies of the mutant viral genome in plasma in the macaques inoculated with both of the wild-type SIVmac239 molecular clone DNA and the mutant SIVmac239G216S molecular clone DNA. In case of direct sequencing of the PCR products (indicated by direct), ++ indicates detection of both the wild-type and the mutant at comparable levels, + indicates detection of the wild-type predominantly and the mutant slightly, and − indicates detection of the wild-type only. In case of sequencing clones (indicated by clones), the ratio of the number of the mutant clones to the number of the sequenced clones is shown.

Table I. *MHC-I and MHC-II Alleles of Macaques Used in This Study*

Animal[a]	Father	MHC-I *Mamu-A & B* RSCA pattern[b]	MHC-II *Mamu-DRB & DQA* alleles[c]
Naive control			
N1	R90-088	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
			*DRB(AB112040), DRB*W2603, DRB*W402, DQA1*0502*
N2	R90-120	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB(Z26165), DRB(AB112039), DRB(AB112043), DQA1*06(MM76195)*
N3	R94-027[d]	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB*W2104, DRB*W2603, DRB*W606, DQA1*0502*
N4	R90-010	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB*0321, DRB*0323, DRB*W606, DQA1*05(M76227)*
Vaccinee			
V1	R90-088	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
			*DRB*W2505, DRB(AB112046), DRB(AB124813), DQA(AB124814)*
V2	R20-120	90-120-Ib	*DRB*W2002, DRB*W2501, DQA1*0502*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V3	R90-120	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V4	R94-027	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			*DRB*W2505, DRB(AB112046), DRB(AB124813), DQA(AB124814)*
V5	R94-027	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
			DRB(AB112043), DRB(AB112047)
V6	R94-027	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
V7	R94-027	*2	*DRB1*0316, DRB*W2507, DQA1*01(M76202)*
			*DRB*W2104, DRB*W2603, DRB*W606, DQA1*0502*
V8	R90-010	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB1*0316, DRB*W2507, DQA1*09(M76200)*
Breeder			
R90-088	unknown	*1	*DRB1(Z26148), DRB*W502, DQA1*03(M76230)*
R90-120	unknown	90-120-Ia	*DRB1*1007, DRB1(Z26137), DQA1*03(M76228)*
		90-120-Ib	*DRB*W2002, DRB*W2501, DQA1*0502*
R90-010	unknown	ND	*DRB*W2104, DRB*W2603, DQA1*0502*
			*DRB1*0316, DRB*2507, DQA1*01(M76202)*

[a]The underlined macaques showed control of SIV replication.
[b]MHC-I *Mamu-A* and *Mamu-B* alleles and haplotype compositions of macaques were examined by RSCA and sequencing of cloned cDNA. The haplotype 90-120-Ia derived from macaque R90-120 consists of three *Mamu-A* alleles (*Mamu-A120-1*, *Mamu-A120-4*, and *Mamu-A120-5*) and four *Mamu-B* alleles (*Mamu-B120-1*, *Mamu-B120-6*, *Mamu-B120-8*, and *Mamu-B120-9*). The haplotype 90-120-Ib derived from macaque R90-120 consists of two *Mamu-A* alleles (*Mamu-A120-2* and *Mamu-A120-3* [= *Mamu-A*05*]) and five *Mamu-B* alleles (*Mamu-B120-2*, *Mamu-B120-3*, *Mamu-B120-4*, *Mamu-B120-5* [= *Mamu-B*36*], and *Mamu-B120-7*). Macaques N1 and V1 shared an RSCA pattern of a haplotype derived from R90-088 (*1). Macaques N3, V6, and V7 shared an RSCA pattern of a haplotype not derived from R90-120 (*2).
[c]MHC-II *Mamu-DRB* and *Mamu-DQA* alleles were analyzed by DGGE and sequencing of cDNA. The determined alleles are shown. Each number in parentheses indicates the corresponding accession number for the nt sequence of the allele that has not yet been designated.
[d]The father of macaque R94-027 is macaque R90-120.

cific $CD8^+$ T cell responses. The remaining one (naive control macaque N2) showed a detectable level of $Gag_{206-216}$-specific $CD8^+$ T cell responses at week 3 after challenge, although the level was low (2.5×10^2 cells/million PBMCs). These results strongly suggest that the $Gag_{206-216}$ epitope is restricted by an MHC-I molecule derived from the 90–120-Ia haplotype.

Diminished Replicative Ability of the CTL Escape Variant SIV. We then explored the hypothesis that the escape mutation selected by the vaccine-induced $Gag_{206-216}$-spe-

cific CTL resulted in a loss of viral fitness. We constructed a molecular clone of the escape mutant SIV, referred to as SIVmac239G216S, with a mutation resulting in the L to S substitution at the 216th aa in Gag. The mutant SIV was replication competent in vitro but showed lower levels of proliferation kinetics in PBMC culture compared with the wild-type SIVmac239 (Fig. 5 A). To compare the SIVmac239G216S replication kinetics with the wild-type in macaques, two macaques (M1 and M2, neither of them descended from macaque R.90-120) were coinoculated intramuscularly with 5 mg of the SIVmac239 molecular clone DNA (pBRmac239) and 5 mg of the SIVmac239G216S molecular clone DNA (pBRmac239G216S; Fig. 5, B and C). Both viral genomes were detected at comparable levels in plasma from both of the macaques at week 1 after the inoculation. After that, however, the mutant SIVmac239G216S disappeared and the wild-type SIVmac239 became dominant. Neither of the macaques showed $Gag_{206-216}$-specific $CD8^+$ T cell responses at week 3 (not depicted). These results indicate that the L to S change at the 216th aa in Gag is disadvantageous for SIV replication in the absence of $Gag_{206-216}$-specific $CD8^+$ T cell responses in macaques.

Discussion

In this study, we present evidence indicating that vaccine-induced CTLs control SIVmac239 replication in rhesus macaques. Each of the macaques that controlled viral replication had a mutation in Gag leading to an aa change in a CTL epitope by week 5 after challenge, reflecting strong CTL-induced selective pressure. This finding lends support to the notion that epitope-specific CTL responses played a central role in the control of replication of the SIVmac239 challenge virus because it was difficult to detect the challenge virus at week 5 after challenge.

Among the 12 macaques used in the challenge experiment, 8 macaques descended from macaque R.90-120 and 4 of them shared an MHC-I haplotype, 90-120-Ia. Among the four, not the naive (N2) but the three vaccinees (V3, V4, and V5) controlled SIV replication and selected for the same $Gag_{206-216}$-specific CTL escape variant with L to S change at the 216th aa in Gag. Therefore, we examined the reproducibly selected escape variant SIVmac239G216S intensively and found that in the absence of $Gag_{206-216}$-specific $CD8^+$ T cell responses, its replication efficiency is diminished compared with the wild-type SIVmac239 in vivo as well as in vitro. The rapid selection of the escape variant with lower viral fitness in the vaccinees with $Gag_{206-216}$-specific CTLs indicates that the vaccine-induced CTLs exerted strong immune pressure leading to clearance of the wild-type SIVmac239.

