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Multiplicity of Virus-Encoded Helper T-Cell Epitopes Expressed on FBL-3 Tumor Cells

MICHIHIRO IWASHIRO,[1] TOKUKAZU KONDO,[2] TAKEYUKI SHIMIZU,[2] HIDEO YAMAGISHI,[2] KATSUTOSHI TAKAHASHI,[3] YUJI MATSUBAYASHI,[1] TOHRU MASUDA,[1] AKIRA OTAKA,[4] NOBUTAKA FUJII,[4] AKINORI ISHIMOTO,[5] MASAAKI MIYAZAWA,[6,7] MICHAEL N. ROBERTSON,[7] BRUCE CHESEBRO,[7] AND KAGEMASA KURIBAYASHI[1]*

Institute for Immunology, Faculty of Medicine,[1] Departments of Biophysics[2] and Chemistry,[3] Faculty of Science, Department of Pharmaceutical Manufacturing, Faculty of Pharmaceutical Science,[4] and Institute for Virus Research,[5] Kyoto University, Kyoto 606, and Department of Pathology, Tohoku University School of Medicine, Sendai 980,[6] Japan, and Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840[7]

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To identify retroviral antigenic determinants recognized by CD4$^+$ T helper cells during tumor rejection, we established four noncytolytic, helper-type, CD4$^+$ T-cell clones by limiting dilution cultures of mixed lymphocyte-tumor cultures from mice immune to a Friend virus-induced tumor, FBL-3. Among these, three T helper cell clones were isolated from C57BL/6 mice and the fourth was isolated from a (BALB/c × C57BL/6)F$_1$ mouse. All these clones proliferated in response to the immunizing FBL-3 tumor cells in a major histocompatibility complex class II-restricted manner. Each clone expressed a distinct T-cell receptor with a characteristic combination of α and β chains. The localization of helper T-cell determinants on viral proteins was analyzed with recombinant vaccinia viruses expressing Friend murine leukemia virus (F-MuLV) *gag* or *env* genes or shorter fragments of the *env* gene. Epitopes recognized by these T-cell clones were mapped to at least two distinct portions in the *env* region of the F-MuLV genome. These epitopes were identified more precisely with synthetic peptides derived from the F-MuLV envelope protein sequence. One of these epitopes was common to Friend and Moloney MuLVs and was located in the N-terminal region of the gp70 glycoprotein at amino acids 122 to 141. The second epitope, which was recognized in the context of hybrid I-E$^{b/d}$ major histocompatibility complex class II molecule, was located close to the C-terminal end of gp70 at amino acids 462 to 479. In addition, a possible third epitope was located in the N-terminal half of the gp70 sequence and differed from the first epitope in that it was not cross-reactive with the Moloney MuLV envelope protein.

Murine leukemia virus (MuLV)-induced tumor cells express viral proteins that can function as important antigens during tumor rejection (3, 12, 14, 24, 37, 38, 48). To identify antigenic epitopes recognized by tumor-specific effector cells, extensive research has focused on the proteins encoded by Friend (F), Moloney (M), Rauscher, and Gross MuLVs (2, 8, 20, 25, 27, 34, 35, 40, 49). Several studies have revealed that both *gag* and *env* gene products of MuLV can act as target molecules for either tumor-specific cytotoxic T lymphocytes (CTL) or CD4$^+$ T cells of helper phenotypes (Th) (8, 12, 20, 25, 27, 35, 37, 48). Immunization of mice with a syngeneic MuLV-induced tumor often elicits potent effector T-cell populations, most notably CTL. Induction of the effector T cells may result in rejection of the tumor by the immunized host animal itself or adoptive transfer into tumor-bearing hosts (28, 35, 36, 46). We have reported that an FBL-3 F virus (FV)-induced tumor elicited a dominant CTL population that expressed a T-cell receptor (TCR) encoded by a single combination of α- and β-chain genes and recognized by an anti-idiotypic monoclonal antibody (MAb) (29). Although the CTL population seems to play a dominant role in MuLV-induced tumor rejection, it has been demonstrated that noncytolytic CD4$^+$ Th cells also participate as an effector cell population in antitumor immune responses (13, 16, 47). We have also reported that spontaneous recovery from FV-induced leukemia and protective immunity against FV infection were both associated with the successful priming of Th cells (10, 30–33, 39). However, neither the function of Th cells nor the viral antigens recognized by Th cells generated during the immune responses to retrovirus infection or retrovirus-induced tumor cells have been well clarified at the clonal level, probably because of the difficulty in establishing Th clones in these systems (3, 27).

