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Rheumatic Fever: an animal model for a human disease

F.F. Alcantara, E. Postol, E.R. Alencar, J. Kalil, L. Guilherme

Immunology Laboratory Heart Institute (InCor), University of Sao Paulo Medical School and Department of Allergy and Clinical Immunology, University of Sao Paulo - School of Medicine, Brazil

flaviofalcantara@yahoo.com

Rheumatic fever is an inflammatory autoimmune condition following 3 % of non treated group A streptococci pharyngitis of certain M serotype strains. In addition to a cellular heart valve attack, anti-basal ganglia autoantibodies arise later in the autoimmune process targeting caudate and subthalamic nuclei antigens, being responsible for the neurologic symptoms of Sydenham's chorea. Mammalian lysoganglioside and N-acetyl-beta-D-glucosamine (GlcNAc), the dominant epitope of the group A streptococcal (GAS) carbohydrate are the probable molecules involved in neurologic damage. Antibodies targeting both heart antigens and M proteins were found in blood of affected patients. Antigenic mimicry between streptococcal M protein epitopes and heart components have been proposed as the triggering factor leading to the heart autoimmune attack. The understanding of the disease process and therapeutic advances has been hampered by the lack of an adequate animal model for the disease. We have injected Lewis rats with streptococcus recombinant M1 protein 500 µg on day 0 followed by 500 µg boost on day 7 and sacrifice on day 21, in order to reproduce a recently described animal model of rheumatic fever [Quinn, A. et al, 2001, Infect.Immun. 69(6):4072-78]. Rat hearts were subjected to histopathological analyzes. Spleen and lymph node lymphocyte cells, as well as sera, were harvested and probed against ABC domains, AB domains (N-terminus), or C domain (C-terminus) of the M1 complete protein and myosin or control proteins. We have obtained specific lymphoproliferative responses against selected M protein fragments and specific cardiac proteins, as seen in our previous results with patient samples [Guilherme et. al, 1995, Circulation 92:415-20]. We are currently using the rat-immunized cells for FACS analysis to study their phenotypic profile and cytokine production. The aim of our studies is to map the minimal M protein epitope(s) responsible for rheumatic fever after immunization with different recombinant M protein fragments.

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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

Daisuke Sugahara, Sachiyo Tsuji-Kawahara,* and Masaaki Miyazawa

Department of Immunology, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511, Japan

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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

* Corresponding author. Mailing address: Department of Immunology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan. Phone and fax: 81-72-367-7660. E-mail: skawa@immunol.med.kindai.ac.jp.

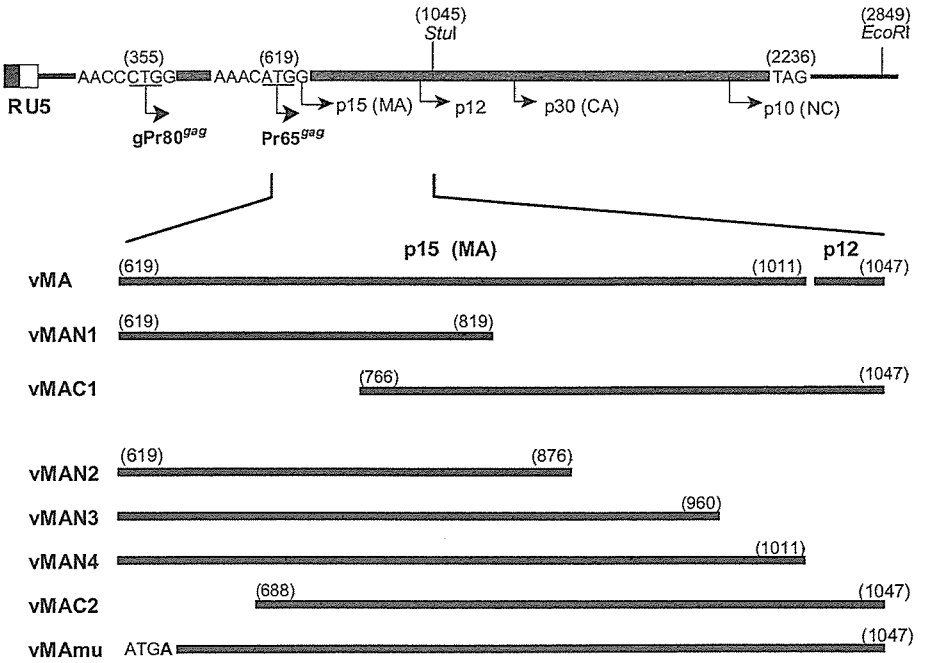

FIG. 1. Schematic representation of the F-MuLV *gag* gene and strategies for construction of the rVVs expressing portions of the MA protein. Base numbers of the *gag* gene in parentheses are given according to the published sequence of F-MuLV FB29 (39).

(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.

To further narrow the region containing the protective Th epitope, rVVs expressing full-length MA (vMA; positions 619 to 1047), the N-terminal half (vMAN1; positions 619 to 819), or the C-terminal half (vMAC1; positions 766 to 1047) were constructed (Fig. 1), and (B6 × A)F_1 mice, which are susceptible to FV infection, were immunized twice with one of these rVVs before FV infection. As shown in Fig. 2, when immunized with the rVV expressing the entire $Pr65^{gag}$/$gPr80^{gag}$ (r9-28B), more than 80% of the (B6 × A)F_1 mice recovered from the initial development of splenomegaly by 6 weeks and survived longer than 12 weeks after inoculation with 1,500 SFFU of FV. On the other hand, all the control mice given the rVV expressing influenza virus HA or His tag-conjugated HS1 showed enlargement of the spleen shortly after the FV challenge, and about half of them died by 12 weeks postchallenge,

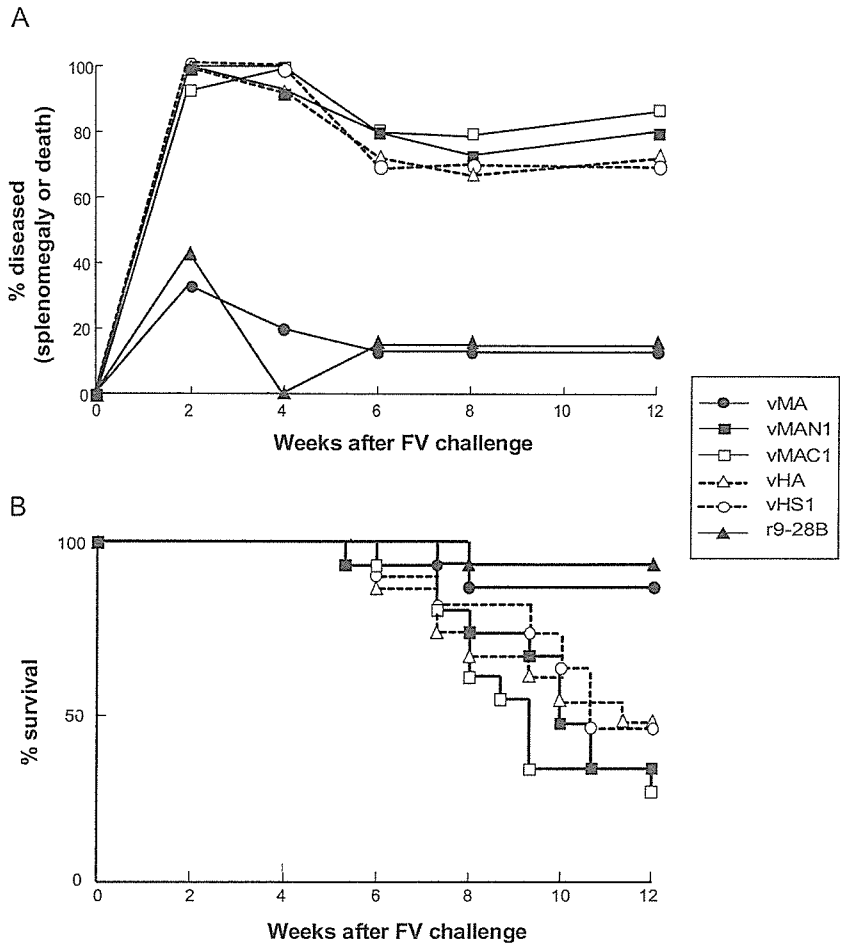

FIG. 2. Induction of protective immunity against FV infection with the MA region of F-MuLV Gag. Each group of (B6 × A)F_1 mice (10 to 15 per group) was immunized twice with one of the rVVs shown. Four weeks after the second immunization, mice were injected intravenously with 1,500 SFFU of FV and then 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