The emergence of escape variants depends on the balance between CTL-induced immune pressure and viral fitness costs (32). Viral escape from CTLs during the acute phase of natural immunodeficiency virus infections has been observed in Tat, Nef, Vpr, and Env (33–36). Escape variants with mutations in the structural protein Gag have been also reported (37), but it has been shown that they mostly diminish viral fitness and require multiple additional compensatory mutations to restore their replicative competence (38–41). Indeed, the $Gag_{206-216}$-specific CTL escape variant selected in macaques V3, V4, and V5 diminished viral replication. Therefore, our results suggest that the vaccine-induced CTLs were crucial to the rapid containment of replication of the challenge virus and selected for the virus with diminished replicative ability. Without compensatory mutations, the crippled virus might be easily controlled by the immune system.

The macaques used in our challenge experiment were non-Indian rhesus and the setpoint plasma viral loads in the naive control group might be lower than those usually observed in SIVmac239-infected Indian rhesus. However, the viral loads are higher than those typically observed in untreated humans infected with HIV-1 and equivalent to viral loads seen in SIVsmE660-infected Indian rhesus (16, 42). Indeed, all of the naive animals failed to control the virus replication after SIVmac239 challenge, indicating that CTLs are unable to contain and clear the virus in natural SIVmac239 infections of our non-Indian rhesus macaques. Thus, this study provides clear evidence demonstrating that vaccine induction of effective CTLs that can cripple the virus can result in the containment of replication of a neutralization-resistant, highly pathogenic immunodeficiency virus that is unable to be contained in the natural chronic course of infections. In conclusion, our results show that vaccine-induced CTLs can control SIVmac239 replication and indicate that induction of highly effective CTLs might be critical for the vaccine-based containment of immunodeficiency virus replication.

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Identification of a Protective CD4$^+$ T-Cell Epitope in p15^{gag} of Friend Murine Leukemia Virus and Role of the MA Protein Targeting the Plasma Membrane in Immunogenicity

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Recent studies have demonstrated an essential role of Gag-specific CD4$^+$ T-cell responses for viral control in individuals infected with human immunodeficiency virus type 1. However, little is known about epitope specificities and functional roles of the Gag-specific helper T-cell responses in terms of vaccine-induced protection against a pathogenic retroviral challenge. We have previously demonstrated that immunization with Friend murine leukemia virus (F-MuLV) Gag proteins protects mice against the fatal Friend retrovirus (FV) infection. We report here the structure of a protective T helper cell (Th) epitope, (I)VTWEAIAVDPPP, identified in the p15 (MA) region of F-MuLV Gag. In mice immunized with the Th epitope-harboring peptide or a vaccinia virus-expressed native full-length MA protein, FV-induced early splenomegaly regressed rapidly. In these mice, FV-infected cells were eliminated within 4 weeks and the production of virus-neutralizing antibodies was induced rapidly after FV challenge, resulting in strong protection against the virus infection. Interestingly, mice immunized with the whole MA mounted strong CD4$^+$ T-cell responses to the identified Th epitope, whereas mice immunized with mutant MA proteins that were not bound to the plasma membrane failed to mount efficient CD4$^+$ T-cell responses, despite the presence of the Th epitope. These mutant MA proteins also failed to induce strong protection against FV challenge. These data indicate the importance of the properly processible MA molecule for CD4$^+$ T-cell priming and for the resultant induction of an effective immune response against retrovirus infections.

Defining the immune mechanisms that facilitate resistance to viral infections is vital for the rational development of preventative and therapeutic modalities against virus-induced diseases. Substantial evidence indicates that virus-specific CD4$^+$ T helper (Th) cells play a key role in the control of many different viral infections (reviewed in references 14 and 36). In mouse models, maintenance of CD8$^+$ cytotoxic T-cell (CTL) responses and control of viremia have been demonstrated to depend on virus-specific CD4$^+$ T cells during chronic viral infections (1, 28, 57, 62). In addition, cooperation between antigen-specific CD4$^+$ T cells and neutralizing antibody (Ab)-producing B cells is required for long-term virus control in lymphocytic choriomeningitis virus infections (43, 53). With regard to immunosuppressive retrovirus infections, activation of virus-specific CTL responses alone is largely ineffective in inducing protection against simian immunodeficiency virus (SIV) infection (12, 49, 60). In contrast, adoptive transfer of autologous CD4$^+$ T cells results both in the induction of virus-specific CTL responses and in the production of neutralizing Abs, with long-term anti-SIV control (56). Thus, the development and maintenance of functional CTL and B-cell responses that are aided by the activation of virus-specific CD4$^+$ T cells might be required for effective protection against chronic virus infections. However, the precise nature of the virus-specific CD4$^+$ T cells that contribute to effective antiviral immunity remains unclear. More recently, an inverse association between human immunodeficiency virus type 1 (HIV-1)-specific CD4$^+$ T-cell responses and plasma viral load has been demonstrated in long-term nonprogressors and individuals treated with highly active antiretroviral therapy (22, 26, 42, 46, 47). Intriguingly, in such HIV-1-infected individuals, strong Gag-reactive CD4$^+$ T-cell responses were detected in association with a high level of HIV-1-specific CTL responses.

The Gag protein of retroviruses is a major viral component and is relatively conserved in its structure among various isolates and between retroviruses of different host species in comparison with the Env protein. Broadly cross-reactive Th epitopes, as well as CTL epitopes, have been identified in conserved regions of retroviral Gag proteins (11, 29, 48, 58). Finally, by use of a mouse model of Friend retrovirus (FV) infection, it has been found that immunization with *gag* gene products induces CD4$^+$ T-cell-mediated protective immunity (32), although the precise epitopes involved have not been identified. Given these observations, there is compelling evidence indicating that Gag-specific CD4$^+$ T cells are effective in controlling retrovirus infections, and therefore they may be potential targets for the development of effective antiretrovirus vaccines.