In this study, the characteristics of four Th clones specific for the FV-induced tumor cell line FBL-3 were examined, and the locations of the epitopes recognized by these clones were mapped with recombinant vaccinia viruses (rVV) expressing F-MuLV *gag* or *env* genes and synthetic peptides. The composition of TCR α and β genes expressed on these Th clones was also analyzed.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) (*H-2^b*), BALB/c (*H-2^d*), and their hybrid F$_1$ mice were bred and maintained in laminar flow hoods at the Experimental Animal Facility, Faculty of Medicine, Kyoto University. Both male and female mice were used between 8 and 12 weeks of age.

Cell lines. FBL-3 and MBL-2 induced in B6 mice by FV and M-MuLV, respectively, were passaged as described previously (29). Tumor cell line FT-5 was established in the Institute for Immunology, Kyoto University, from B6 mice by injecting the neonates with FV according to the protocols reported previously (19). The tumor cells were taken from spleens and adapted to tissue culture from the first transplant

* Corresponding author.

generation. All these cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Df45 is a cell clone derived from Fisher rat embryo fibroblasts expressing both components of FV (spleen focus-forming virus [SFFV] and F-MuLV) and the transfected murine class I, *H-2D^b*; B2 is a clone of Fisher rat embryo cells expressing only transfected *H-2D^b*. These two cell lines were kindly donated by F. Lilly (40). These transfectant cell lines were maintained in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum and 1 mg of geneticin (G418; Sigma Chemical Co., St. Louis, Mo.) per ml.

Immunization of mice with FBL-3 tumor cells. Both male and female mice were inoculated with 5×10^6 FBL-3 tumor cells by subcutaneous injection into the dorsal region. After complete tumor regression, mice were given up to four intraperitoneal booster doses of 5×10^6 tumor cells at 2-week intervals.

Generation of FBL-3-specific Th clones. Mixed lymphocyte-tumor cell culture and limiting dilution (LD) cultures were performed by previously reported methods (29). In brief, 1×10^5 irradiated tumor cells were mixed with 5×10^6 immune spleen cells in complete medium and incubated for 5 days at 37°C in 5% CO_2 and air. Complete medium consisted of RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. LD of mixed lymphocyte-tumor cells (one cell per well) was done in the presence of 2.5 ng of human recombinant interleukin-2 (TGP-3; Takeda Pharmaceutical Industries Ltd., Osaka, Japan) per ml. Growing colonies were screened by flow cytometry for the surface expression of either CD8 or CD4. All the CD4$^+$ T cells of the LD cultures were expanded and tested to determine whether they were specifically reactive to FBL-3 or autoreactive, that is, responded to syngeneic spleen cells without adding the irradiated tumor cells. Consequently, four CD4$^+$ T-cell clones were found to be specifically reactive to FBL-3, while most other CD4$^+$ clones were autoreactive. Each of the four clones was recloned at 0.5 cell per well. Those Th clones were passaged every 7 days together with irradiated syngeneic spleen cells (20 Gy) and FBL-3 tumor cells (100 Gy) in the presence of human recombinant interleukin-2.