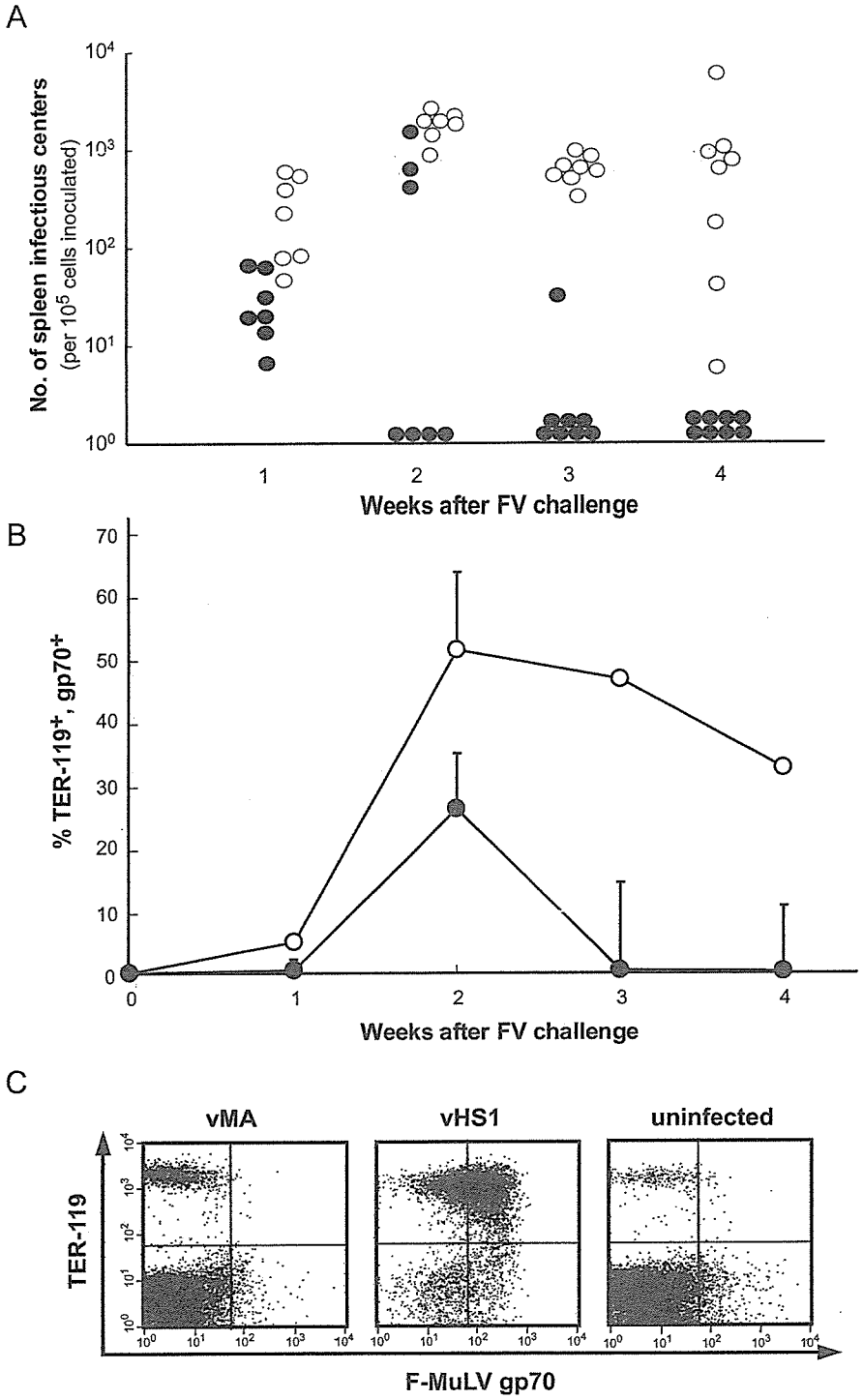

FIG. 3. Changes in the numbers of FV-infected cells in vMA-immunized mice detected by the infectious center assay (A) and fluorescence-activated cell sorter analyses (B and C). (B6 × A)F_1 mice were immunized twice with vMA (●) or vHS1 (○), and 4 weeks later they were challenged with 1,500 SFFU of FV. 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.

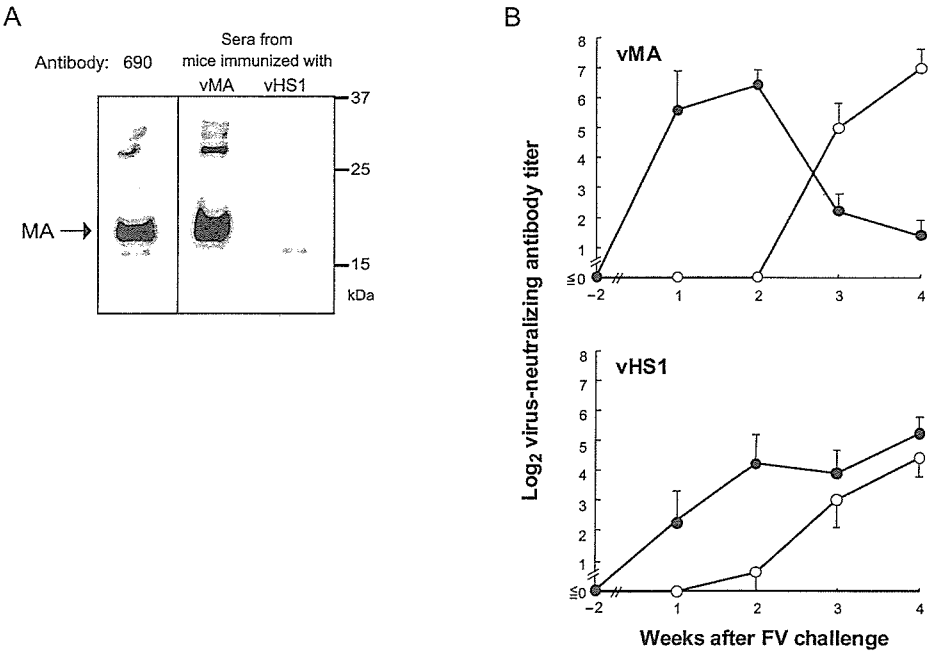

FIG. 4. Presence and titers of anti-MA and virus-neutralizing Abs in sera from vMA-immunized mice. Serum samples were collected from vMA- or vHS1-immunized mice at 2 weeks after the final immunization (−2) and at 1, 2, 3, and 4 weeks after FV challenge. (A) Detection of MA-reactive Abs in sera from immunized 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-

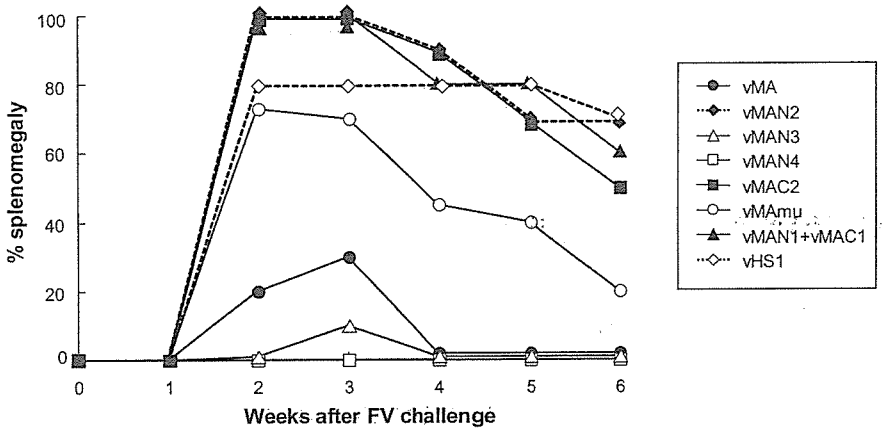

FIG. 5. Localization of protective epitopes within the MA by using rVVs. The portions of F-MuLV MA expressed by the rVVs used are diagramed in Fig. 1. The ability of each rVV to induce protective immunity against FV infection was analyzed by immunizing (B6 × A)F_1 mice (10 per group) and challenging 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,