FV is an immunosuppressive retrovirus complex that induces fatal erythroleukemia in adult immunocompetent mice. Since the cell surface receptors, intracellular signaling, and host factors controlling virus replication and host immune responses have been well characterized, infection with this retrovirus represents a useful model in which to study both acute and persistent viral infections, as well as virus-host interactions

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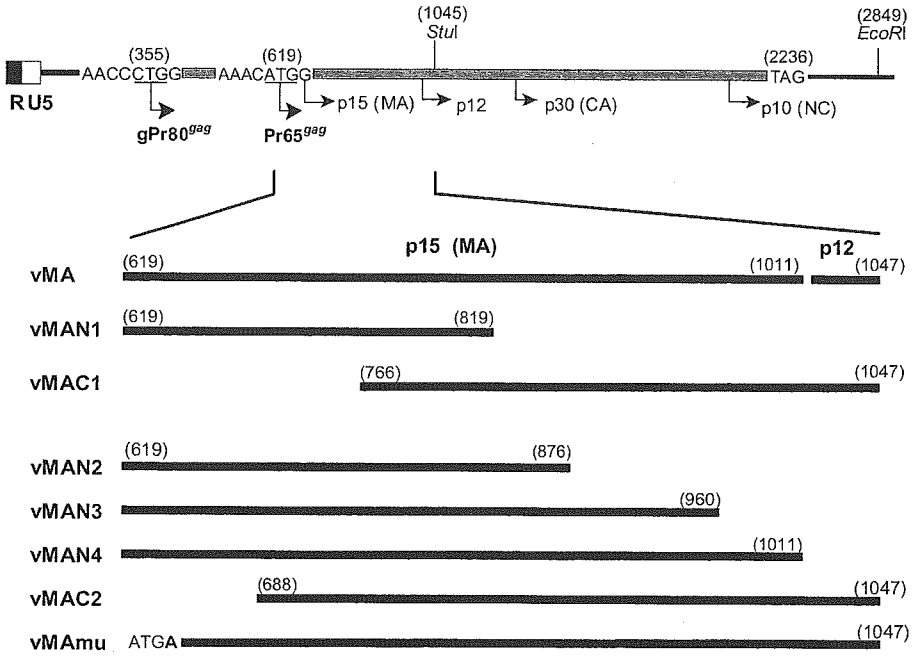

FIG. 1. Schematic representation of the F-MuLV *gag* gene and strategies for construction of the rVVs expressing portions of the MA protein. Base numbers of the *gag* gene in parentheses are given according to the published sequence of F-MuLV FB29 (39).

(reviewed in references 8 and 13). The replication-competent helper component of FV, Friend murine leukemia virus (F-MuLV), contains the immunological determinants necessary for anti-FV immune responses, while the replication-defective spleen focus-forming virus (SFFV) is required for the pathogenicity of FV complex in adult mice (21, 34). FV induces rapid splenomegaly because the SFFV envelope protein binds to the erythropoietin receptor on erythroid precursor cells, causing false proliferation signals. Susceptible animals develop acute and severe splenomegaly after FV inoculation, and unresolved infection leads to leukemic death within several weeks after challenge.

In order to understand and characterize the role of Gag-specific $CD4^+$ T cells in protective immunity against retrovirus infections, we attempted here to identify a Th epitope in the MA protein of F-MuLV Gag and investigated the possible association of Gag-primed $CD4^+$ T-cell responses with host protection. Furthermore, we examined structural features of the MA required for the induction of efficient cellular and humoral immune responses in vivo. The results provide new insights into different accessibilities for antigen presentation of the membrane-bound and unbound MA proteins and underscore their importance in vaccine development for retrovirus infections.

MATERIALS AND METHODS

Mice and virus. Female C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). A/WySnJ mice were originally purchased from The Jackson Laboratory (Bar Harbor, Maine). (B6 × A)F_1 mice were bred and maintained at the animal facility, Kinki University School of Medicine, and these mice, aged 8 to 16 weeks at the time of immunization, were used for the experiments described below. A stock of B-tropic FV was originally given by Bruce Chesebro, Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases (Hamilton, Mont.), and the stock used in the present study was prepared from infected BALB/c mice as a 20% spleen homogenate as described previously (31). For virus challenge, mice were injected in the tail vein with 1,500 spleen focus-forming units (SFFU) of FV complex in 0.5 ml of phosphate-buffered balanced salt solution (PBBS) containing 2% fetal bovine serum. After virus challenge, mice were observed daily, and the number of surviving mice was counted. The development of splenomegaly was monitored by palpation as described elsewhere (32). In some experiments, moribund mice were killed by cervical dislocation and spleen weights were measured to compare the results of palpation to actual spleen weights. Spleens weighing >0.5 g were consistently marked as palpable splenomegaly. All the animal experiments were approved and performed under relevant guidelines of the Japanese government and of Kinki University.

Construction of rVVs expressing the F-MuLV *gag* genes. Recombinant vaccinia viruses (rVVs) were constructed by the standard homologous recombination method using transfer plasmids based on pSC11 (6). Fragments of a *gag* gene from an infectious molecular clone of F-MuLV, FB29 (39) (GenBank accession no. Z11128), that were cloned into rVV are shown in Fig. 1. An rVV, r9-28B, expressing the entire $gPr80^{gag}$ and $Pr65^{gag}$ proteins has been described previously (32). All fragments of MA were fused with a polyhistidine metal-binding peptide (His tag) at their C termini so that their expression could be visualized with an anti-His tag Ab. For construction of rVVs expressing the His tag-conjugated proteins, a derivative of pSC11 (pSC11-His) was newly generated by inserting a His tag sequence and the multiple cloning site from the pcDNA3.1/V5-His vector (Invitrogen Corp., Carlsbad, Calif.) into a StuI site of pSC11-SS as described elsewhere (17). All DNA fragments encoding portions of F-MuLV MA (Fig. 1) were synthesized by PCR using pairs of oligonucleotide primers with additional sequences to generate the restriction enzyme site at their 5′ ends. After digestion with the corresponding restriction enzymes, PCR-amplified fragments were inserted in frame into the multiple cloning site of pSC11-His, which allowed fusion of the MA gene fragments to the N-terminal end of the His tag. The resultant plasmids were used to generate rVV vMAs. An unmyristylated form of the MA in which the N-terminal glycine required for protein myristylation (44) was replaced with an alanine was created by site-directed mutagenesis. To introduce a glycine-to-alanine point mutation, the following oligonucleotide was used as the sense primer: 5′-CCCCGTCGACCATGGCCCAGGCTGTT-3′. The PCR product amplified with the mutagenic primer pair and with the plasmid harboring the whole F-MuLV *gag* gene (32) as the template was inserted into pSC11-His as described above to generate an rVV vMAmu. Nucleotide sequences of all the cloned DNA fragments were confirmed, and the protein expression from the newly constructed rVV was detected by Western blotting and/or immunofluo-

rescent staining with an anti-His Ab (Santa Cruz Biotechnology, Santa Cruz, Calif.). A control rVV expressing the influenza virus hemagglutinin gene (vHA) has been described previously (51). As another control, an rVV, vHS1, expressing His tag-conjugated HS1, a hematopoietic-cell-specific intracellular molecule, was made by inserting a cDNA fragment encoding the N-terminal part (residues 1 to 204) of human HS1 (24) (GenBank accession no. H16663) into pSC11-His. Mice were inoculated with 10^7 PFU of an rVV via tail scratch (32), followed by an intravenous injection with the same amount of the identical virus 2 weeks later as a booster. Four weeks after the booster immunization, the mice were challenged with FV complex by intravenous inoculation.