TCR cDNA cloning and sequencing. The cDNA of poly(A)$^+$ RNA from each Th clone was linked to an *Eco*RI adaptor and cloned into the bacteriophage λgt11 vector. Screening was done with the Cα (TT11) and Cβ (86T5) probes (7, 17). Positive clones were subcloned into the plasmid pHSG399 vector, and the inserts of the clones carrying a fragment longer than 1.3 kb were completely sequenced. Nucleotide sequences were determined by the dideoxy-chain termination method with the universal M13 primer M4, reverse primer RV, or Cα- and Cβ-specific primers (18, 42). The nucleotide sequences of TCR α and β cDNA were compared with the EMBL, GenBank, and DDBJ data bases, and V and J gene segments were assigned from the deduced amino acid sequence (22, 23).

Preparation of rVVs. The VV expression vector pSC11-SS was developed by inserting a synthetic oligonucleotide that carried *Sal*I and *Stu*I cloning sites and stop codons placed in all three reading frames into the unique *Sma*I site of pSC11 (4) and was kindly provided by B. Moss, Laboratory of Viral Diseases, National Institutes of Health, Bethesda, Md. rVVs expressing the entire *env* gene (10) and the gPr80^{gag} and Pr65^{gag} genes (31) of F-MuLV and the influenza virus hemagglutinin gene (45) have been described previously.

To construct an rVV that expressed the *env* gene of F-SFFV, plasmid BT4-1a3 containing a molecular clone of SFFV genome in pBR322 (50) was digested with *Kpn*I, and a *Bam*HI linker was inserted after blunting the DNA ends. A *Bam*HI-digested fragment that carried the entire SFFV *env* gene and a part of the long terminal repeat from this modified plasmid was inserted into the unique *Bam*HI site of the VV expression vector pSC11-SB (31), and the resultant plasmid was used to generate the VV-SFFV *env* (32). Expression of the SFFV envelope protein gp55 on infected cell surfaces was confirmed by membrane immunofluorescence with MAb 514 (5).

To express portions of the F-MuLV *env* gene in rVV, plasmid p3-14B that contained an infectious molecular clone of F-MuLV strain 57 in a modified pUC19 (44) was digested with *Bam*HI and *Sca*I, and a 1.7-kb fragment containing the 3′ portion of the *env* gene and the 5′ portion of the long terminal repeat was cloned into the polylinker site of pUC19, forming p7-2. From plasmid p7-2, a 0.9-kb *Bam*HI-*Pst*I fragment containing a portion of the *env* gene was isolated and recloned into the polylinker site of pBLUESCRIPT-KS, forming p5-1. This plasmid was then cut with *Sca*I at the polylinker site, the DNA ends were blunted with the Klenow enzyme, and the modified plasmid was ligated to a *Sal*I linker, forming p8-2. The 0.8-kb *Bam*HI-*Bam*HI fragment containing the remaining 5′ portion of the F-MuLV *env* gene was isolated from p3-14B and inserted into the unique *Bam*HI site of p8-2, forming p11-14, which contained the partial *env* gene from the initiation site to the *Pst*I site at position 7419 (Fig. 1). A *Sal*I-digested fragment containing the partial *env* gene from p11-14 was cloned into the unique *Sal*I site of pSC11-SS, and the resultant plasmid was used to generate rVV MR1.

Plasmid p8-2 was also digested with *Bst*EII, the DNA ends were blunted, and it was then ligated to the *Sal*I linker, forming p12-19. After the 0.8-kb *Bam*HI-*Bam*HI fragment from p3-14B was inserted, this plasmid was cut with *Sal*I, and a 1.4-kb fragment that contained the portion of the *env* gene from the initiation site to the *Bst*EII site at position 7151 was cloned into pSC11-SS (Fig. 1). The resultant plasmid was used to generate rVV MR2. To further remove the 3′ portion of the *env* gene, p5-1 was digested with *Hin*cII and religated, and a plasmid that lost the 0.5-kb *Hin*cII-*Hin*cII fragment was selected. The 0.8-kb *Bam*HI-*Bam*HI fragment containing the 5′ portion of the F-MuLV *env* gene was then inserted into this plasmid, forming p48-1, which contained the portion of the *env* gene from the initiation site to the *Hin*cII site at position 6926 (Fig. 1). The *Sac*I site of p48-1 was converted similarly to a *Sal*I site, and the 1.2-kb *Sal*I-*Hin*cII fragment was then inserted into pSC11-SS. The resultant plasmid was used to generate rVV MR3.