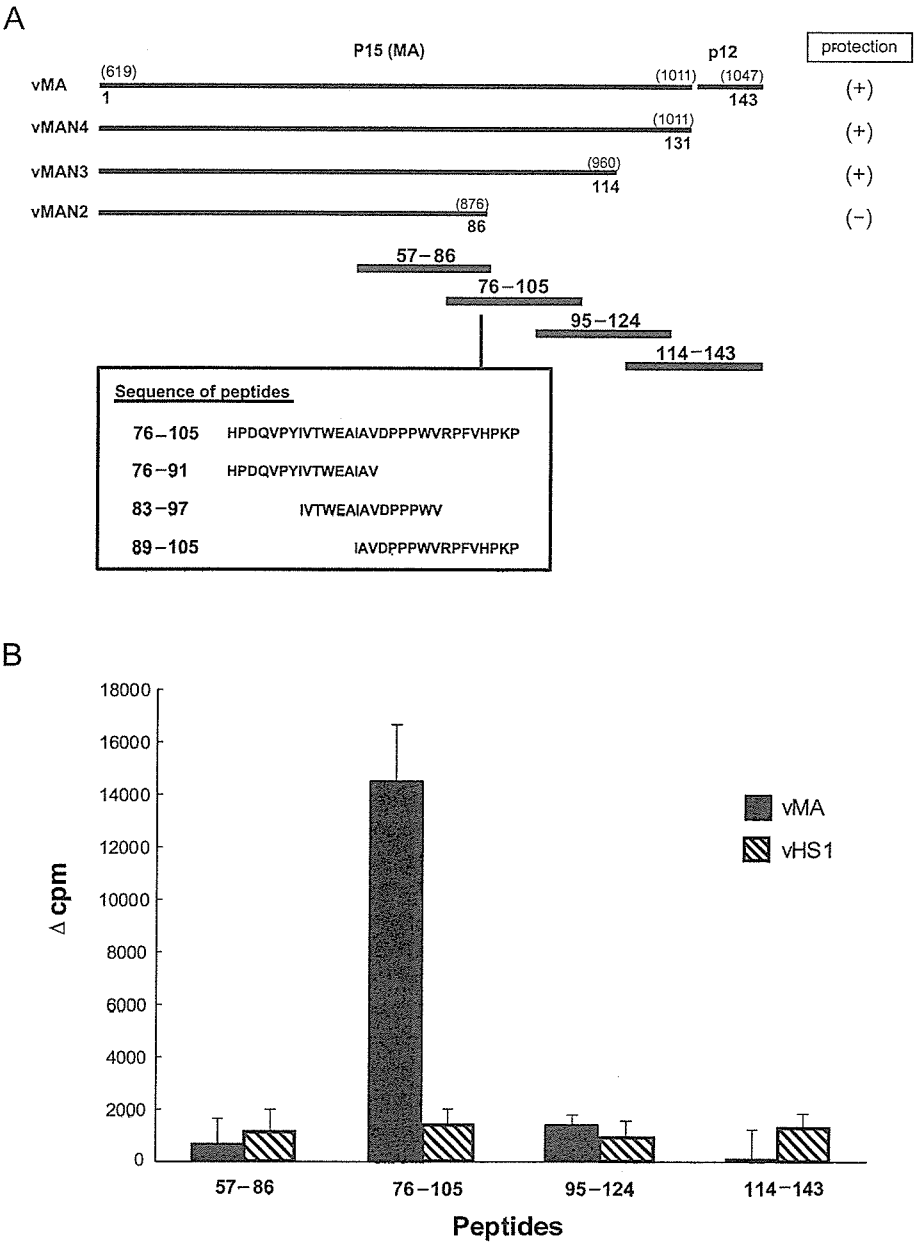

FIG. 6. Identification of a T-cell-stimulating epitope in MA by use of synthetic peptides. (A) The position of each peptide tested is shown along the schematic representation of the truncated MA proteins used for the mapping experiment represented by Fig. 5. Numbers in parentheses are base positions 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-

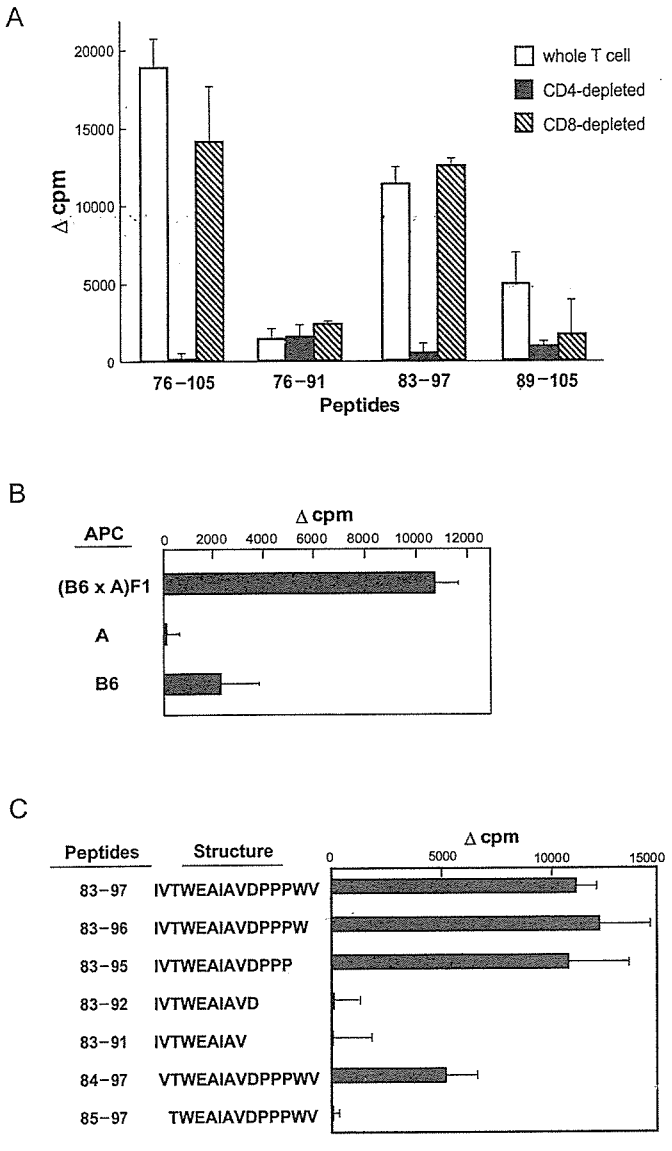

FIG. 7. Fine specificities and MHC restriction of primed $CD4^+$ T cells prepared from vMA-immunized mice. (A) Spleen T cells prepared from vMA-immunized mice were depleted of either $CD4^+$ or $CD8^+$ T cells by the magnetic cell sorting system. Unseparated T cells or purified T-cell subsets were incubated with each indicated peptide and with syngeneic spleen cells as APC, and antigen-specific proliferative responses were measured. Amino acid sequences of the peptides used here are shown in Fig. 6A. In this experiment, T cells from six immunized mice were pooled before the depletion of each subset. (B) T cells pooled from two to three vMA-immunized (B6 × A)F_1 mice were stimulated with peptide 83-97 and irradiated spleen cells prepared from F_1, B6, or A mice, and proliferative responses were analyzed as above. (C) T cells pooled from two vMA-immunized mice were stimulated with each indicated peptide and the syngeneic APC, and proliferative responses of T cells were measured. All data shown here are representative of two to four independent experiments with essentially identical results.

ments might have an influence on their immunogenicity. To prove this assumption, we first examined the intracellular localization of the mutant MA proteins by staining them with an anti-His Ab. The native MA protein expressed by vMA infection was present throughout the cells in a diffuse distribution and also localized at the plasma membrane (Fig. 9), as expected. In contrast, the mutant form of full-length MA, which lacked the site of myristylation, expressed by vMAmu infection, was localized more prominently in the nucleus than in the cytoplasm. In addition, the fluorescent intensity in vMAmu-infected cells was relatively low at the edges of the cells in comparison with that in vMA-infected cells. Interestingly, the MA protein from which the N-terminal 24 residues had been deleted, expressed by vMAC2 infection, was localized largely in the nucleus, which implies that, in addition to the glycine residue, the N-terminal short region of F-MuLV MA (as shown for HIV-1 MA [61, 63]) is necessary for its localization at the plasma membrane. The mutant MA proteins expressed by vMAmu and vMAC2 were observed by fluorescence at levels comparable to that of the native MA protein expressed by vMA infection, and a quite similar result showing the almost equal level of their expression was also obtained by Western blot analysis (data not shown).

To confirm that these changes in intracellular localization of the MA protein do affect its immunogenicity, we next compared the abilities of these MA mutants to prime the antigen-specific $CD4^+$ T cells (Fig. 10). T cells from vMAmu-immunized mice showed moderate proliferative responses when stimulated with the epitope-harboring peptide 76-105, but the response level was significantly lower than that of T cells taken from vMA-immunized mice. T cells prepared from vMAC2-immunized mice proliferated only marginally in response to stimulation with the Th peptide, showing no significant difference from the response of T cells from vHS1-immunized control mice. These results indicated that targeting of the MA protein to the plasma membrane might be critical for induction of efficient priming of MA-specific $CD4^+$ T cells.