Synthetic peptides and immunization. Locations within MA and sequences of the peptides used in this study are shown in Fig. 6A and 7C. Overlapping 30-mer or 9- to 17-mer peptides covering the C-terminal half of the F-MuLV MA were ordered from QIAGEN K. K. (Tokyo, Japan). Lyophilized powder of each purified peptide was dissolved in Dulbecco's phosphate-buffered saline and emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco, Detroit, Mich.). Mice were immunized once subcutaneously in the abdominal wall with multiple split doses for a total of 100 μl of emulsion containing 50 μg of a peptide; 4 weeks later, they were challenged with FV complex. Control mice were given the same amount of CFA emulsified with phosphate-buffered saline without any peptide.

Flow cytometry. Spleen tissue was dissociated in PBBS containing 2% fetal bovine serum, and a single-cell suspension was prepared as described elsewhere (16). Cells were incubated with 10 μg of anti-mouse CD16/CD32 (BD Biosciences PharMingen, San Diego, Calif.)/ml to prevent test Abs from binding to Fc receptors. For detection of erythroblasts infected with F-MuLV, spleen cells were incubated with R-phycoerythrin-conjugated TER-119 (BD Biosciences PharMingen) and biotinylated monoclonal Ab (MAb) 720 followed by fluorescein isothiocyanate-conjugated streptavidin (BD Biosciences PharMingen). TER-119 is specific for late erythroblasts and mature erythrocytes (23), and MAb 720 reacts specifically to F-MuLV gp70 but not to any other mouse retrovirus (45). Cells were also stained with isotype-matched control Abs. Dead cells were excluded from analyses by staining with 7-aminoactinomycin D (Beckman Coulter, Marseille, France), and viable cells were analyzed for specific staining with a FACScalibur (Becton Dickinson Immunocytometry Systems, Franklin Lakes, N.J.).

Infectious center assays. Infectious center assays were performed as described previously (31). Briefly, spleen cell suspensions prepared from mice challenged with FV complex were serially diluted, plated in triplicate onto monolayers of *Mus dunni* cells, and then cocultured for 2 days. After fixation with methanol, F-MuLV-infected cell foci were stained with MAb 720, visualized by using the avidin-biotinylated peroxidase complex (ABC; Vector Laboratories, Burlingame, Calif.), and counted under a magnifier.

Assays for virus-neutralizing Abs. Mice were bled of 100 μl from the retroorbital sinuses under ether anesthesia before immunization, at 2 weeks after the last immunization, and once a week after FV inoculation. The details of the assay for F-MuLV-neutralizing Abs have been described previously (31, 32).

Assays for T-cell proliferative responses. Proliferative responses of T cells against MA peptides were analyzed at 3 weeks after immunization with each rVV. The assay method has been described elsewhere (15, 32). Briefly, nylon wool-passed T cells were prepared from the spleen, and irradiated (4,000 rads) syngeneic or parental spleen cells were used as antigen-presenting cells (APC). The T cells (5×10^5) were incubated with the APC (5×10^5) and each synthesized peptide (20 μM) in a total volume of 200 μl. Three days later, the cells were pulsed with [^{3}H]thymidine (Amersham Biosciences, Piscataway, N.J.) added at 1.0 μCi per well; its uptake was measured 18 h later with a scintillation counter (Perkin-Elmer Applied Biosystems, Foster City, Calif.). All data are expressed as the mean difference in counts per minute (Δcpm, calculated as the average incorporation of [^{3}H]thymidine by cultures stimulated with a peptide minus that by unstimulated cultures). In some experiments, proliferative responses of T-cell subsets were analyzed. Nylon wool-passed spleen T cells were incubated with an anti-CD4 or anti-CD8 MAb conjugated with magnetic microbeads and were passed through a separation column placed in a magnetic sorter I (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the cell fractions was determined by flow cytometry after staining with appropriate fluorescence-labeled MAbs as described under "Flow cytometry" above. Each preparation contained less than 0.5% of the depleted cell type.

Western blotting. For some mice immunized with an rVV, production of anti-MA Abs in sera was analyzed by immunoblotting. F-MuLV particles were purified from the culture supernatant of *M. dunni* cells chronically infected with FB29 as described previously (15, 33). The purified virus particles (8 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gradient gel (PAGEL AE6000; ATTO Corp., Tokyo, Japan) electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking with 10% skim milk, the membranes were incubated with a 1:10 dilution of each serum sample, followed by another incubation with a horseradish peroxidase-conjugated anti-mouse immunoglobulin (Ig) Ab (Zymed, South San Francisco, Calif.). MA proteins were visualized by using an enhanced chemiluminescence detection system (Amersham Biosciences) as described elsewhere (52). MAb 690, directed against F-MuLV MA (30), was used as a positive control for detection of blotted MA proteins.

Analysis of intracellular localization of His tag-conjugated MA proteins by confocal microscopy. CV-1 cells were infected with a low titer of rVV and incubated overnight so that isolated infectious plaques were visible. The cells were fixed for 10 min in 3.7% formaldehyde, permeabilized by 0.4% Triton X-100, and blocked with 5% goat serum. His tag-conjugated MA proteins were stained with the anti-His Ab, followed by incubation with a fluorescein isothiocyanate-conjugated anti-rabbit Ig Ab (Southern Biotechnology, Birmingham, Ala.). The stained samples were scanned with an LSM 5 PASCAL laser confocal microscope (Carl Zeiss, Berlin, Germany).

Statistical analyses. Survival data were expressed by the Kaplan-Meier method, and the Mantel-Haenszel log rank test was employed for comparison of survival curves using GraphPad Prism (GraphPad Software, Inc., San Diego, Calif.). Student's *t* test was used for comparison of data for T-cell proliferative responses and frequencies of spleen infectious centers between experimental groups.

RESULTS

Early protection against FV infection in mice immunized with F-MuLV MA. The F-MuLV *gag* gene codes for two alternatively translated polyproteins, $Pr65^{gag}$ and $gPr80^{gag}$ (9). $Pr65^{gag}$ is the precursor to virion core structural proteins and is myristylated on the N-terminal glycine and proteolytically cleaved into four proteins (p15, p12, p30, and p10) during virion maturation. The glycosylated cell surface Gag protein, $gPr80^{gag}$, contains the entire amino acid sequence of $Pr65^{gag}$ plus a leader sequence (Fig. 1). We previously showed that protective immune responses against FV infection mediated by $CD4^+$ T cells were induced by immunization with the full-length *gag* gene products. Moreover, immunization with an rVV expressing full-length $Pr65^{gag}$ (positions 619 to 2849) or with an rVV expressing an N-terminal portion of Gag (positions 355 to 1047) representing the 5′ leader sequence, the entire MA, and a short N-terminal fragment of p12, elicited similarly efficient protection, suggesting that the major protective epitope might be located within a *gag* gene product encoded by the segment corresponding to positions 619 to 1047 (32). However, since it has been shown that two overlapping CTL epitopes are located within the leader peptide (7, 25, 54), it is not certain whether the MA protein alone can be protective.

