Peptide-induced immune protection of CD8\(^+\) T cell-deficient mice against Friend retrovirus-induced disease

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Abstract

CD8\(^+\) CTLs and virus-neutralizing antibodies have been associated with spontaneous and vaccine-induced immune control of retroviral infections. We previously showed that a single immunization with an \(env\) gene-encoded CD4\(^+\) T cell epitope protected mice against fatal Friend retrovirus infection. Here, we analyzed immune cell components required for the peptide-induced anti-retroviral protection. Mice lacking CD8\(^+\) T cells were nevertheless protected against Friend virus infection, while mice lacking B cells were not. Virus-producing cells both in the spleen and bone marrow decreased rapidly in their number and became undetectable by 4 weeks after infection in the majority of the peptide-immunized animals even in the absence of CD8\(^+\) T cells. In the vaccinated animals the production and class switching of virus-neutralizing and anti-leukemia cell antibodies were facilitated; however, virus-induced erythroid cell expansion was suppressed before neutralizing antibodies became detectable in the serum. Further, the numbers of virus-producing cells in the spleen and bone marrow in the early stage of the infection were smaller in the peptide-immunized than in unimmunized control mice in the absence of B cells. Thus, peptide immunization facilitates both early cellular and late humoral immune responses that lead to the effective control of the retrovirus-induced disease, but CD8\(^+\) T cells are not crucial for the elimination of virus-infected cells in the peptide-primed animals.

Introduction

Understanding the types of immune responses associated with and responsible for effective control of viral infection is pivotal for the development of antiviral vaccines. We, along with other researchers, have been studying the requirements of different immune cell components and their regulation by host genetic factors utilizing the mouse model of Friend retrovirus infection. Friend retrovirus complex (FV) is composed of replication-competent Friend murine leukemia virus (F-MuLV) and defective spleen focus-forming virus (SFFV), the latter of which induces rapid growth and terminal differentiation of infected erythroid progenitor cells (1, 2). FV is known to induce fatal erythroleukemia associated with severe immuno-suppression when inoculated into immunocompetent adult mice of susceptible strains (1, 3). Genotypes at both MHC class I and class II loci, along with those at a non-MHC locus located on chromosome 15, affect spontaneous immune resistance against FV-induced disease development which is phenotypically manifested by the regression of early splenomegaly and clearance of viremia (1, 4–8). As predicted, requirements of both CD4\(^+\) and CD8\(^+\) T cells for the above
spontaneous resistance have been demonstrated through antibody-mediated depletion of T cell subsets and through the blocking of T cell responses by administration of anti-MHC class II antibodies (9, 10). Further, different roles of CD4+ and CD8+ T cells and of virus-neutralizing antibodies have been demonstrated for immune protection against FV infection induced with a live attenuated vaccine (11, 12).

We previously showed that a single immunization with an 18-mer peptide that contains a single CD4+ T cell epitope identified within the env gene product SU of F-MuLV induces strong protective immunity against fatal FV infection in susceptible strains of mice (13, 14). In peptide-immunized (B10.A x A.BY)F1 mice, the vast majority of virus-producing cells were eliminated from the spleen between 8 and 12 days after FV challenge, and the SFFV-induced early splenomegaly regressed rapidly. Production and class switching of virus-neutralizing antibodies roughly coincided with the above reduction in the number of virus-producing cells in the spleen (13), suggesting the possible importance of virus-neutralizing antibodies in the vaccine-induced confinement of FV infection. However, since the activation of both CD4+ and CD8+ cytotoxic effector cells and of NK cells was detectable prior to the decrease of virus-producing cells in peptide-immunized (BALB/c x C57BL/6)F1 (CB6F1) mice (14), it was also possible that the cellular responses, rather than the antibodies, were mainly responsible for the control of FV-induced disease development conceivably through the destruction of virus-producing cells. Moreover, since CD8+ CTLs and NK cells were activated in comparable degrees both in peptide-immunized and unimmunized animals after FV infection (14), their actual extents of contribution to the peptide-induced immune protection remained unclear.

To directly evaluate the role of each separate immune cell component in peptide-induced protection against FV infection and to compare the effector mechanisms induced by the peptide immunization with those induced by previously described live attenuated vaccines (11, 15), we performed the protection experiments on the highly susceptible strain of mice that lacked either CD8+ T or B lymphocytes.

**Methods**

**Mice**

BALB/c-AJcl and CB6F1 mice were purchased from Japan SLC, Inc., Hamamatsu, Japan. (B10.A x A.BY)F1 mice were those described previously (13). Breeding pairs of BALB/c-J-B2m<sup>tm1Unc</sup> and C57BL/6J (B6)-<sup>B2m<sup>tm1Unc</sup> mice carrying homozygous disruption of the β2-microglobulin gene (β<sup>−/−</sup>) were purchased from the Jackson Laboratory, Bar Harbor, ME, USA, and F<sub>1</sub> crosses were produced at the Animal Facilities, Kinki University School of Medicine. Phenotypic lack of CD8+ T cells in the produced F<sub>1</sub> crosses was confirmed by bleeding each mouse from the tail vein and staining peripheral blood with a mixture of fluorescence-labeled anti-CD4 and anti-CD8 mAbs as described in the following section.