DISCUSSION

We have demonstrated here, for the first time, efficient protection against a pathogenic retrovirus infection through the priming of Gag-specific $CD4^+$ T cells with an MA peptide. Immunization with the rVV expressing native MA alone was sufficient to protect mice from FV-induced disease development, and a protective epitope was present within residues 83 to 97 of MA. T cells primed in vivo with the native MA protein proliferated in vitro when stimulated with the minimal peptide 83-95 (IVTWEAIAVDPPP), and reactivity was completely abolished when $CD4^+$ T cells were depleted, indicating that the protective epitope is recognized by $CD4^+$ T cells. A hybrid class II molecule expressed on $H\text{-}2^{b/a}$ cells is required for the presentation of this epitope. Of note, this peptide is highly immunogenic, since a single vaccination with the epitope-harboring peptide was sufficient to protect mice against FV-induced disease. As in the previously demonstrated case of protection against FV infection induced by priming $CD4^+$ T cells with an Env-derived single-epitope peptide (16, 31), multiple effector mechanisms might have been activated upon FV infection in mice immunized with the MA-derived peptide. In agreement with our results, it has been shown that HIV-specific $CD4^+$ T cells in long-term nonprogressors are mainly directed against p17, the N-terminal HIV Gag protein analogous to F-MuLV MA (42). Thus, the high immunogenicity of

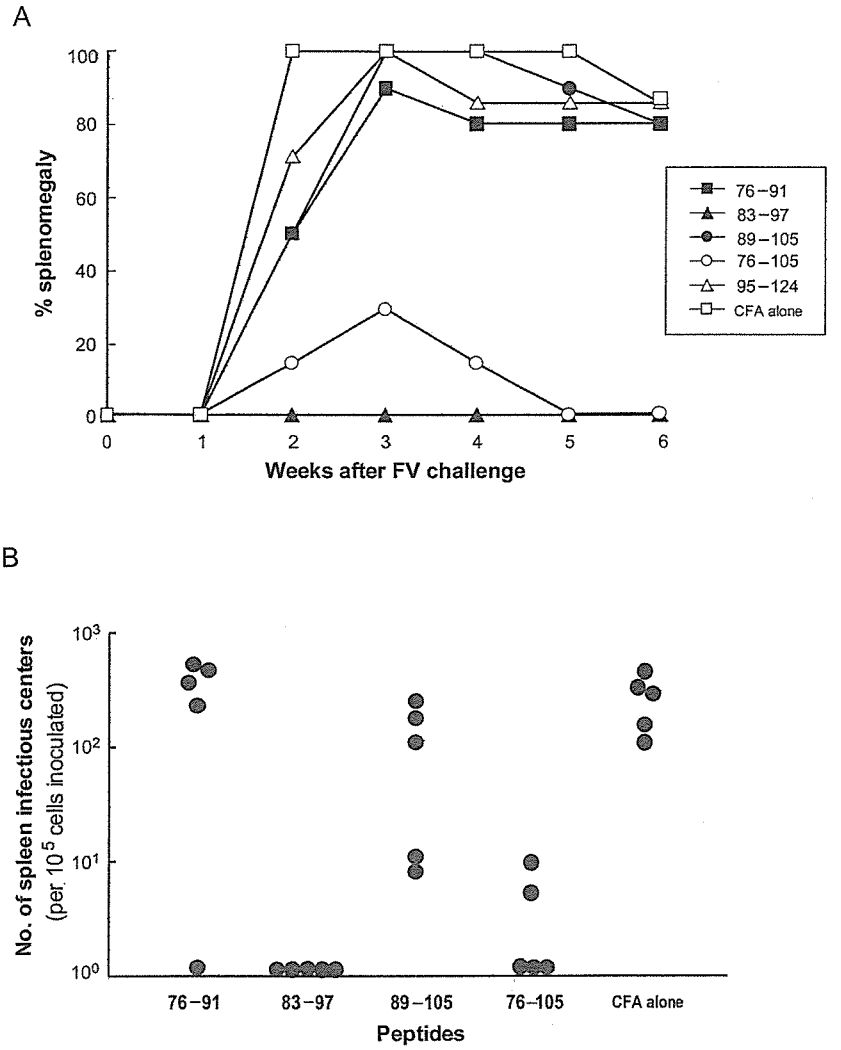

FIG. 8. Protection against FV infection induced by immunization with the Th epitope-harboring peptide alone. Mice (eight per group) were immunized once with 50 μg of one of the peptides shown or with CFA alone, followed by challenge with 1,500 SFFU of FV. (A) Splenomegaly over a 6-week period after challenge was observed as an indicator of FV-induced disease. (B) Frequencies of spleen infectious centers were determined at 4 weeks after FV challenge (five mice per group). The differences between CFA-injected and peptide 83-97- or 76-105-immunized mice were significant ($P < 0.01$ by Student's t test).

MA might be a common feature among diverse retroviruses. In support of this, broadly reactive Th epitopes were also identified in the MA region by using macaques chronically infected with SIV (48). Taken together, these data suggest that MA might be a potential target for the development of effective antiretrovirus vaccines.

In all the mice immunized with native MA expressed from the rVV or with the Th epitope-harboring peptide alone, virus-producing cells became undetectable by 4 weeks after FV challenge. The immune effector functions exerted by the virus-specific $CD4^+$ T cells might be very diverse (36), including helper functions provided for B- and $CD8^+$ T-cell responses, production of antiviral cytokines, and direct cytolysis. Our results here have shown that the class switching of virus-neutralizing Abs from IgM to IgG after FV challenge is remarkably accelerated in vMA-immunized mice. MA-specific Abs were detected by Western blotting before FV challenge in the sera of mice immunized with the rVV expressing native MA, but they were incapable of neutralizing F-MuLV (Fig. 4). Thus, the presence of a dominant neutralizing epitope(s) within MA is unlikely. Since the neutralizing Abs were produced in vMA-immunized mice only after FV challenge, it is more likely that the MA-primed $CD4^+$ T cells may have provided a helper function to Env-reactive B-cell responses. Alternatively, the production of Abs directed against the cell surface $gPr80^{gag}$ might have been facilitated after FV challenge by help from the MA-primed $CD4^+$ T cells. Prevention of cell-to-cell transmission of retroviruses by anti-$gPr80^{gag}$ Abs has been demonstrated (41).

The virus-specific $CD4^+$ T cells might also have been required for the maintenance of functional virus-specific CTL responses, as has been shown in other reports (1, 28, 40, 53). However, the mechanisms by which Gag-specific $CD4^+$ T cells may fulfill this role is poorly understood. In HIV-1-infected

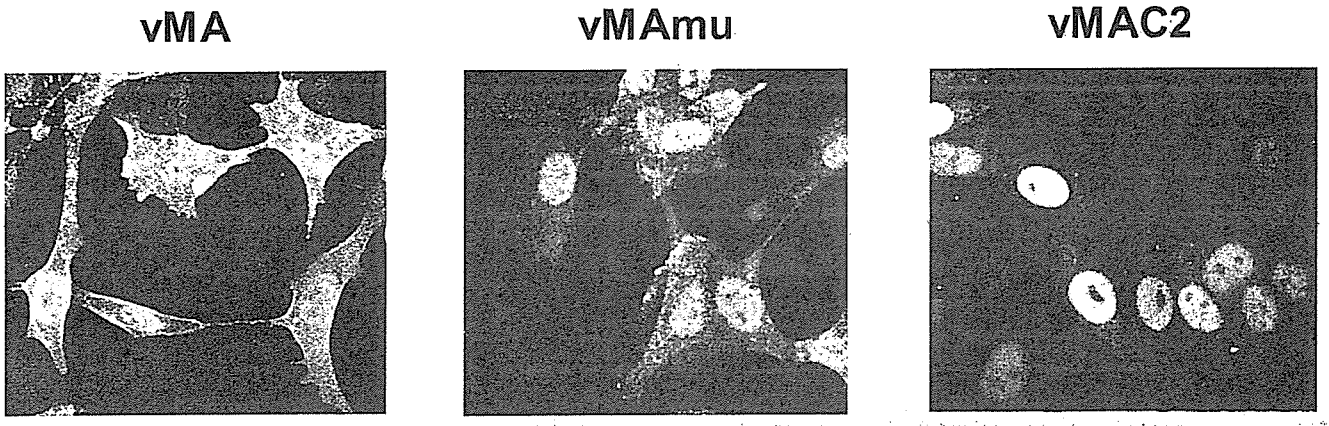

FIG. 9. Intracellular localization of mutant MA proteins. CV-1 cells were infected with vMA, vMAmu, or vMAC2; 24 h later, they were fixed and permeabilized. His tag-conjugated MA proteins were visualized with an anti-His tag Ab. The experiments were performed three times with essentially identical results.

individuals, enhanced $CD4^+$ T-cell responses have been associated with a higher level of virus-specific CTL responses and lower viral loads (22, 46). In this regard, we and others (7, 25, 54) previously found the presence of overlapping CTL epitopes in the leader sequence upstream of the ATG start codon for $Pr65^{gag}$, but not in the MA region, although it has not been determined whether these epitopes are protective or not. However, our previous work clearly demonstrated that the rVV expressing $Pr65^{gag}$ without the leader sequence was as effective as the rVV that expressed $gPr80^{gag}$, indicating that the CTL epitope in the leader sequence is not a requisite for protection against FV infection. Thus, the $CD4^+$ T cells primed with MA might have induced rapid responses of Gag-specific CTL, as well as Env-specific B cells, but the possible importance of Gag-specific CTL responses, if any, has yet to be identified.