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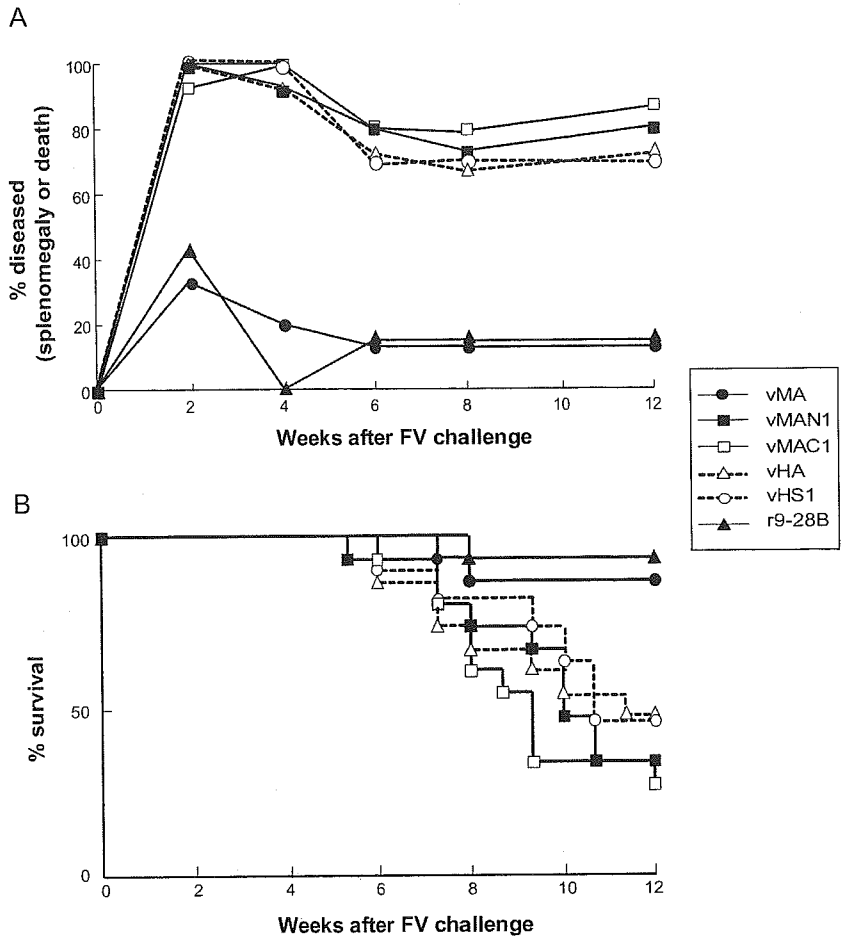

FIG. 2. Induction of protective immunity against FV infection with the MA region of F-MuLV Gag. Each group of (B6 × A)F_1 mice (10 to 15 per group) was immunized twice with one of the rVVs shown. Four weeks after the second immunization, mice were injected intravenously with 1,500 SFFU of FV and then monitored for the development of splenomegaly and death. (A) The incidence of disease at each time point was calculated by adding the numbers of mice that had splenomegaly (>0.5 g) and mice that had died. (B) Survival curves of the groups of mice examined. Statistically significant differences ($P < 0.05$) between the upper two (▲, ●) and the lower four survival curves were confirmed by the Mantel-Haenszel log rank test.

results comparable to those observed for nonimmunized mice of the same strain. As expected, significant protection was observed in mice immunized with vMA, expressing the entire MA plus the short fragment of p12 but not the leader peptide, confirming the predicted existence of a protective epitope(s) in the MA region. Surprisingly, however, neither the N-terminal (vMAN1) nor the C-terminal (vMAC1) half of MA induced significant protection against FV infection. At 12 weeks postchallenge, the spleens of the surviving mice were weighed, and the results corresponded well with those of palpation, indicating that the individuals shown as diseased in Fig. 2A were indeed leukemic. The incidences of splenomegaly at 6 weeks after challenge were well correlated with the incidence of leukemic death or splenomegaly at 12 weeks after challenge. In addition, in the previous experiments performed with the same or similar mouse strains and ≥1,500 SFFU of FV, recovery from splenomegaly present at 6 to 7 weeks postchallenge has rarely been observed (31, 32). Therefore, in subsequent experiments, splenomegaly was monitored, as an indicator of the FV-induced disease, over a period of 6 weeks postchallenge. Furthermore, since mice immunized with vHA or vHS1 showed similar incidences of disease development, the rVV-expressing His tag-conjugated HS1 was subsequently used as the negative control.

Kinetics of the development of early protective immunity in mice immunized with the MA proteins. To elucidate how rapidly antiviral protection developed as a result of immunization with full-length MA, we prepared spleen cells from vMA-immunized mice at early time points after FV challenge and examined the numbers of virus-producing cells by infectious center assays (Fig. 3A). In both groups of mice, those immunized with vMA and those immunized with vHS1 (negative control), cells producing infectious F-MuLV particles were detected in the spleen at 1 week after FV challenge, but the average number of spleen infectious centers was significantly lower in vMA- than in vHS1-immunized mice (3.3×10^1 versus 2.7×10^2 per 10^5 nucleated cells, respectively). In control mice, spleens were dramatically enlarged, reaching a peak average weight of 1.96 g at 3 weeks postchallenge, and average numbers of spleen infectious centers increased and remained high