B6-Igh<sup>−6<sup>m1Cgm</sup></sup> mice carrying homozygous disruption of the membrane exon of the Ig µ-chain gene (µ-chain membrane exon-targeted: µ<sub>MT</sub>|µMT) and thus lacking B cells (16) were also purchased from the Jackson Laboratory. To induce the µ-chain disruption into BALB/c background, a cross-intercross production of a congenic strain was performed as follows: the B cell-deficient B6 male mice were mated with BALB/c female mice and F<sub>1</sub> crosses carrying heterozygous disruption of the µ-chain membrane exon were obtained. These heterozygous F<sub>1</sub> crosses were cross-mated, and F<sub>2</sub> mice carrying the homozygous µ gene disruption were selected by performing both genetic and phenotypic analyses as described below. The homozygous disruption of the µ-chain membrane exon in the resulting F<sub>2</sub> crosses was confirmed by PCR analyses as follows: genomic DNA was prepared from the tail tip of each mouse using DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. Oligo-DNA primers (5′ primer: 5′-TCTATCGCTTCTTGAGCAG-3′, 3′ primer: 5′-TACAGCTAGCTGTCTTGAG-3′) were prepared based on the sequence information on the knockout cassette (16) and were used for PCR amplification of genomic DNA fragments. PCR products were separated by electrophoresis in a 4% agarose gel and were visualized under a UV light after ethidium bromide staining. In addition to the above genetic analyses, peripheral blood was stained with a mixture of fluorescence-labeled anti-CD3 and anti-CD19 mAbs, and multicolor flow cytometric analyses were performed as described in the following section. Male F<sub>2</sub> mice carrying homozygous disruption of the µ-chain membrane exon and thus lacking B cells were mated with BALB/c female mice again, and this cross-intercross mating procedure was repeated seven times. After the seventh cycle of crossing and intercrossing, the resultant BALB/c-background mice possessing homozygous disruption of the µ-chain gene were maintained by sister–bother mating, and CB6F1 mice lacking B cells were produced by crossing the B6-Igh<sup>−6<sup>m1Cgm</sup></sup> and the above-established BALB/c-µ<sub>MT</sub>|µMT mice.

For immune protection experiments, both male and female mice aged 8–11 weeks at the time of immunization were used throughout the present study. All the animal experiments were approved by the Animal Experiment Committee and performed under the guidelines of Kinki University.

**Viruses and their inoculation**

A stock of B-tropic FV complex was originally given by Bruce Chesebro, Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, Hamilton, MT, USA. The stock used in the present study has been described (14, 17). SFFV and F-MuLV titers of the FV stock were determined as described previously (13, 18). For inoculation into CB6F1 mice, a dilution of the virus stock prepared with phosphate-buffered balanced salt solution (PBBS) containing 2% fetal bovine serum (FBS) was injected intravenously into the tail vein. Infected mice were observed at least twice a day and the number of surviving mice was determined. The development of splenomegaly was monitored by palpation as described (5, 17, 19). In some experiments, moribund mice were killed by cervical dislocation and spleen weights were measured to compare the results of palpation with actual spleen weights. Spleens weighing >0.5 g were consistently marked as palpable splenomegaly. Mice found dead were...
dissected, and their spleen weight was measured to confirm leukemic death.

**Peptide synthesis and immunization**

The peptides used for detailed mapping of the CD4+ T cell epitope were synthesized by Fmoc chemistry and purified, and their molecular weight confirmed by quad-polar mass spectrometry as described previously (20–22). Peptides used for immune protection experiments were ordered from Qiagen K. K. (Tokyo, Japan). For immunization each peptide was dissolved in PBBS and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI, USA). Mice were injected intradermally with 100 μl of the emulsion given as multiple split doses into the abdominal wall. Control mice were given an emulsion of PBBS and CFA that did not contain any peptide.

**T cell proliferation assays**

Two T cell clones, F5-5 and FP7-11 (20, 23), specific for the Eb/d-env epitope of F-MuLV env gene product were maintained as described previously (20). For examination of proliferative responses, 2 × 10^5 spleen cells irradiated with 40 Gy γ-ray were mixed with 2 × 10^5 T cells and various concentrations of a peptide in a well of 96-well microculture plates. After 48 h of incubation at 37°C, each culture was pulsed with 18.5 kBq [3H]thymidine (Du Pont NEN, Boston, MA, USA) for the final 18 h. Cells were harvested onto a glass fiber filter, and incorporated radioactivity was measured with a microplate scintillation counter (TopCount, Packard Instrument Co., Meriden, CT, USA). For the calculation of relative stimulatory effect of each peptide, the concentration of peptide i (μM) required to induce 50% of the maximum proliferative response (ED_{50}) was divided with ED_{50} (μM) of the peptide in question (22, 23). In the present study proliferative responses were measured for a range of peptide concentrations between 0.01 and 20 μM in 2-fold dilutions, and peak responses (>30 000 counts per minute) were observed by stimulation with 1 μM of peptide i. ED_{50} of peptide i was 0.2 μM.

**Assays for virus-neutralizing antibodies**

The in vitro assays for quantitative measurement of F-MuLV-neutralizing antibodies have been described elsewhere in detail (5, 13, 17, 19). Mice were bled from the tail vein under ether anesthesia and sera separated were stored at −30°C until use. Stock of an infectious molecular clone of F-MuLV, FB29 (24), was prepared from a high-producer clone of chronically infected *Mus dunni* cells. Serial 2-fold dilutions of sera were made with PBBS containing 1% FBS and mixed with an appropriate dilution of the F-MuLV stock and inoculated to *Mus dunni* cells in 24-well plates. Control wells were inoculated with the virus dilution admixed with the diluent alone. Two days later, foci of F-MuLV-infected cells were visualized with mAb 720 (18) and counted under a dissecting microscope. Neutralizing titers were determined by the reciprocals of maximum dilutions that gave a reduction in the number of F-MuLV-infected cell foci to <25% of those in the control wells. IgG titers were determined by treating each serum sample with 0.05 M 2-mercaptoethanol whereas IgM titers were calculated by dividing the neutralizing titers of the untreated sera by the corresponding IgG titers (6).