Gag-specific $CD4^+$ T cells may also have direct roles in the control of FV infection through their possible cytotoxic activities and production of antiviral cytokines. Direct cytotoxic activities of $CD4^+$ T cells have been described in a number of viral infections (2, 19, 36, 59). A previous study with the FV-infected mouse model, in which direct cytotoxic activities of Env-specific $CD4^+$ T cells were detected, also supported those observations (16). Among $CD4^+$ T-cell clones established from HIV-1-infected individuals with vigorous Gag-specific responses, some displayed virus-specific cytotoxic activities (35, 55). Furthermore, $CD4^+$ T cells have been shown to directly control virus replication by production of gamma interferon in FV infection (10, 18). Thus, $CD4^+$ T cells primed with the MA protein might have contributed to the observed protection against FV infection through multiple effector functions.

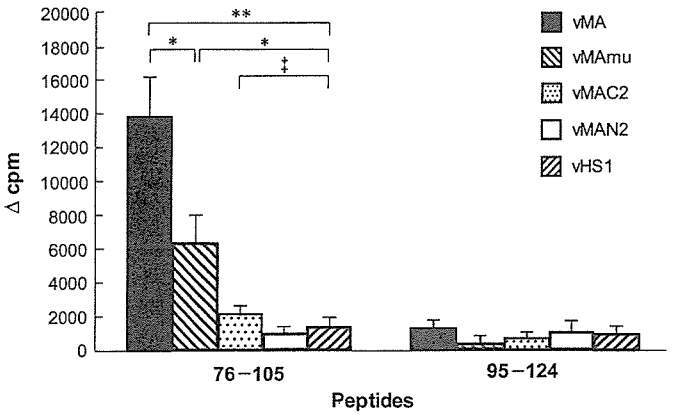

FIG. 10. Different abilities of mutant and truncated MA proteins at priming $CD4^+$ T cells. Proliferative responses of T cells obtained from mice immunized with each rVV were tested by stimulation with peptide 76-105. Each result is expressed as the mean Δcpm for data obtained from four to five mice. Error bars, standard errors of the means. The experiments were performed twice with essentially identical results. Statistically significant differences were observed by Student's t test (*, $P < 0.05$; **, $P < 0.001$). ‡, no significant difference ($P > 0.05$).

The present study has also provided useful information on the structural requirements for effective priming of virus-specific $CD4^+$ T-cell responses by the MA protein. T cells primed in vivo with native MA (vMA) proliferated when stimulated with the Th epitope-harboring peptide 76-105. In contrast, full-length MA lacking the N-terminal myristylation site (vMAmu) and the MA from which the N-terminal 24 residues had been deleted (vMAC2), despite carrying the whole Th epitope, induced only moderate or marginal T-cell responses, respectively, when used to prime T cells in vivo. Of note, their different abilities to elicit the $CD4^+$ T-cell response were well correlated with their efficacies in inducing protection against FV infection in vivo. There was also a correlation between the observed degree of localization of the MA protein at the plasma membrane and its ability to elicit T-cell proliferation and immune protection: By the destruction of the myristylation site, the degree of localization of the MA protein at the plasma membrane was diminished, and the MA lacking the N-terminal 24 residues localized predominantly in the nucleus. These results indicate that the N-terminal region of F-MuLV MA, not just the myristylation site, is responsible for its subcellular localization. A highly basic domain between MA residues 17 and 31 in HIV-1, besides the myristylation signal, has been implicated in membrane binding of the Gag polyprotein (61, 63), and there is a corresponding basic region present between MA residues 17 and 34 in F-MuLV.

Efficient priming of $CD4^+$ T cells by virally encoded proteins is dependent on sufficient levels of antigen expression and delivery of the protein-derived peptides to the MHC class II (MHC II) compartment. Although there is evidence for the activation of $CD4^+$ T cells by viral-DNA-encoded proteins (20, 32, 36), the epitopes displayed on cytoplasmic proteins are usually presented by MHC class I (MHC I) molecules to $CD8^+$ T cells. Epitopes presented by MHC II molecules to $CD4^+$ T cells are mainly derived from extracellular "foreign" proteins taken into cells by endocytotic activities and then degraded in endosomal vesicles. Although retroviral MA is a cytoplasmic protein, there must be some mechanisms for MA to gain access to the cellular site of protein processing involved in peptide presentation on MHC II. It is possible that accumulation of MA at the plasma membrane may result in the formation of aggregates that might be engulfed into phagosomes by mechanisms similar to autophagocytosis. As another possibility, some portions of the MA molecule might be exposed on the outside of the viral envelope, since neutralizing Abs reactive to MA have been detected in some retrovirus infections (3, 5, 38, 50). In addition, there may be an alternative mechanism for the presentation of foreign antigens to $CD4^+$ T cells, in which APC such as macrophages take up whole rVV-infected cells or their fragments by phagocytosis, and the degraded MA is presented on MHC II molecules through the conventional class II pathway. However, the last possibility is unlikely to be the main pathway for MHC II presentation of rVV-generated MA antigens, since the mutant MA proteins expressed by infection of vMAmu and vMAC2, which carried the Th epitope and were detected at a level comparable to that of the native MA expressed by vMA infection, nevertheless failed to induce strong enough $CD4^+$ T-cell proliferation and full protection against FV infection. Therefore, the targeting of MA to the plasma membrane may provide this protein with efficient access to the cellular site for processing and presentation through MHC II pathways, which facilitates induction of the observed immune responses through more-efficient antigen-specific activation of $CD4^+$ T cells. In support of this hypothesis, a recent study demonstrated that a chimeric HIV-1 $p55^{gag}$ protein forced to traffic to the MHC II compartment elicited strong cellular and humoral immune responses in immunized mice (27).

In summary, the results presented here provide compelling evidence that a retrovirus MA peptide is capable of inducing a strong $CD4^+$ T-cell-mediated immune response, which results in effective protection against virus challenge. It will be interesting to design future studies to explore whether there are functional differences between Env-primed and Gag-primed $CD4^+$ T cells, which may be of importance for the development of an effective antiretrovirus vaccine strategy. In addition, the finding that the binding of the MA protein to the plasma membrane is associated with its stronger immunogenicity may lead us to considerations of practical importance about the appropriate immunogenic forms of cytoplasmic proteins when they are considered as candidates for virus-based vaccines.

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Both T and non-T cells with proliferating potentials are effective in inducing suppression of allograft responses by alloantigen-specific intravenous presensitization combined with suboptimal doses of 15-deoxyspergualin

Hideo Tahara[a,b], Norimasa Iwanami[b,1], Nobutada Tabata[b,2], Haruo Matsumura[b], Takeshi Matsuura[a], Takashi Kurita[a], Masaaki Miyazawa[b,*]

[a]*Department of Urology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan*
[b]*Department of Immunology, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511, Japan*

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Abstract

In an MHC class I-disparate combination of mouse strains, a single intravenous injection of donor spleen cells combined with 10 suboptimal doses of 15-deoxyspergualin (DSG) administration was effective in inducing donor-specific suppression of cytotoxic T-lymphocyte (CTL) responses and prolonged survival of the relevant skin allograft. Proliferative potentials of the donor spleen cells were requirement for the induction of suppressed allospecific responses, but both highly purified T cells and non-T cells were equally effective to induce the suppression of CTL responses by intravenous injection. These results have shown that, although working on different mechanisms, DSG is as effective as FK506 or rapamycin in inducing allograft tolerance when used at suboptimal doses along with the donor-specific intravenous presensitization, and an immune mechanism other than well-characterized veto T cells is working in this model in suppressing alloreactive CTL precursors.
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Keywords: Donor-specific intravenous presensitization; 15-deoxysupergualin; Skin graft; Tolerance; Veto cell

1. Introduction

The favorable effects of pretransplantation transfusion of donor blood, often referred to as donor-specific transfusion (DST), on allograft survival has been well recognized (for recent reviews see [1,2]). Mechanisms of the effects of DST and influences of different transfusion protocols on the prolongation of graft survival and on recipient immune functions have been studied using various rodent models [3–11]. However, contrasting outcomes can still be observed depending on the degree of disparity at different loci in major histocompatibility complex (MHC) [1,2,11]. In mice, highly reproducible suppression of allograft responses can be induced by a single or repeated intravenous injection(s) of donor spleen cells (donor-specific intravenous presensitization: DSP) in combination with or without suboptimal doses of an immunosuppressive reagent [12–18]. Compelling evidence has indicated that DSP alone suppresses donor antigen-specific proliferative and cytokine-producing responses of recipient T-cells, but cytotoxic T-lymphocyte (CTL) induction is suppressed only by a combination of DSP with antibody-mediated $CD8^+$ T-cell depletion or administration of suboptimal doses of FK506 or rapamycin [12–17]. Cells required

Abbreviations: B6, C57BL/6; Con A, concanavalin A; CTL, cytotoxic T-lymphocyte; DSG, 15-deonxysupergualin; DSP, donor-specific intravenous presensitization; DST, donor-specific transfusion; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MMC, mytomycin C; PBBS, phosphate-buffered balanced salt solution; R-PE, R-phycoerythrin.