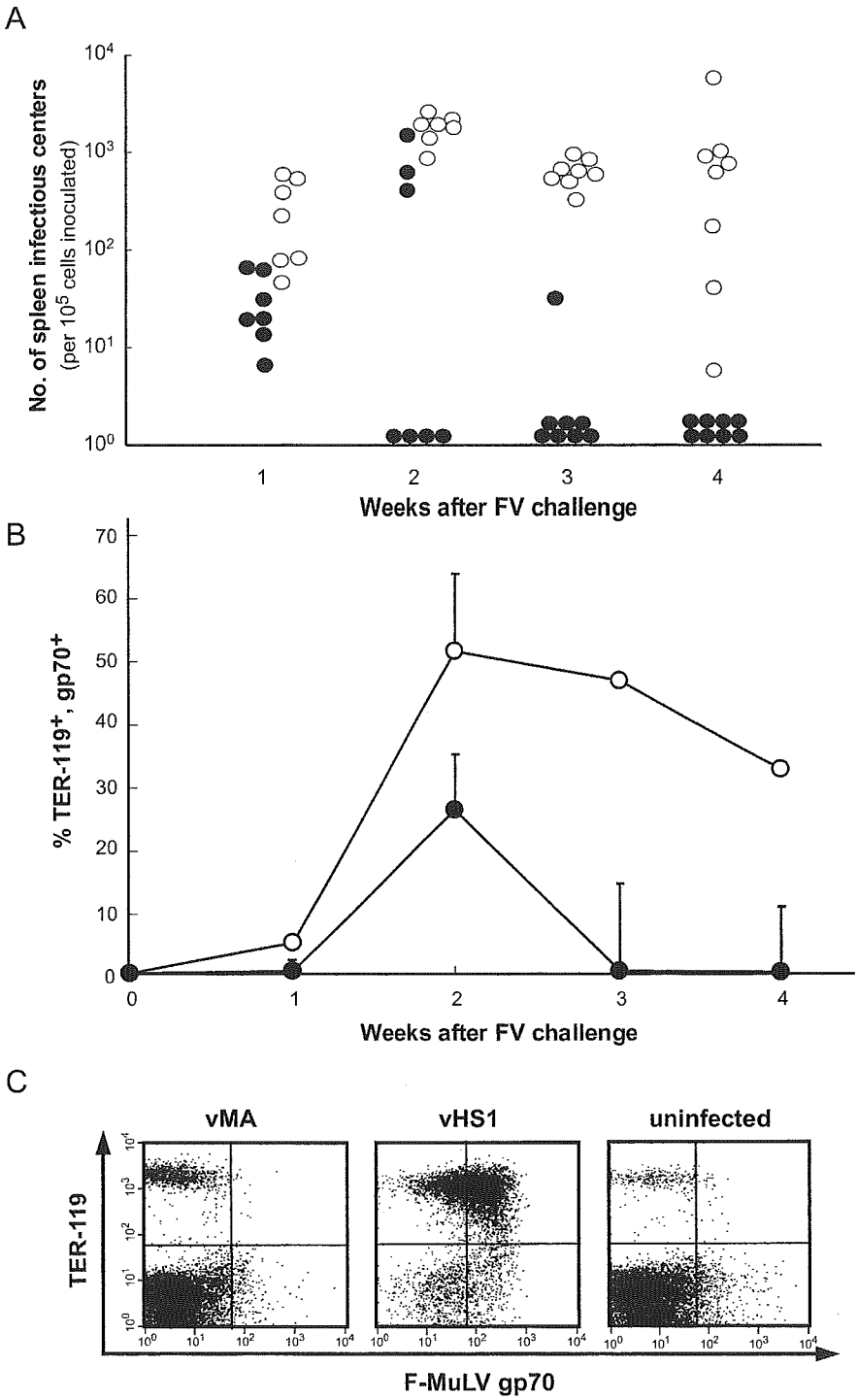

FIG. 3. Changes in the numbers of FV-infected cells in vMA-immunized mice detected by the infectious center assay (A) and fluorescence-activated cell sorter analyses (B and C). (B6 × A)F_1 mice were immunized twice with vMA (●) or vHS1 (○), and 4 weeks later they were challenged with 1,500 SFFU of FV. A group of seven to eight mice was sacrificed at each of the indicated time points, and spleen cells were prepared for analysis. (A) Frequencies of spleen infectious centers were determined by infecting the indicator cells and staining infected foci with a MAb against F-MuLV gp70. Results are shown on a logarithmic scale. Significant differences ($P < 0.05$ by Student's t test) between the two groups were observed at all time points tested. (B) FV-infected erythroblasts in mice immunized with vMA (●) or vHS1 (○) were stained with a combination of MAb 720 and TER-119. Each data point represents the mean percentage of cells stained with both the MAbs ± the standard error of the mean. (C) Representative pattern of staining for each group of mice observed at 4 weeks postchallenge.

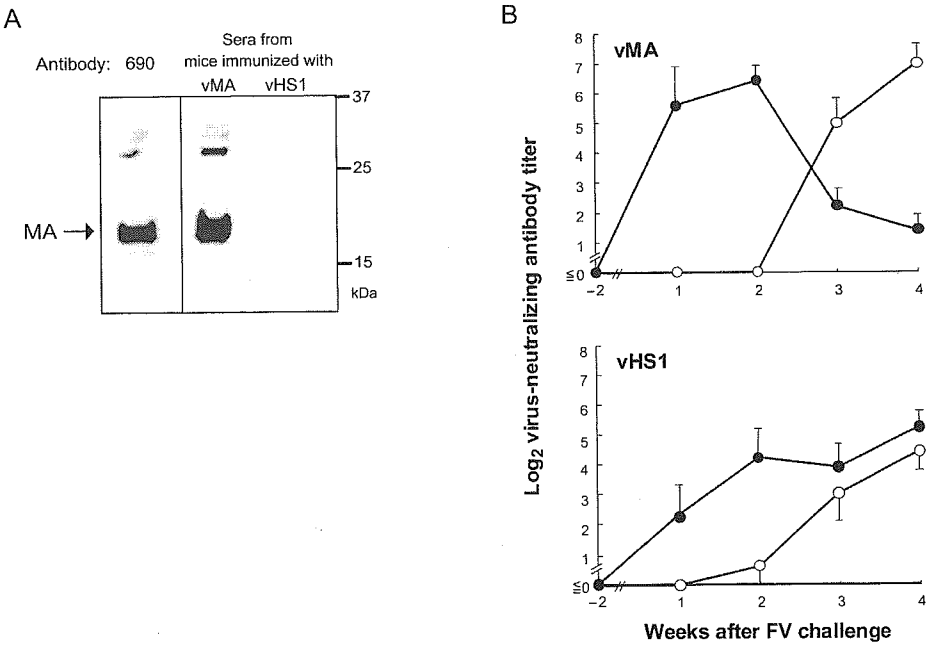

FIG. 4. Presence and titers of anti-MA and virus-neutralizing Abs in sera from vMA-immunized mice. Serum samples were collected from vMA- or vHS1-immunized mice at 2 weeks after the final immunization (−2) and at 1, 2, 3, and 4 weeks after FV challenge. (A) Detection of MA-reactive Abs in sera from immunized mice at 2 weeks after the final immunization but before challenge. In this experiment, serum samples from two to three mice were pooled and used for Western blot analysis. MAb 690, directed against F-MuLV MA, was used as a positive control. Data shown here are representative of two repeated experiments. (B) Titers of IgM (●) and IgG (○) F-MuLV-neutralizing Abs. Each data point is the mean titer from five separate serum samples ± the standard error of the mean, on a logarithmic scale.

through 4 weeks postchallenge. On the other hand, in vMA-immunized mice, the number of virus-producing cells rapidly decreased after 2 weeks postchallenge, eventually becoming undetectable at 4 weeks postchallenge.