Plasmid p7-2 was also used to reconstitute the entire *env* gene by inserting the 0.8-kb *Bam*HI-*Bam*HI fragment, and the *Sac*I site of this plasmid was similarly converted to a *Sal*I site, forming p20-8. The internal *Bst*EII-*Bst*EII fragment was removed from p20-8 to make a 0.68-kb in-frame deletion of the *env* gene, forming p53-1. A 1.9-kb *Sal*I-digested fragment containing the internally deleted *env* gene was then inserted into the *Sal*I site of pSC11-SS, and the resultant plasmid was used to generate rVV MR4 (Fig. 1).

The structures of the *env* genes that were reconstructed from separate fragments were confirmed by restriction enzyme digestion and DNA sequencing around the sites of ligation. All rVVs were produced by standard homologous recombination techniques (4, 31) and plaque purified three times.

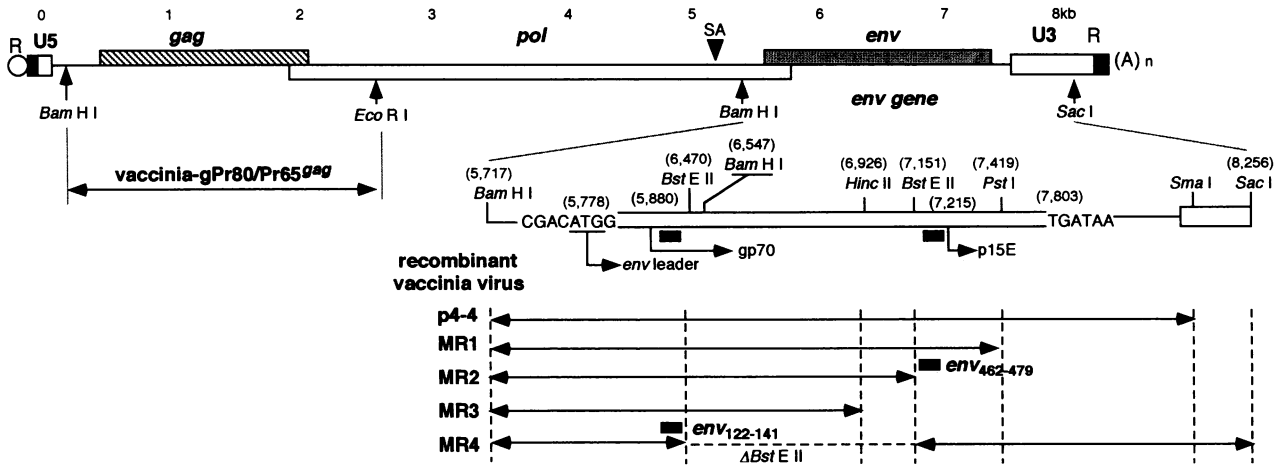

FIG. 1. F-MuLV genome and construction of the rVVs. Details are described in Materials and Methods. The locations of two Th determinants identified in this study are indicated by closed rectangles together with the amino acid positions. SA, splicing acceptor site.