*Corresponding author. Tel.: +81-72-367-7660; fax: +81-72-367-7660.
E-mail address: masaaki@med.kindai.ac.jp (M. Miyazawa).
[1] *Present address:* Laboratory of Immune System Development, RIKEN Research Center for Allergy and Immunology, Tokushima, Japan.
[2] *Present address:* Department of Pediatrics, Kinki University School of Medicine, Osaka-Sayama, Japan.

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for the induction of the suppressed allograft responses by DSP are shown to be radio-sensitive [12] and belong to T-cell populations [12,15], indicating possible roles of veto cells. In the present study, we investigated the efficacy of DSP combined with suboptimal doses of 15-deoxysupergualin (DSG), an immunosuppressive reagent that has mechanisms of action quite different from those of FK506 or rapamycin, in suppressing allograft responses. In addition, we further analyzed cell populations effective in inducing the suppression of allospecific cellular immune responses by highly purifying T and non-T cells using magnetically labeled antibodies.

2. Objectives

The objectives of the present study were (1) to examine if DSG that has mechanisms of action quite different from those of previously tested FK506 and rapamycin is effective in inducing the suppression of allograft responses when used at suboptimal doses in combination with DSP; and (2) to identify a donor cell type(s) and its antigenic and proliferative characteristics required for the effective suppression of allospecific cellular immune responses when used for DSP.

3. Materials and methods

3.1. Mice

C57BL/6 (B6, $H2^b$), B10.QBR ($H2^{bq4}$), and BALB/c ($H2^d$) mice were purchased from Japan SLC, Hamamatsu, Japan, and kept and maintained in animal facilities at Kinki University School of Medicine under specific pathogen-free conditions. B6 and B10.QBR mice differ at the class I D locus, B6 possessing the D^b and B10.QBR the D^q allele, but share the same alleles at other MHC loci. The following experiments were performed under relevant guidelines of the Japanese government and the University, and were approved by the Animal Experiment Committee of the School of Medicine.

3.2. Recipient manipulation and skin grafting

(−)-15-deoxyspegualin was kindly provided by Nippon Kayaku Co., Ltd., Tokyo, Japan as Gusperimus trihydrochloride. A suboptimal dose of 1 mg/kg was given intraperitoneally to recipient B6 mice 10 times at 2-day intervals starting from 8 days before transplantation (Fig. la). For donor-specific presensitization, spleen cells were prepared aseptically from B10.QBR mice and red cells were removed as described in Refs. [14–17,19]. Single-cell suspension was prepared by passing it through sterile nylon mesh, and 1×10^7 cells were injected intravenously into each B6 recipient mouse at 7 days before skin grafting. Skin grafts were prepared by removing 7×7-mm sheets from the tail of B10.QBR mice under ether anesthesia, and transplanted onto the back of each recipient B6 mouse according to the techniques described in Ref. [20]. Bandages were removed on day 7 after transplantation, and graft tissue was observed daily. Skin grafts were determined to be rejected when >80% of the tissue became necrotic as described previously [14–17]. Some recipient mice were killed at 14 days after grafting by cervical dislocation and the spleen was removed for CTL and mixed lymphocyte reaction (MLR) assays as described previously [14–17,19].

3.3. Tissue culture media and reagents

For preparation of spleen cells phosphate-buffered balanced salt solution (PBBS) supplemented with 2% fetal bovine serum (FBS) was used as described in Ref. [19]. To remove erythrocytes, spleen cells were treated with the Tris-buffered ammonium chloride solution, and rinsed three times with PBBS [19]. For tissue culture RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10% heat-inactivated FBS selected for low background stimulation was used. Concanavalin A(Con A), lipopolysaccharide from *Escherichia coli* 0111:B4(LPS), and mytomycin C (MMC) were purchased from Sigma Chemical Co., St. Louis, Missouri.

3.4. CTL and MLR assays

To generate CTL effector cells, 5×10^6 B6 spleen cells were mixed with 1.5×10^6 X-ray (4000 rad)-irradiated B10.QBR or BALB/c stimulator cells, and cultured for 5 days in each well of 24-well tissue culture plates with 2 ml culture medium as described above. Target cells were prepared by stimulating B10.QBR or BALB/c spleen cells with 5 μg/ml Con A for 48 h, and labeling them with 3.7 MBq ^{51}Cr (NEN Life Science Products, Inc., Boston, Massachusetts) per 10^6 cells as described previously [19]. The resultant effector and target cells were mixed at indicated ratios, and the release of ^{51}Cr into the supernate from the 1×10^4 target cells per well of 96-well culture plates in 4 h was measured by using a gamma counter. Levels of antigen-specific target cell lysis were calculated by using a standard formula [19], with maximum release determined by adding 1% Triton X-100 into wells of labeled target cells. For MLR assays, 5×10^5 responder B6 spleen cells were mixed with 4×10^5 4000-rad-irradiated stimulator spleen cells in each well of 96-well tissue culture plates. After culturing for the indicated period, [^{3}H]thymidine (Amersham Life Science, Buckinghamshire, England) was added at 18.5 kBq/well, and the culture was continued for an additional 8 h. Incorporation of [^{3}H]thymidine was measured by using a microplate scintillation counter (TopCount, Packard

Instruments, Meriden, Connecticut) as described previously [19]. The magnitude of antigen-specific proliferation was shown in Δcpm calculated by using a standard formula [19].

3.5. Modification of donor spleen cells and analyses of their antigenicity

B10.QBR spleen cells were prepared as described above, and either irradiated at 1500 or 4500 rad in an X-irradiator, or incubated with 500 μg/ml MMC for 60 min at 37 °C. The treated cells were washed three times with PBBS containing 2% FBS and used as donor cells for DSP. To analyze possible changes in their antigenicity, the above-treated B10.QBR spleen cells, along with untreated ones, were used as stimulator cells and mixed with naive B6 spleen cells in MLR assays as described in Section 3.4. Mixed cultures were incubated for 3, 5 and 7 days, and [^{3}H]thymidine incorporation was measured as described above. Proliferating potentials of the above-treated spleen cells were tested by stimulating them with 5 μg/ml Con A or 10 μg/ml LPS, and [^{3}H]thymidine incorporation was measured at 1, 2, 3, 5 and 7 days after the beginning of culture as described above. Cell surface expression of class I D^q molecules on treated and untreated B10.QBR spleen cells was also analyzed by flow cytometry using an anti-D^q monoclonal antibody (clone KH117, PharMingen, San Diego, California) as described previously [19].

3.6. Purification of T and non-T cells from the spleen

Donor spleen cells were separated into CD90$^+$ T and CD90$^-$, CD45R$^+$ non-T cell populations by using a magnetic cell sorting system. Magnetic microbeads-conjugated anti-mouse CD90 (Thy 1.2) and anti-mouse CD45R (B220) antibodies and CS depletion and VS+ separation columns were purchased from Miltenyi Biotec, Berigische Gladbach, Germany and used according to the manufacturer's instructions. To purify CD90$^+$ T cells, spleen cells were first depleted of B220$^+$ cells and CD90$^+$ cells were positively selected from the B220-depleted population. To purify B220$^+$ non-T cells, spleen cells were first depleted of CD90$^+$ T cells, and B220$^+$ cells were selected from the CD90$^-$ population. Purities of each cell population were confirmed by flow cytometric analyses by using a FACSCaliblur (Becton-Dickinson Immunocytometry Systems, San Jose, California). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD3 and R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD19 antibodies and their isotype-matched controls were purchased from PharMingen.

3.7. Statistical analyses

Averages of graft survival periods and magnitudes of proliferative responses between groups of mice were compared by Student's t test. Average percentages of specific lysis at five different effector-to-target ratios were compared between groups as curves by using Hotelling's T^2 test.

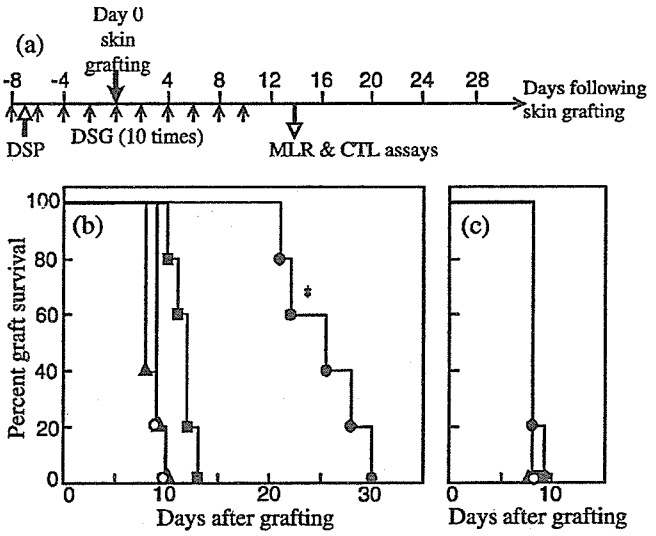

Fig. 1. Experimental protocols and time-courses of graft rejection. (a) Mice were injected with DSG and donor spleen cells as indicated, and received a skin graft. (b) Time-courses of the rejection of B10.QBR skin graft in untreated B6 mice (○), B6 mice treated with DSP alone (▲), those treated with DSG administration alone (■), or those treated with the combination of DSP and DSG administration (●). *, $P<0.001$. (c) Time-courses of the rejection of BALB/c skin graft in untreated and treated B6 mice. Symbols used are the same as those in b.