**Infectious center assays**

These assays were performed as described previously (13, 17). Briefly, spleen and bone marrow tissues were dissociated in PBBS containing 2% FBS, and a single-cell suspension was prepared by passing each dissociated tissue through a nylon mesh. Cells were stained with a combination of the following mAbs, washed three times with PBBS containing 2% FBS and 0.05% NaN₃ and stained with 20 μg ml⁻¹ 7-aminonacetoxybenzyne D (7-AAD). 7-AAD was used to exclude dead cells (26). The mAbs and their final concentrations used in the present study were: chroom conjugated anti-mouse CD3 (hamster IgG, PharMingen, San Diego, CA, USA) at 0.5 μg per 10^6 cells, FITC-conjugated anti-mouse CD4 (rat IgG2b, Seikagaku Corporation, Tokyo, Japan) at 0.5 μg per 10^6 cells, PE-conjugated anti-mouse CD8 (rat IgG2a, Caltag Laboratories, Burlington, CA, USA) at 1 μg per 10^6 cells, PE-conjugated anti-mouse CD19 (rat IgG2a, PharMingen) at 1 μg per 10^6 cells, FITC-conjugated anti-mouse CD69 (hamster IgG, PharMingen) at 1 μg per 10^6 cells and allophycocyanin-conjugated anti-mouse TER-119 (PharMingen) at 0.2 μg per 10^6 cells. TER-119 reacts with a molecule associated with glycopentin A, and marks the late erythroblasts and mature erythrocytes, but not burst-forming and colony-forming units of erythroid cells (27). Biotinylated mAb 720 (IgG1) and 514 (IgM) used for the detection of F-MuLV gp70 and SFFV gp55, respectively, on infected cell surfaces has been described (13, 17). mAb 34 (IgG2b) reactive with the p15 (MA) protein (28) was similarly purified and biotinylated to detect cell-surface expression of the gag gene products (19). All staining reactions were performed in the presence of 0.25 μg per 10^6 cells anti-mouse CD16/CD32 (PharMingen) as described previously (25) to prevent the binding of mAb to FcR-expressing cells. Isotype-matched control antibodies were either purchased from the same suppliers or prepared as purified and biotinylated Ig of an irrelevant specificity as described (25), and staining patterns obtained with the negative-control antibodies were used to draw demarcation lines between cells positively stained and
those not stained. Multicolor flow cytometric analyses were performed with a Becton Dickinson FACSCalibur and CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Mature erythrocytes and dead cells were excluded from the analyses by setting a polygonal gate in the dot plots showing intensities of forward scatter and fluorescence for 7-AAD.

**Titration of serum antibody reactive to the surface of FV-induced leukemia cells**

Sera were serially diluted between 1/4 and 1/256 with PBBS and 100 µl of each dilution was incubated with 10^6 FV-induced leukemia cells Y57-2C (H2b/Cb). Characteristics of the leukemia cell line used in the present study have been described (14). After washing twice with PBBS containing 2% FBS, bound IgM and IgG were differentially detected by incubating the cells either with FITC-conjugated anti-mouse IgM (µ-chain specific, Southern Biotechnology Associates, Inc., Birmingham, AL, USA) at 5 µg per 10^6 cells or with FITC-conjugated anti-mouse IgG (y-chain specific, Zymed Laboratories, Inc., South San Francisco, CA, USA) at 1.5 µg per 10^6 cells, respectively, for 20 min. Stained cells were washed three times before being examined by flow cytometry as described above.

**Purification of T cells and their transfer**

Purification of T cell subsets from the spleen of naive, immunized and/or FV-infected mice was performed by using mAb-conjugated magnetic microbeads and a magnetic cell sorter I (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Spleen cells were first treated with Tris-buffered ammonium chloride solution to lyse erythrocytes, and incubated with anti-B220 mAb-conjugated magnetic beads to remove B cells by passing through a negatively selecting CS column. To purify CD4+ T cells, B220− cells were then incubated with anti-CD8 mAb-conjugated magnetic beads, passed through a CS column to remove CD8+ cells and then incubated with anti-CD4 mAb-conjugated microbeads to positively select CD4+ cells by passing through a VS column. Multicolor flow cytometric analyses revealed that the resultant cell preparation was >99% CD4+. CD8+ cells were similarly purified from B200− cells by removing CD4+ cells and positively selecting CD8+ cells. This preparation was 97–98% CD8+ in repeated experiments. Percentages of CD4+ and CD8+ T cells in the spleen after FV inoculation were determined by flow cytometry in CB6F1 mice immunized with peptide i. To reconstitute the full number of T cells that belonged to each subset in the immunized mice, unimmunized recipient mice were injected intravenously with 2 × 10^7 to 2.5 × 10^7 CD4+ or CD8+ T cells per mouse.

**Depletion of CD4+ T cells**

Anti-mouse CD4 mAbs were purified from culture supernatant of the hybridoma cell line GK 1.5 (29) as described previously (13, 25). Control rat myeloma IgG was purchased from Zymed Laboratories, Inc. The amount of the mAbs required for complete depletion of CD4+ T cells from CB6F1 mice was determined by intravenously administering the purified mAb and monitoring the number of CD4+ and CD8+ cells in the spleen by flow cytometry. The schedule of mAb administration finally adopted was as follows: CB6F1 mice were immunized with 3 µg per mouse of peptide i emulsified in CFA. Three weeks later, mice were intravenously given 125 µg per mouse purified anti-CD4 mAb. Five additional intravenous doses of 125 µg per mouse anti-CD4 mAb were given 2, 4, 6, 9 and 18 days after the first administration. The negative-control rat IgG was given to a separate group of mice on the same schedule. Mice were inoculated with 150 spleen focus-forming units (SFFU) FV 7 days after the beginning of the mAb administration. Peripheral blood was collected from three representative animals at each time point through the tail vein at post-infection day (PID) 3, 6, 10 and 13, and flow cytometric analyses were performed to confirm the absence of CD4+ T cells.

**Statistical analyses**

Differences in survival curves expressed by the Kaplan–Meier method were compared by a Mantel–Haenszel logrank test using GraphPad Prism 3 (GraphPad Software, Inc., San Diego, CA, USA). The numbers of mice that developed or lacked splenomegaly were compared between the immunized and unimmunized groups by Fisher's exact test. Average numbers of infectious centers between experimental groups and anti-leukemia cell antibody titers were compared by Mann-Whitney's U-test because these values were not expected to follow a Gaussian distribution. Differences in IgM and IgG titers of virus-neutralizing antibodies were compared by paired t-test. Spleen weights and percentages of TER-119+, gp70+ cells in the spleen and bone marrow between the immunized and unimmunized groups of mice were compared by Student's or Welch's t-test depending on whether the variances of the compared samples were estimated to be equal or not.