The spleen cells prepared as described above were also subjected to flow cytometric analyses using a combination of MAb 720 and TER-119. The latter marks late erythroblasts and mature red blood cells but not erythroid burst-forming units or CFU. The changes in the frequency of erythroblasts expressing F-MuLV gp70 were in good correlation with the changes in the numbers of spleen infectious centers (Fig. 3B), and the number of FV-infected cells in vMA-immunized mice was markedly reduced by 4 weeks postchallenge, as shown by the representative pattern of staining obtained at that time (Fig. 3C). These results suggested that immunization with vMA suppressed both viral replication and proliferation of FV-infected erythroid cells.

We next studied the kinetics of production of F-MuLV-neutralizing Abs in vMA-immunized and control mice. Before FV challenge, MA-reactive Abs were detected in sera obtained from vMA-immunized mice, but not in those obtained from vHS1-immunized mice, by Western blotting (Fig. 4A). Nevertheless, virus-neutralizing Abs were not detectable in any sera from vMA-immunized mice before challenge (Fig. 4B). After FV challenge, higher levels of virus-neutralizing IgM Abs were detected in vMA-immunized mice than in vHS1-immunized control mice. Further, IgM-to-IgG class switching of neutralizing Abs was observed in vMA-immunized mice at 3 weeks postchallenge, but neutralizing Abs remained IgM dominant in control mice during the observation period until 4 weeks postchallege.

Localization of protective epitopes in MA by expression of longer fragments. Since the results shown in Fig. 2 (in which neither the N-terminal nor the C-terminal half of MA induced resistance to FV infection) raised the question of whether two separate epitopes in MA might be necessary for full protection, we next attempted to immunize mice simultaneously with vMAN1 and vMAC1. However, contrary to our expectation, the combination of vMAN1 and vMAC1 was also unable to induce protective immunity (Fig. 5). Therefore, we decided to construct additional rVVs by deleting even shorter fragments from the vMA whose protective efficacy has been proven. Immunization with vMAN3 or vMAN4, which expressed MA with a short deletion in the C-terminal end or the entire MA without the p12 fragment, respectively, induced significant protection (Fig. 5). On the other hand, vMAN2, expressing MA with a longer C-terminal deletion, did not induce early regression of the splenomegaly that had developed immediately after FV challenge, indicating that at least one of the putative protective epitopes is localized between positions 876 and 960 of the F-MuLV *gag* gene, which encodes residues 86 to 114 of MA.

Although a longer segment of the N-terminal region of MA was included for comparison with vMAC1, vMAC2 did not induce significant protective immunity (Fig. 5). These results suggested the possibility that vMAC1 was ineffective not because of its lack of a protective epitope but because of inappropriate processing or presentation of an existing epitope. Since N-terminal myristylation is a common feature of retro-

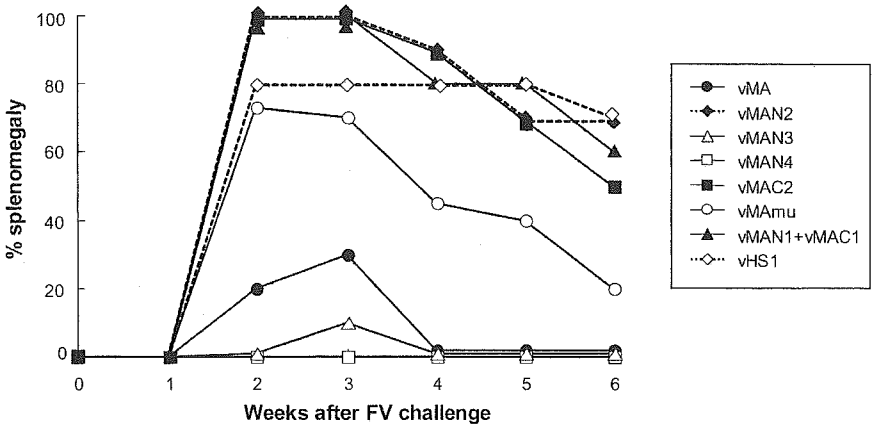

FIG. 5. Localization of protective epitopes within the MA by using rVVs. The portions of F-MuLV MA expressed by the rVVs used are diagramed in Fig. 1. The ability of each rVV to induce protective immunity against FV infection was analyzed by immunizing (B6 × A)F_1 mice (10 per group) and challenging them with FV. The development of splenomegaly over a 6-week period after challenge was observed as an indicator of FV-induced disease.

viral MA that is necessary for stable association with the plasma membrane (4, 37, 44), an MA protein that is unmyristylated due to the lack of its N-terminal glycine is predicted not to bind efficiently to the plasma membrane and to remain primarily in the cytoplasm. As expected, immunization with vMAmu, expressing the whole MA protein in which the N-terminal glycine had been replaced with an alanine, was unable to prevent effectively the development of early splenomegaly in FV-infected mice (Fig. 5). Among mice immunized with vMAmu, 70% developed early splenomegaly by 2 weeks postchallenge, and half still carried an enlarged spleen until 4 weeks postchallenge. Thus, vMAmu appeared to show low immunogenicity in spite of possessing the full MA sequence, suggesting that myristylation of the MA protein at the N terminus might influence its immunogenicity.

Determination of a protective Th epitope with synthetic peptides. Studies with the rVVs described above showed that a putative protective Th epitope should be present between residues 86 and 114 of MA. To identify the precise structure of the protective Th epitope, proliferative responses of T cells primed with vMA were analyzed by in vitro stimulation with overlapping 30-mer peptides covering the C-terminal half of MA, which should contain a protective epitope (Fig. 6A). Spleen T cells primed with the full-length MA protein showed proliferative responses only when stimulated with a peptide corresponding to residues 76 to 105 of MA, not when stimulated with any other peptide (Fig. 6B). When T cells from control mice immunized with vHS1 were stimulated, all the peptides tested induced only marginal levels of proliferation.

In a previous study, the T cells primed by immunization with Gag antigens were mainly $CD4^+$ (32). Therefore, we next examined the antigen-specific proliferative responses of T-cell subsets. As expected, CD4-depleted T cells prepared from vMA-primed mice showed no proliferative response to stimulation with peptide 76-105, whereas CD8-depleted T cells proliferated at a level comparable to that of whole-spleen T cells (Fig. 7A). To further pursue the structure of the Th epitope, we additionally synthesized overlapping peptides of 15- to 17-mer lengths covering residues 76 to 105 (Fig. 6A) and analyzed their abilities to stimulate vMA-primed T cells. Whole T-cells proliferated in response to stimulation with a 15-mer peptide representing residues 83 to 97 (IVTWEAIAVDPPPWV) of MA; this proliferative response was totally abolished by the depletion of $CD4^+$, but not of $CD8^+$, T cells (Fig. 7). The peptide representing residues 76 to 91 was ineffective, and peptide 89-105 induced only marginal proliferation of vMA-primed T cells.