4. Results

4.1. Prolongation of skin graft survival by DSP combined with suboptimal doses of DGS in the MHC class I-disparate combination

When B6 mice were treated either by a single DSP or 10 suboptimal doses of DSG injection alone, no significant prolongation of graft survival was observed (Fig. 1b). However, in B6 mice treated with the combination of an intravenous injection of B10.QBR spleen cells and 10 suboptimal dose of DSG injection, skin grafts from the MHC class I-incompatible B10.QBR mice survived significantly longer than those transplanted onto untreated B6 mice. Skin grafts transplanted from the third party BALB/c mice were rejected within 9 days regardless of the treatment of recipient B6 mice (Fig. 1c). These results showed that DSG is as effective as FK506 and rapamycin in inducing the elongation of allograft survival when used at suboptimal doses in combination with DSP.

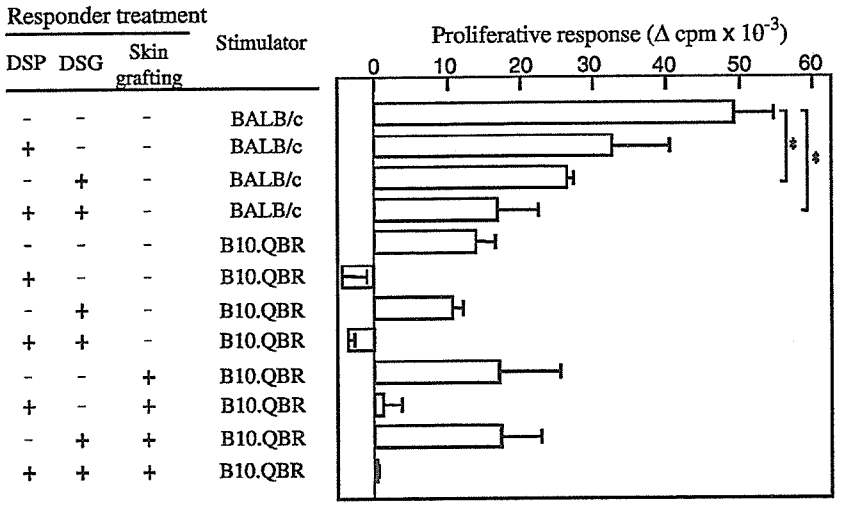

Fig. 2. Proliferative responses of untreated and treated B6 spleen cells to irradiated BALB/c or B10.QBR spleen cells. [^{3}H]thymidine incorporations were measured on days 3, 5 and 7 after the beginning of the MLR responses, and peak responses were always observed on day 5. Thus, average Δcpm values ($n=3$) at day 5 are shown with bars representing S.E.M. *, $P<0.04$.

4.2. Suppression of MLR and CTL responses in B6 mice treated with DSP and/or DSG

When spleen cells from treated and untreated B6 mice with or without skin grafting were tested for allospecific proliferative responses, administration of 10 doses of DSG alone significantly weakened MLR responses to the third party BALB/c stimulator cells, but vigorous proliferative responses were still observed even after DSP with B10.QBR spleen cells plus DSG administration (Fig. 2). In accordance with the previous observations, DSP alone, but not DSG administration alone, completely abolished the ability of B6 spleen cells to respond in MLR assays to MHC class I-disparate B10.QBR simulator cells, and this unresponsiveness was retained in the DSP-treated mice even after being grafted

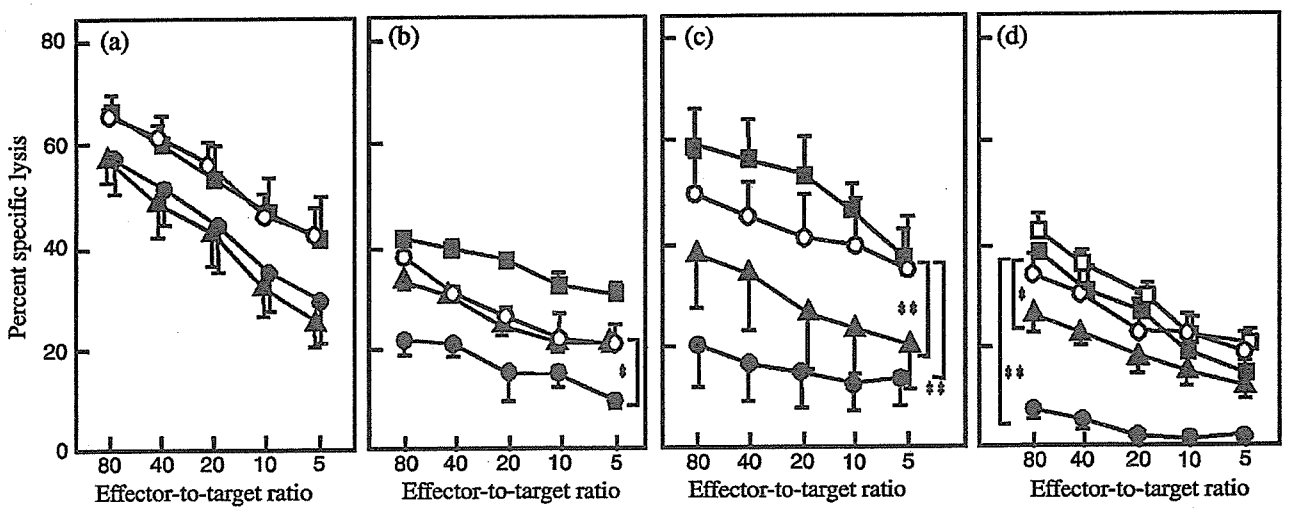

Fig. 3. CTL responses exerted by in vitro-stimulated effector cells derived from untreated and treated B6 mice. (a) Effector cells were induced by in vitro stimulation with irradiated BALB/c spleen cells, and target cells used were labeled BALB/c blast cells. (b–d) Effector cells were induced by in vitro stimulation with irradiated B10.QBR spleen cells, and target cells used were labeled B10.QBR blast cells. In a and b effector cells were prepared from untreated B6 mice (○), B6 mice treated with DSP alone (▲), those treated with DSG administration alone (■), or those treated with the combination of DSP and DSG administration (●). In c B6 mice were treated as above (shown with the same symbols), and received a B10.QBR skin graft. In d effector cells were prepared from untreated B6 mice (○), B6 mice injected with MMC-treated B10.QBR spleen cells plus DSG (▲), those injected with 1500 rad-irradiated B10.QBR spleen cells plus DSG (■), those injected with 4500 rad-irradiated BIO.QBR spleen cells plus DSG (□), or those injected with the combination of untreated BIO.QBR spleen cells and DSG administration (●). Each data point represents the mean from 3 to 6 repeated experiments, and bars represent S.E.M. *, $P<0.03$ by Hotelling's T2 test; **, $P<0.001$ by the same test.

with B10.QBR skin. However, CTL responses against B10.QBR target cells were not significantly affected by DSP alone (Fig. 3b). Only in the group of mice treated with the combination of a single DSP and suboptimal doses of DSG administration, CTL responses to B10.QBR target cells were significantly reduced. Unchanged killing activity of the in vitro-stimulated spleen cells prepared from the treated mice against BALB/c target cells (Fig. 3a) confirmed the alloantigen-specificity of the suppression of CTL activities. Furthermore, significant suppression of CTL activities against B10.QBR target cells was also observed in the B6 mice treated with the combination of DSP and DSG administration even after they received a B10.QBR skin graft (Fig. 3c). CTL activities detected in the untreated control mice and those of the mice treated only with DSG administration were apparently enhanced after skin grafting (Fig. 3b,c), reflecting in vivo priming of CTL precursor cells through alloantigen presentation. However, this priming effect was not observed in mice treated with the combination of DSP and DSG administration. Interestingly, CTL activities detected from the mice treated with a single DSP alone were also significantly lower than those in the control mice when tested after skin grafting (Fig. 3c), probably reflecting the contribution of $CD4^+$ T helper cell functions in the induction of CTL effector cells, which is reduced in the DSP-treated mice (Fig. 2). These data indicated that a significant proportion of CTL precursors were unable to be primed in vivo with the relevant alloantigen when treated with DSP, especially in combination with suboptimal doses of DSG administration.