**Results**

**Suppression of the early growth of FV-infected erythroid cells and prevention of leukemic death in highly susceptible CB6F1 mice by immunization with a single-epitope CD4+ T cell vaccine**

Mice of BALB/c background are extremely susceptible to FV-induced disease, and CB6F1 mice all died within 60 days after infection with only 15 SFFU of FV without showing any signs of spontaneous recovery (Fig. 1a). This was striking because even (B10.A X AWySn)F1 mice that have been used as a strain typically susceptible to FV infection have shown mortality rates of 70–80% at 90–100 days after inoculation with 15 SFFU FV (1, 5). Therefore, the following immune protection experiments were performed in CB6F1 mice with 150 SFFU of FV to ensure that peptide-induced immune responses protect this highly susceptible strain of mice from doses of FV large enough to kill all immunized animals. The efficacy of peptide i in priming CD4+ T cells in vivo has been demonstrated by the establishment of CD4+ T cell clones reactive to this peptide from the peptide-immunized CB6F1 mice (23), and by more pronounced expansion of CD4+ T cells in the spleen after FV challenge in the peptide-immunized than in the unimmunized control CB6F1 mice (14). To directly demonstrate the priming of CD4+ T cells in peptide-immunized...
CB6F1 mice, CD4+ and CD8+ T cells were purified from CB6F1 mice at 3 weeks after a single immunization with peptide i, and re-stimulated in vitro in the presence of syngeneic, γ-irradiated spleen cells as antigen-presenting cells (APCs). As shown in Fig. 2(a), CD4+ T cells purified from the immunized CB6F1 mice proliferated vigorously when stimulated with 1 μM of peptide i, while the proliferative responses of CD8+ T cells purified from the control mice given CFA alone were below the background level even when stimulated with 20 μM of the same peptide. As controls, CD8+ T cells purified either from the peptide-immunized or unimmunized control mice showed no significant proliferative responses even when stimulated with 20 μM peptide i.

To determine the minimal amount of the peptide that is required for the effective induction of protective immunity against FV infection, three different amounts of peptide i were given as a single intradermal immunization to CB6F1 mice, and immunized mice were challenged with 150 SFFU FV. Since most of the unimmunized CB6F1 mice died by PID 45 (Fig. 1a), infected mice were either dissected soon after their death or killed at PID 45, and their spleen weight was measured. As shown in Fig. 1(b), a single immunization with 3 μg (1.7 nmol) per mouse of peptide i was as effective as 10 μg per mouse of the same peptide, and only one mouse among the ten that were given 1 μg peptide i developed splenomegaly after FV infection. Thus, in the following experiments, 3–10 μg per mouse of peptide i was used as a sufficiently large protective dose.

FV-induced early splenomegaly is caused by the rapid growth and differentiation of SFFV-infected erythroid progenitor cells. As shown in Fig. 1(c), the percentages of TER-119+ erythroid cells among nucleated spleen cells of FV-infected CB6F1 mice were either immunized once with 10 μg (5 nmol) of peptide i in CFA or given CFA alone and inoculated with FV 4 weeks later. Each data point shows mean ± SEM calculated by using four to five individual mice per group. (d) Changes in serum titers of virus-neutralizing IgM and IgG antibodies after FV infection. CB6F1 mice were either immunized once with 10 μg per mouse peptide i in CFA or given CFA alone, and challenged with 150 SFFU FV 4 weeks later. Each data point shows mean ± SEM calculated by using seven to eight individual mice per group. Serum titers of F-MuLV-neutralizing IgM and IgG were compared by paired t-test: *IgM titers are significantly higher than IgG titers at P < 0.05; **P < 0.0001; †, IgG titers are significantly higher than IgM titers at P < 0.05; ‡, P < 0.01.
cells, and the resultant erythroblasts and maturing red cells are marked by mAb TER-119 (27). Thus, bursting of the TER-119+ erythroid cells was observed in the unimmunized control mice starting from PID 7, following the slow initial increase of the same cell population (Fig. 1c). On the other hand, the number of TER-119+ erythroid cells in the spleen started to decrease between PID 5 and 7 in the CB6F1 mice that had been immunized once with peptide i. Virus-neutralizing antibodies in the serum were not detectable at PID 7 in FV-infected CB6F1 mice regardless of whether they had been immunized with peptide i or not (Fig. 1d). In the CB6F1 mice given CFA without a peptide (CFA alone) were used as controls. Data shown are averages + SEM of triplicate cultures, and the experiments were performed three times with essentially the same results. (b) Sequences of peptide i and its truncated derivatives, and their relative efficiency to stimulate FV-specific T cell clones. Representative data obtained with clone F5-5 are shown, while the data obtained with clone FP7-11 were consistent with those presented here. ED50 of the full-length i was 0.2 μM. (c) Protection of CB6F1 mice against FV infection with the truncated peptide. CB6F1 mice (n = 10 per group) were immunized once with 5 nmol per mouse of peptide i13, 25 nmol per mouse of i12, or 25 nmol per mouse of i11. Control mice were given CFA emulsion containing no peptide. Four weeks later, they were inoculated with 150 SFFU FV and followed for their survival. (d) CB6F1 mice (n = 22 per group) were immunized once with 3 μg per mouse of peptide i and repeatedly injected with the anti-CD4 mAb (D) or control rat IgG (O). Four weeks after immunization, these mice and a group of unimmunized control mice (●) were inoculated with 150 SFFU FV and followed for their survival.

Protection against FV disease correlates with CD4+ T cell stimulation

To identify the minimal effective sequence of the peptide vaccine, a series of truncated peptides were compared for their in vitro T cell-stimulating and in vivo protection efficacies (Fig. 2b and c). It was clear that the C-terminal Arg residue was indispensable for the recognition of this epitope by T cells. In fact, a 17-mer peptide, i–R, that lacked only the C-terminal...
Arg totally lost the ability to stimulate CD4+ T cell proliferation in vitro, while another 17-mer, i17, that retained the Arg residue but lacked the N-terminal His kept the ability, albeit less efficiently than peptide i, to stimulate the T cells. When N-terminal residues were further removed from the 18-mer i and their efficacy to stimulate the CD4+ T cells was examined through the range of concentrations between 0.01 and 20 μM, the 13-mer (i13) retained the T cell-stimulating activity and showed a stimulatory effect comparable to peptide i17, while the 12-mer (i12) showed a stimulatory effect <1/24 of that of the full-length peptide i. Peptide i11 did not induce significant proliferation even when as much as 20 μM was added to the culture. In line with this result, the 13-mer retained the ability to induce protection against FV challenge in immunized CB6F1 mice, while the 12-mer did not protect the same strain of mice against FV-induced disease even when five times more molecules were administered (Fig. 2c).