To analyze major histocompatibility complex (MHC) molecules involved in the presentation of the $CD4^+$ T-cell epitope, T cells from the (B6 × A)F_1 mice previously immunized with vMA were stimulated in vitro with the 15-mer peptide plus APC prepared from either B6, A, or F_1 mice. The primed T cells showed an $H\text{-}2^{b/a}$-restricted proliferative response, but both parental APC were ineffective at presenting the peptide (Fig. 7B), indicating the possibility that peptide 83-97 may be presented by either hybrid $A^{b/k}$ or $E^{b/k}$ class II molecules. To further investigate whether residues 83 to 97 (IVTWEAIA VDPPPWV) were the minimal structure for inducing the peptide-specific T-cell responses, several shorter peptides covering residues 83 to 97 were synthesized and tested for their abilities to stimulate vMA-primed T cells (Fig. 7C). The results showed that the 13-mer peptide spanning residues 83 to 95 (IVTWE AIAVDPPP) was sufficient to elicit vMA-primed T-cell responses of similar strength to those elicited by the 15-mer peptide. Elimination of the N-terminal I, as represented by peptide 84-97, significantly diminished the induced proliferative responses, and elimination of the two N-terminal residues from the 15-mer peptide totally abolished the stimulating potential. These results indicated that the 12-mer sequence VTWEAIAVDPPP, as the longest possibility, constituted the core structure of the T-cell epitope.

We next investigated whether immunization with the single Th epitope could induce effective protection against FV infection. Mice given a single immunization with either peptide 83-97 or peptide 76-105, but not those given any other peptide tested, showed a lack of development of splenomegaly or very rapid regression of the splenomegaly induced by FV challenge (Fig. 8A). In mice immunized with peptide 83-97 or 76-105,

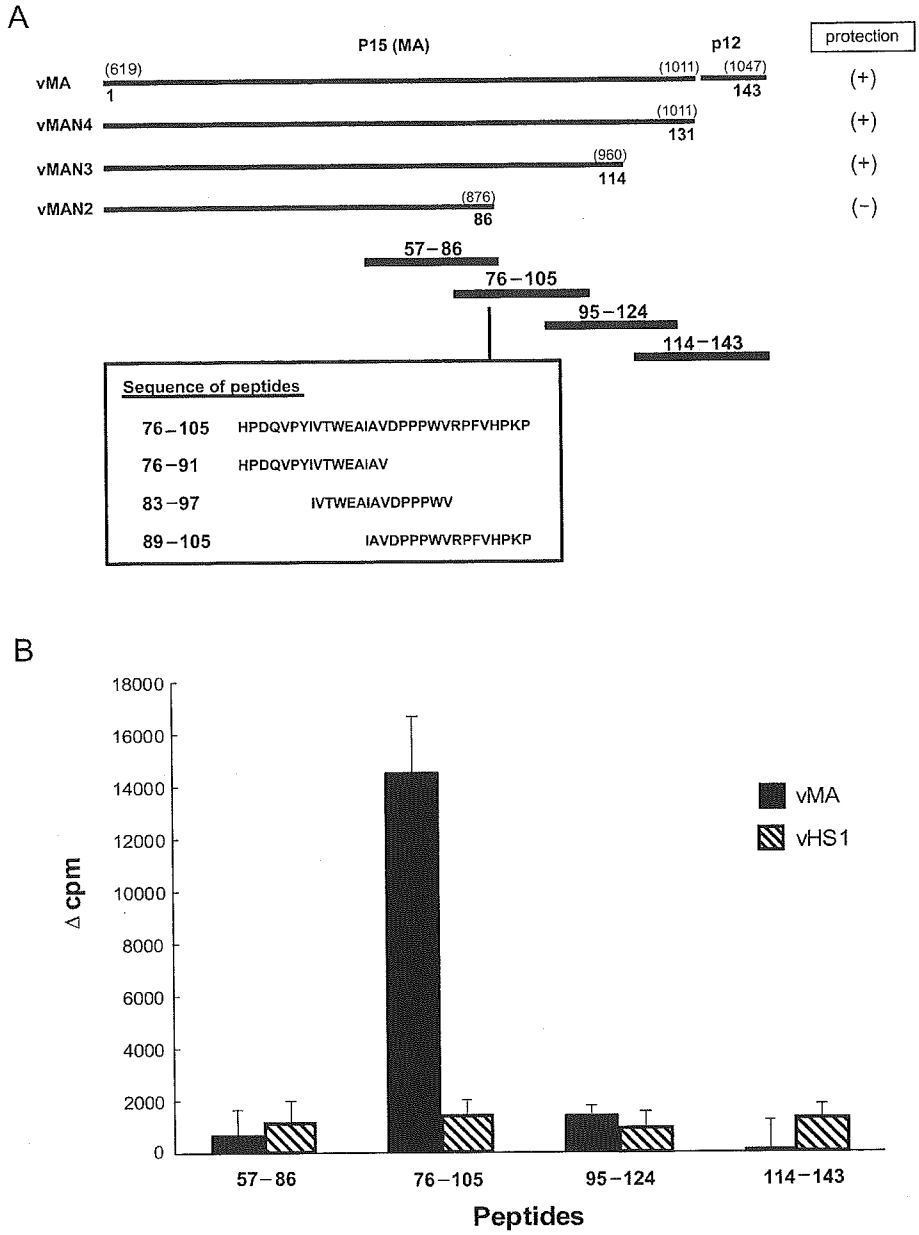

FIG. 6. Identification of a T-cell-stimulating epitope in MA by use of synthetic peptides. (A) The position of each peptide tested is shown along the schematic representation of the truncated MA proteins used for the mapping experiment represented by Fig. 5. Numbers in parentheses are base positions in the *gag* gene; other numbers are amino acid positions starting from the initial methionine for $Pr65^{gag}$. (B) Spleen T cells prepared from vMA- or vHS1-immunized mice at 3 weeks after immunization were cultured with one of the synthetic peptides shown (20 μM) and with syngeneic irradiated spleen cells as APC; their proliferative responses were measured by [^{3}H]thymidine incorporation. Each result is the mean Δcpm for data obtained from five separate mice. Error bars, standard errors of the means. The experiments were performed twice with essentially identical results.

F-MuLV-producing cells in the spleen were either undetectable or detectable at very low frequencies at 4 weeks after FV challenge, as evaluated by infectious center assays (Fig. 8B). These results indicated that the Th epitope present between residues 83 and 97 of MA is sufficient to induce protective immune responses against FV infection.

Correlation between intracellular localization and immunogenicity of mutant MA proteins. The results described above indicated again that vMAC1, which definitely carried the protective Th epitope, was nevertheless not protective, because of a reduced immunogenicity of the otherwise antigenic C-terminal portion of the MA protein. Substantial evidence suggests that the N-terminal region of retroviral MA, the amino-terminal glycine residue and basic sequences close downstream, is responsible for the targeting of MA to the plasma membrane (4, 37, 44, 61, 63). The results of the protection experiments with vMAC2 and vMAmu (Fig. 5) further suggested that the targeting of the MA molecule to different subcellular compart-