4.3. Characterization of cellular requirements for the suppression of allograft responses by DSP

To characterize cell properties required for the induction of suppressed allograft responses by DSP, donor spleen cells were treated with several different procedures that modulated their proliferating potentials and antigenicities before intravenous injection. Irradiation (either 1500 or 4500 rad) or MMC treatment of donor spleen cells almost completely abolished their proliferative potentials after Con A or LPS stimulation: no significant incorporation of [^{3}H]thymidine into the treated cells was observed at any one of the four time-points at which the measurements were performed between 1 and 7 days after the beginning of cell cultures. When used as stimulator cells to induce proliferative responses of naive B6 spleen cells in vitro, B10.QBR spleen cells irradiated with 1500 or 4500 rad of X-ray showed significantly reduced antigenicity. They induced only 25% and 15% of peak Δcpm values at day 5 after the begin-ning of the MLR assays, respectively, in comparison with untreated B10.QBR cells, while the stimulatory potential of MMC-treated spleen cells was not significantly reduced. No significant changes in the levels of cell surface expression of D^q molecules between untreated, irradiated, and MMC-treated B10.QBR spleen cells were observed by flow cytometric analyses (data not shown). Interestingly, when used for DSP along with suboptimal doses of DSG, 1500 rad-irradiated spleen cells induced statistically significant but only marginal suppression of MLR responses, and 4500 rad-irradiated spleen cells were not effective at all in inducing the suppression (Table 1). However, MMC-treated B10.QBR spleen cells induced significant suppression of MLR responses when injected intravenously along with DSG administration. When CTL responses of the in vitro-stimulated spleen cells prepared from the untreated and treated B6 mice were compared, irradiated and MMC-treated B10.QBR spleen cells did not induce the suppressive effect that was observed when untreated B10.QBR spleen cells were injected (Fig. 3d), although the injection of MMC-treated B10.QBR spleen cells resulted in slightly reduced CTL activities in comparison with those exerted by the stimulated spleen cells of control untreated mice. These results, especially those with MMC-treated donor cells, indicated that proliferating potentials of donor cells, not just the presence of the relevant alloantigen on their surfaces, are required for the suppression of CTL responses by DSP plus DSG administration.

4.4. Both T and non-T cells were effective in inducing the suppressed allospecific cellular immune responses

To further characterize the donor cells that are involved in the induction of the suppression of allograft responses by DSP and DSG administration, spleen cells used for DSP were separately purified into $B220^-$, $CD90^+$ T and $CD90^+$, $B220^-$ non-T cell populations using a magnetic cell sorter (Fig. 4a). The obtained T-cell population was >98% $CD3^+$ and almost completely devoid of $CD19^+$ B cells, while the non-T cell population was almost completely free from contaminating T cells and comprised of 94% B cells. A single injection of both T and non-T cell populations in combination with suboptimal doses of DSG administration completely abolished the donor antigen-specific proliferative responses in the treated B6 mice (Fig. 4b). Further, CTL responses against B10.QBR target cells were significantly reduced in mice injected with the donor T or non-T cell population (Fig. 4c). As measured by the induction of suppressed alloantigen-specific CTL responses, the purified T cell population was as effective as the whole spleen cells, and purified non-T cells were even more effective and induced almost total suppression of the CTL responses.

5. Discussion

Several different mechanisms have been proposed for the induction of allograft tolerance by DST or DSP in

Table 1
Effects of donor cell irradiation or MMC treatment on the suppression of MLR responses after DSP plus DSG administration

Donor cell treatment	Proliferative responses of recipient spleen cells (Δcpm)	Significance in difference from the control
1500 rad irradiated	7820±528	$P=0.007$
4500 rad irradiated	18 714±2520	NS
MMC treated	2384±468	$P=0.004$
None	−1031±528	$P=0.003$
Control (No DSP)	13 947±2190	

Δcpm values are shown as mean±S.E.M.
NS, not significant.

rodent models. In rats tolerized by DST for heterotopic heart transplantation, recipient immune cells still infiltrated into graft tissues, but cytokine production from the immune cell infiltrated was deeply impaired [1]. Generation of an inhibitory signal from DST-primed host immune cells upon secondary presentation of donor antigens has been reported. In a model using class I L^d-specific T-cell receptor transgenic mice, a single dose of DSP with L^d-expressing spleen cells induced a significant deletion of the donor antigen-reactive T cells, and activated a regulatory subset of T cells in the recipient, which resulted in permanent survival of skin allografts [10]. The above regulatory T cells have been recently characterized as a novel CD4- and CD8- double negative population that exerts Fas-mediated killing of $CD8^+$ effector cells [18]. DST combined with anti-CD154 (CD40 ligand) antibody administration is shown to be effective in tolerance induction for islet grafts, and this model also is apparently mediated by the induction of $CD4^+$ regulatory T cells [2]. For the induction of the above tolerance by DST plus anti-CD154 antibody administration, small resting B cells are sufficient, and T cells are not required in the DST preparation.

In the MHC class I-incompatible model similar to the present study, however, radio-sensitive T cells in the donor cell inoculum is shown to be required for the induction of prolonged skin graft survival associated with reduced CTL precursor frequencies [12,15], suggesting that donor T cells in the DSP preparation may function as veto cells. In the H2 class I-disparate model

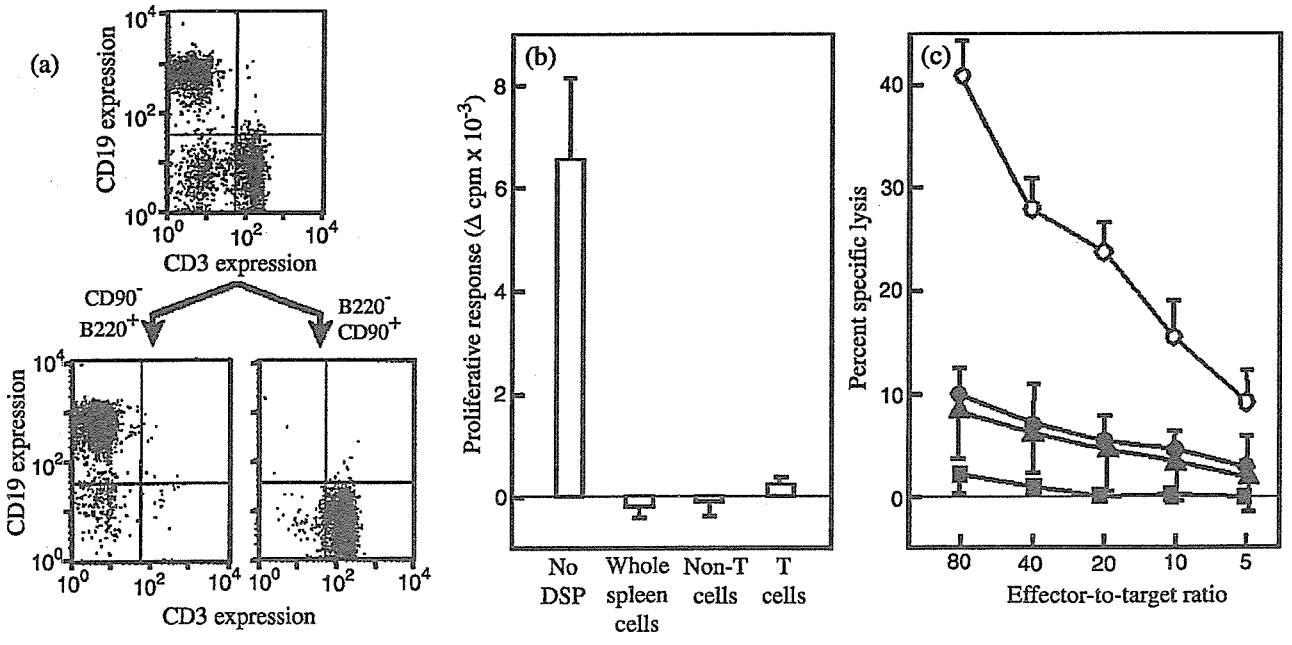

Fig. 4. Identification of cell types in DSP preparation effective in suppressing allospecific cellular immune responses. (a) Flow cytometric analyses confirming purity of injected T and non-T cell populations. (b) Proliferative responses of untreated and treated B6 spleen cells upon stimulation with irradiated B10.QBR spleen cells. Each data show mean±S.E.M. calculated with data obtained from 3 to 6 individuals per group. (c) CTL responses exerted by in vitro-stimulated effector cells prepared from untreated B6 mice (○), B6 mice injected with the purified T cells plus DSG (▲), those injected with the purified non-T cells plus DSG (■), or those injected with the whole B10.QBR spleen cells plus DSG (●). Each data point represents the mean from 3 to 6 repeated experiments, and bars represent S.E.M.