The requirement of CD4+ T cells for the peptide-induced immune protection was further confirmed by depleting CD4+ T cells from vaccinated CB6F1 mice. The adopted schedule of the antibody administration resulted in undetectable CD4+ T cells in the spleen in separately examined uninfected animals for a period equivalent to PID 0–14, and lack of CD4+ T cells in the peripheral blood was confirmed in the vaccinated and infected group on PID 3–13 (data not shown). Antibody-induced depletion of CD4+ T cells abrogated the efficacy of peptide immunization, and CD4+ T cell-depleted animals died even more rapidly than the unimmunized control mice (P < 0.05). Injection of the control rat IgG did not affect the protective efficacy of the peptide vaccine, and ~80% of the peptide-immunized CB6F1 mice that had been given the control antibody survived past PID 60 (Fig. 2d).

**Peptide-induced immune protection against FV-induced disease in CB6F1 mice genetically lacking a single component of the immune system**

To examine possible effectiveness of the peptide immunization in mice genetically lacking either CD8+ T or B cell components of the immune system, we produced CB6F1 mice with a homozygous disruption of the β2m gene or of the Ig μ-chain gene. The absence of CD8+ T or B lymphocytes, respectively, was confirmed by flow cytometric analyses of the spleen and PBMCs (data not shown). In accordance with the prior experiments (Figs 1 and 2), >80% of the wild-type CB6F1 mice were protected against FV infection when immunized with peptide i. Protective efficacy of the 13-mer peptide, i13, was further confirmed, and the development of early splenomegaly was prevented in 70% of the CB6F1 mice given i13 (Fig. 3a). Surprisingly, when CB6F1-β2m−/− mice lacking CD8+ T cells were immunized with peptide i, only <30% of the immunized mice developed splenomegaly and >70% survived until PID 100 in repeated experiments (Fig. 3). The observed survival curves were not significantly different between the peptide-immunized wild-type and β2m−/− groups (P > 0.4), indicating a similar effectiveness of the peptide vaccine both in the presence and absence of CD8+ T cells. On the other hand, when the mice of the same susceptible CB6F1 background that lacked B cells due to the homozygous μMT mutation were immunized with peptide i, they developed splenomegaly and all died by PID 100, indicating crucial roles of B cells for the peptide-induced immune protection. Interestingly, however, the temporal changes in the incidences of splenomegaly and leukemic death delayed significantly in repeated experiments in the peptide-immunized, B cell-deficient mice compared with those in the unimmunized control mice of the same deficiency (Fig. 3c and f). The delay in the development of splenomegaly in the peptide-immunized μMT/μMT mice was also substantiated by flow cytometric enumerations of FV-infected erythroid cells: at PID 7, 28.2 ± 3.7% (n = 5) of the nucleated spleen cells were positive for both TER-119 and F-MuLV gp70 in the unimmunized control mice, while the proportion of the TER-119+/gp70+ cells in the spleen was significantly smaller (P < 0.05) 12.1 ± 8.2% (n = 5) in the peptide-immunized μMT/μMT mice. The effect of peptide immunization was more striking in the bone marrow where the percentage of TER-119+, gp70+ cells in the unimmunized mice was 11.1 ± 4.4%, while that of the peptide-immunized mice was 0.72 ± 0.22% (P < 0.03) at PID 7. These results indicate some functions of non-B cells in delaying the FV-induced disease development.

**Elimination of FV-producing cells from the spleen and bone marrow in the β2m−/− mice immunized with peptide i**

We next compared the numbers of FV-infected cells between peptide-immunized and unimmunized control mice using infectious center assays. The relative ratio in the number of FV-producing cells in the spleen between the peptide-immunized and unimmunized mice started to decrease at PID 8 as observed in the previous experiments (13, 14), and FV infectious centers became undetectable by our assays by PID 28 in peptide-immunized wild-type mice (Fig. 4a). The lack of detectable FV-producing cells in the spleen of all the tested, peptide-immunized CB6F1 mice was confirmed by seeding the cells prepared from the entire spleen (>10^9) of each animal as infectious centers at PID 28. The number of FV-producing cells in the bone marrow was also significantly lower in the peptide-immunized wild-type mice than in the unimmunized control mice at PID 8, 14 and 21, and became undetectable at PID 28 (Fig. 4d). At PID 28, 2.1 × 10^7 bone marrow cells were tested from each mouse and no infectious centers were detectable by our assays in any of the examined animals. In the CB6F1-β2m−/− mice, the numbers of FV-producing cells in the spleen and bone marrow were significantly lower in the peptide-immunized than in unimmunized control mice at PID 14 and 28 (Fig. 4b and e), in accordance with the observed effectiveness of the peptide immunization in preventing the FV-induced disease development in the absence of CD8+ T cells (Fig. 3). It should be noted that in seven of the nine immunized animals tested at PID 28 no infectious centers were detectable even when the cells of the entire spleen were inoculated into the culture. However, there were also individuals among the peptide-immunized β2m−/− mice in which FV-producing cells were still detectable in the spleen or bone marrow at PID 28 (Fig. 4b and e), while such cells were not detectable in any of the immunized wild-type mice tested at the same time point. These results imply that CD8+ T cells were not necessarily required but may play some roles in the elimination of virus-infected cells in peptide-immunized CB6F1 mice.
In accordance with the lack of protection against FV-induced disease development, virus-producing cells constantly increased between PID 5 and 21 in the spleen and bone marrow of the CB6F1-lMT/lMT mice, regardless of whether the hosts were immunized with peptide i or not. Interestingly, however, the numbers of virus-producing cells both in the spleen and bone marrow were significantly lower in the peptide-immunized, B cell-deficient mice than those in the unimmunized control mice of the same deficiency at PID 8 (Fig. 4f). This observation is consistent with the significant delay in the development of early splenomegaly and leukemic death (Fig. 3), and smaller numbers of TER-119+ and viral gp70+ FV-infected erythroid cells in the spleen and bone marrow in peptide-immunized, B cell-deficient CB6F1 mice.

**Fig. 3.** Effects of immunization with peptide i on the development of FV-induced disease in CB6F1 mice lacking CD8+ T or B cells. Wild-type CB6F1 mice (a and d), CB6F1 mice lacking CD8+ T cells due to homozygous targeting of the β2m gene (b and e) and CB6F1 mice lacking B cells due to homozygous targeting of the membrane exon of Ig μ-chain gene (c and f) were either immunized with 10 μg per mouse of peptide i in CFA (●) or given CFA alone (○). Another group of the wild-type mice were immunized with 10 μg per mouse of peptide i13 in CFA (A). Four weeks later, they were inoculated with 150 SFFV FV and followed for the development of splenomegaly and leukemic death. In (c), * indicates significant differences in the frequency of splenomegaly between the immunized and control groups (P < 0.001), and in (f), † indicates significant difference between the two survival curves (P = 0.041). The number of animals in each group were: (a) and (d), ●, 10; ○, 10; (b) and (e), ●, 23; ○, 20 and (c) and (f), ●, 12; ○, 16. The experiments were performed twice with essentially identical results.
An increase in the proportion of CD69+ cells among CD4+ T cells was readily detectable (Fig. 5b). The percentages of CD69+ cells among CD4+ T cells in the spleen at PID 7 were significantly higher in the peptide-immunized than in the unimmunized animals, indicating re-activation of peptide-primed T cells upon FV infection (Fig. 5c). The effect of peptide immunization on the induction of CD69 expression was even more pronounced when bone marrow cells were tested (Fig. 5d). Interestingly, the CD69+ population among CD8+ T cells also showed a significant increase when peptide-immunized and unimmunized μMT/μMT mice were compared at PID 7, confirming the previously demonstrated activation of CD8+ cytotoxic cells at PID 7 (14).

Production and class switching of serum antibodies reactive to the surface of FV-induced leukemia cells in the peptide-immunized mice

Although virus-neutralizing antibodies were not detectable in FV-infected animals until PID 14 (Fig. 1d), non-neutralizing anti-FV antibodies might have been produced at earlier time points, and might have contributed to the observed decrease in the number of FV-infected cells in the vaccinated animals, which was evident at as early as PID 7 (Figs 1c and 4). Thus, the possible presence of anti-FV antibody in the serum was examined using FV-induced leukemia cells as indicators. The hemisyngeneic (H2b/b) FV-induced leukemia cells Y57-2C expressed both the F-MuLV gag and env gene products as
well as SFFV gp55 on their surfaces (Fig. 6a). Sera from FV-infected CB6F1 mice bound onto the surface of Y57-2C cells, and geometric means of the fluorescence intensities decreased in proportion to serum dilutions (Fig. 6b). Therefore, at each time point titers of serum antibodies reactive to the surface of the FV-induced leukemia cells, designated hereinafter anti-leukemia cell antibody titers, were determined by dividing geometric means of fluorescence intensities obtained by incubating the indicator cells with a 1/16 dilution of serum samples by the geometric mean of fluorescence intensities obtained with the same dilution of pooled control serum collected from uninfected CB6F1 mice. Interestingly,
serum anti-leukemia cell antibodies were detectable at as early as PID 7, but average titers of these antibodies decreased in the following 3 weeks of infection, and no class switching to IgG was observed in the unimmunized animals (Fig. 6c). In contrast, anti-leukemia cell IgM titers were significantly higher in the peptide-immunized than in the unimmunized control mice at PID 14, and IgG class of anti-leukemia cell antibodies were detectable at PID 21 and 28 in the peptide-immunized animals.

**Role of CD8+ T cells in the induction of Ig class switching of virus-neutralizing and anti-leukemia cell antibodies**

Kinetics of the production and class switching of virus-neutralizing and anti-leukemia cell antibodies in the genetically
modified animals were analyzed between PID 7 and 28. Serum titers of FV-neutralizing IgM and IgG in unimmunized β2m−/− mice were not significantly different from those in the unimmunized wild-type mice (compare Figs 1d and 7a). As in the case of peptide-immunized wild-type mice, production of virus-neutralizing IgM was detected at PID 14 in the serum of peptide-immunized β2m−/− mice genetically lacking CD8+ T cells. However, in contrast to the peptide-immunized wild-type mice, neutralizing Ig titers in the peptide-immunized β2m−/− animals were not significantly higher than their IgM titers even at PID 28, suggesting some roles of CD8+ T cells in facilitating class switching of virus-neutralizing antibodies in FV-infected mice. The role of CD8+ T cells in the induction of IgG class virus-neutralizing antibodies was further confirmed by transferring purified CD8+ T cells from peptide-immunized to unimmunized mice. Unimmunized mice did not possess detectable levels of virus-neutralizing antibodies in their serum at PID 10, and the antibodies were IgM-dominant at PID 20 (Fig. 7b), confirming the results of the kinetic analyses shown in Fig. 1(d). As expected, the recipients of CD4+ T cells from the peptide-immunized and FV-infected mice showed the production of neutralizing IgG at PID 20. Interestingly, the recipients of highly purified CD8+ T cells from peptide-immunized and challenged mice also showed the production of virus-neutralizing IgG, the level of which was comparable to that in the recipients of the CD4+ T cell transfer. As controls, transfer of purified CD4+ or CD8+ T cells from unimmunized control mice into FV-infected CB6F1 mice did not induce significant class switching of virus-neutralizing antibodies even at PID 20 (data not shown). No neutralizing antibodies were detectable in FV-infected μMT/μMT mice regardless of whether they were immunized with peptide i or not.

When anti-leukemia cell antibodies in the sera were tested, unimmunized β2m−/− mice possessed anti-leukemia cell IgM at PID 7 and their titers decreased toward PID 14 as observed in the unimmunized wild-type mice (Fig. 7c). High titers of anti-leukemia cell IgM were also detectable in the peptide-immunized β2m−/− mice at PID 7; however, in contrast to the peptide-immunized wild-type mice, β2m−/− mice did not show a significant increase in the IgG titers between PID 14 and 21, confirming inefficient class switching of both neutralizing and anti-leukemia cell antibodies in the β2m−/− animals.

**Fig. 7.** Titers of virus-neutralizing and anti-leukemia cell IgM and IgG antibodies in sera from the CD8+ T cell-deficient CB6F1 mice and the effect of immune T cell transfer on the production of virus-neutralizing antibody. (a) CB6F1-β2m−/− mice were either immunized with peptide i or given CFA alone and challenged with FV as described. Sera were collected at the indicated time points and their neutralizing IgM and IgG titers were determined. Each data shows mean ± SEM calculated from five to nine serum samples, with statistically significant differences between the paired IgG and IgM titers indicated by *P < 0.03, **P < 0.001. (b) CD4+ and CD8+ T cells were purified from the spleen of peptide-immunized CB6F1 mice at PID 7 and separately transferred into unimmunized control mice at PID 7. Sera were collected 3 (PID 10) and 13 days (PID 20) after cell transfer. Data shown here are mean ± SEM calculated from 4 to 5 serum samples at each time point. Differences between neutralizing IgM and IgG titers were compared by paired t-test: **P < 0.005. (c) Changes in the titers of IgM and IgG anti-leukemia cell antibodies detectable in the sera after FV infection. Each data point shows mean ± SEM calculated by using four to seven mice per group. *, the indicated titer in the immunized mice is significantly higher than that in unimmunized mice at P < 0.05.
In the present study, we attempted to unravel the different roles of CD8+ T and B cells in peptide-induced immune protection against FV-induced disease development by using genetically modified animals of the highly susceptible CB6F1 background. CB6F1 mice immunized only once with the peptide that contained a single CD4+ T cell epitope were protected against fatal FV disease, and the development of early erythroid cell proliferation was prevented by the peptide immunization. The minimum sequence of the peptide required for in vivo protection against FV challenge, VYSQFEKSYRHKR, was the same as that required for CD4+ T cell stimulation in vitro, indicating a close correlation between the peptide’s ability to stimulate CD4+ T cells and its efficacy in inducing protective immunity against FV-induced disease development. The requirement of CD4+ T cells for the peptide-induced immune protection against FV infection was further confirmed by the lack of protection in the vaccinated animals after antibody-induced depletion of CD4+ T cells.

In contrast to the bursting of TER-119+ erythroid cells in the spleen of the unimmunized mice starting from PID 7, the reduction in the number of erythroid cells was observed between PID 5 and 7 in the peptide-immunized CB6F1 mice, before virus-neutralizing antibodies became detectable in the serum (Fig. 1), and the numbers of spleen and bone marrow infectious centers were significantly smaller in the immunized than in the unimmunized control mice at PID 8 (Fig. 4). Although IgM antibodies reactive to the surfaces of FV-infected leukemia cells were detectable at as early as PID 7 (Figs 6 and 7), these antibodies are unlikely to play major roles in the observed suppression of the early growth of FV-infected cells in the vaccinated animals because anti-leukemia cell titers were not significantly different between the immunized and unimmunized groups at PID 7 (Fig. 6). This notion is consistent with the significantly reduced numbers, in comparison with those in unimmunized animals, of infectious centers in the spleen and bone marrow of the vaccinated mice at PID 8 even in the absence of B cells (Fig. 4c and f), and suggests the possibility that cellular immune responses, rather than antibodies, are involved in the regulation of SFFV-induced erythroid cell proliferation. In this regard, significantly larger populations of both CD4+ and CD8+ T cells were activated in the peptide-immunized, B cell-deficient mice in comparison with those in the unimmunized control mice at PID 7 (Fig. 5), and cytotoxic effector functions exerted by CD4+ and CD8+ T cells and those exerted more efficiently by NK cells have been demonstrated at as early as PID 7 in FV-infected CB6F1 mice (14). These non-B effector cells might be involved in the control of SFFV-induced erythroid cell proliferation through direct killing of infected target cells. On the other hand, the numbers of virus-producing cells detected after PID 14 in the spleen and bone marrow were not different between the immunized and unimmunized groups of the B cell-deficient mice (Fig. 4c and f). This is again consistent with the detection of significantly higher titers of virus-neutralizing and anti-leukemia cell antibodies in the serum of vaccinated than in the unimmunized wild-type and β2m−/− animals starting from PID 14 (Figs 1d and 6). These data suggest that the observed elimination of FV-producing cells in the peptide-immunized mice after PID 14 may depend mainly on the production of antibodies. Thus, early prevention of erythroid cell proliferation apparently depends mainly on cellular immune responses, but humoral responses seem to play crucial roles in the elimination of virus-producing cells in the later stage.

As to the relationship and relative importance between virus-neutralizing and anti-leukemia cell antibodies, tempos of the production and class switching of these antibodies after PID 14 were similar in the vaccinated wild-type animals (Figs 1d and 6c). However, in unimmunized animals, significant production of virus-neutralizing IgM was detectable after PID 21 when anti-leukemia cell titers were low and diminishing. Similarly, paradoxical production of virus-neutralizing antibodies in the presence of low anti-leukemia cell antibody titers has been observed in H2aB (A.BY × A/WySn)F1, mice at 20 days after FV infection (Miyazawa, M., Ishihara, C., and Takei, Y. A., unpublished results). It should be noted that although the anti-leukemia cell IgG titers at PID 21 and 28 were low, mean fluorescence intensities were four to five times higher than that obtained with the control serum, which reflects significant shifting of the peaks of fluorescence (Fig. 6b). Thus, it is conceivable that only a small proportion of serum antibodies detectable as anti-leukemia cell antibodies exhibit virus-neutralizing capability especially in the early stage of FV infection, and the proportion of neutralizing antibodies among anti-FV antibodies increases in the later stage. This interpretation also suggests that the production of antibodies reactive to virus-neutralizing epitopes, but not just any anti-FV antibody, depends on T cells. Further studies are required to elucidate the molecular and epitope specificities of neutralizing and anti-leukemia cell antibodies. It can be pointed out that the presence of virus-neutralizing IgM at PID 21 is not effective to reduce the number of FV-infected cells as clearly shown in the case of unimmunized, wild-type CB6F1 mice (Figs 1d and 4). Further, class switching to IgG of virus-neutralizing and anti-leukemia cell antibodies may not be a requisite because the number of FV infectious centers were reduced in the absence of efficient class switching in the peptide-immunized β2m−/− mice (Figs 4 and 7). Thus, the presence of virus-neutralizing and/or anti-leukemia cell IgM at around PID 14 might be crucial in preventing the spread of FV infection to a large enough number of target cells to support progressive infection.

Using partially FV-resistant (B10.A × A.BY)F1 mice and an N-tropic F-MuLV as an attenuated vaccine, Dittmer, Brooks, and Hasenkrug (11) dissected different roles of the immune cell components in protection against FV-induced disease development. Their demonstration of the effectiveness in inducing the recovery from initial splenomegaly of cell transfer from vaccinated to naive animals after depletion of immune CD8+ T cells is in agreement with our results showing the peptide-induced reduction of FV-producing cells in the spleen and bone marrow in the absence of CD8+ T cells (Fig. 4). However, the development of splenomegaly was prevented only when the whole spleen cells or all three sub-populations (CD4+, CD8+ and CD19+) of lymphocytes were transferred from the vaccinated to naive animals in the above live vaccine experiments, while in the present study the development of early splenomegaly was prevented in >70% of the CB6F1-β2m−/− mice after immunization with peptide i. This apparent discrepancy may be due to the difference in challenge dose of
FV (10 000 versus 150 SFFU), or might also be explained by the exaggerated and earlier production of IFN-γ from CD4+ T cells and persistent activation of NK cells in β<sup>−/−</sup>/MT<sup>−/−</sup> mice in comparison with those in their wild-type counterparts (30), as discussed below.

Dittmer, Brooks, and Hasenkrug (11) also showed that passive priming with a virus-neutralizing mAb prior to FV challenge resulted in a significant reduction in the number of virus-producing cells at PID 10 and recovery from the initial development of splenomegaly, although 2.9 × 10<sup>6</sup> infectious centers on average were still detectable in the spleen. This result is partly consistent with our demonstration that B cells are required for the elimination of virus-producing cells from the spleen and bone marrow, especially after PID 14. However, the reported lack of protection after the transfer of B cells from vaccinated to naive mice contrasts with our demonstration of the crucial role of B cells. As they discussed later (12), transferred B cells might not have produced a sufficient level of FV-reactive antibodies until they were re-stimulated upon FV challenge of the recipients. In this regard, the production of virus-neutralizing antibodies in FV-infected mice is dependent on CD4+ T cells (31). Further, we have shown in our previous (13, 17) and the present experiments that peptide-induced priming of CD4+ T cells facilitates both production and class switching of virus-neutralizing and anti-leukemia cell antibodies upon FV infection. Thus, the lack of protection by the transfer of purified B cells alone from vaccinated to naive mice does not necessarily contradict our demonstration of the requirement of B cells for peptide-induced immune protection, especially because the titers of anti-FV antibody had not been determined in the above-reported B cell-transferred animals. The same authors (32) have shown that the presence of virus-neutralizing antibodies at the time of infection is crucial for a vaccine-induced protection of naturally resistant C57BL/6 mice against FV infection.

The observed lack of protection in the μ<sup>MT</sup>/μ<sup>MT</sup> mice might also be caused by the lack of or inefficient priming and/or re-activation of CD4+ T cells due to the absence of B cells as APC. Although successful priming of CD4+ and CD8+ T cells with protein as well as cellular antigens and effective induction of CTL responses have been reported in the μ<sup>MT</sup>/μ<sup>MT</sup> mice (33, 34), the effect of the homozygous μ<sup>MT</sup> mutation on T cell priming can be variable (35). However, since we were using as immunogen the 18-mer peptide which has been shown to prime CD8+ T cells and persistent activation of NK cells in β<sup>−/−</sup>/MT<sup>−/−</sup> mice in comparison with those in their wild-type counterparts (30), as discussed below.

Actual effector mechanisms involved in the observed elimination of FV-infected erythroid cells in the absence of CD8+ T cells (Fig. 4) may include the previously described CD4+ CTLs and NK cells (14), as well as FV-reactive, cytotoxic antibody (38). However, anti-leukemia cell IgMs were detectable in both immunized and unimmunized animals at PID 7 (Figs 6 and 7), excluding the role of these antibodies in controlling the growth of FV-infected cells in the early stage. It should be emphasized that killing activities of CD4+ cytotoxic and NK cells were detectable in the peptide-immunized C6F<sub>1</sub> mice at as early as PID 7, and NK cells were much more efficient than CD8+ CTLs in killing FV-induced leukemia
cells (14). Further, it has been shown that activation of NK cells after lymphocytic choriomeningitis virus infection is prolonged in β2m−/− mice than in the wild-type mice (30). Thus, similarly enhanced activation of NK cells, along with the exaggerated production of IFN-γ from vaccine-primed CD4+ T cells, might have compensated otherwise indispensable CD8+ T cell functions in the peptide-immunized CB6F1-β2m−/− mice. In this regard, the incidences of splenomegaly in unimmunized CB6F1-β2m−/− mice at PID 14 and 21 were significantly lower than those in the unimmunized wild-type mice (P < 0.001), although their survival curves were not significantly different (P > 0.05) (Fig. 4). These observations are consistent with the previously observed activation and killing efficacy of NK cells in FV-infected CB6F1 mice without prior immunization (14), and with the reported enhancement of virus-induced NK cell activity in β2m−/− mice (30).

Taken together, the present study has demonstrated that for efficient immune protection against FV infection with the single-epitope peptide, B cells are more important than CD8+ T cells, but B cell-independent responses, probably exerted by previously demonstrated CD4+ CTLs and NK cells, do play some roles in the earlier stage of FV-induced disease development in suppressing the expansion of FV-infected erythroid cells. Careful comparison of these results and other reports may suggest the possibility that priming of CD4+ T cells with the peptide vaccine might allow the bypassing of the CD8+ T cell activity in β2m−/− mice (30).

References


24 Sito, M., Sola, B., Evans, L. et al. 1986. Hemolytic anemia and erythroleukemia, two distinct pathogenic effects of Friend
MuLV: mapping of the effects to different regions of the viral genome. Cell 47:851.