Proliferation assay. To induce antigen-specific stimulation of Th clones with rVV, B2 cells (2×10^6) were infected overnight in a 6-cm plastic dish with either of the rVVs at a multiplicity of infection of 2, scraped, and added to cultures of Th clones along with syngeneic spleen cells. Proliferation was measured in triplicate cultures in wells of flat-bottomed microculture plates. Irradiated (20 Gy) spleen cells (5×10^5) as antigen-presenting cells (APC) and 1×10^4 irradiated (100 Gy) tumor cells or rVV-infected cells or various concentrations of a synthetic peptide were mixed with 1×10^5 cloned $CD4^+$ T cells in a total volume of 200 μl of complete medium in each well without human recombinant interleukin-2. The plates were incubated at 37°C for 48 h, and 18.5 kBq of [^{3}H]thymidine ([^{3}H]TdR, 74 GBq/mmol; Amersham/Searle, Arlington, Ill.) was added to each well 4 h before the termination of the culture. The cells were then collected onto a glass fiber filter, and radioactivity was measured with a liquid scintillation spectrometer. All the data are expressed here as mean counts per minute ± standard deviation. The significance of [^{3}H]TdR uptake was analyzed by Student's *t* test.

Antibodies. The anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-TCRαβ (H57-597), anti-Thy-1.2, and anti-CD8 MAbs have been described previously (28). Nonpolymorphic rat anti-murine class II (M5/114.15), anti-I-$E^{k,d,p,r}$ (14-4-4S), and anti-I-$A^{b,d,p,q}$ (25-9-17S) MAbs were obtained from the American Type Culture Collection (Rockville, Md.). Anti-major histocompatibility complex (MHC) class II MAbs were used in ascitic form, except for M5/114.15, which was used as a hybridoma culture supernatant fluid for blocking T-cell proliferation.

Peptide synthesis. Peptides encoded within the F-MuLV *env* gene were synthesized starting with each C-terminal amino acid anchored on Wang resin by the 9-fluorenylmethyloxycarbonyl-based solid-phase method, using a model ACT 350 multiple peptide synthesizer (Advanced ChemTech Inc., Louisville, Ken.) (9). The following side-chain-protected Fmoc amino acid derivatives were used: Asp(O^{t}Bu), Glu(O^{t}Bu), Thr(^{t}Bu), Ser(^{t}Bu), Arg(Pmc), Cys(Trt), Asn (Trt), Lys(Boc), His(Trt), Tyr(^{t}), and Gln(Trt). Each amino acid in dimethylformamide was condensed in a stepwise manner by the dicyclohexylcarbodiimide-*N*-hydroxybenzotriazole method (9).

After every amino acid for the peptides was assembled, each peptide-resin was treated with trifluoroacetic acid–thianisole–*m*-cresol–ethanedithiol–H_2O (80:5:5:5:5, vol/vol) at room temperature for 2 h. The deprotected peptide was precipitated from the filtrate with ether, and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in H_2O-CH_3CN. Each crude peptide was purified by high-pressure liquid chromatography on a C-18 reverse-phase column. The column was eluted with a gradient of CH_3CN in aqueous 0.1% trifluoroacetic acid. The desired peak fractions were combined, and the solvent was removed by lyophilization to result in powder. The predicted composition of amino acids was confirmed by amino acid analysis.

Nucleotide sequences. The nucleotide sequence data of TCR reported here will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence data bases with the accession number D12893-D12900.

RESULTS

Isolation and characterization of FBL-3-specific Th-cell clones. The cells of growing colonies in LD cultures were examined for CD4 expression. Although most of the colonies were CTL with a $CD8^+$ phenotype, $CD4^+$ T cells were isolated at a very low frequency (less than 1/500). Among those $CD4^+$ colonies, only four specifically reacted to FBL-3 tumor cells and were recloned for further study. Most of the other $CD4^+$ T cells from LD cultures were autoreactive, that is, they proliferated in response to the syngeneic spleen cells without adding irradiated FBL-3 cells (data not shown). Three of the four FBL-3-specific clones (SB14-31, BL4L-23, and BL4L-30) originated from C57BL/6 (B6) mice. BL4L-23 and BL4L-30 were independent clones derived from a single mouse. F5-5 was cloned from mixed lymphocyte-tumor cells of a (BALB/c × B6)(CB6)F_1 mouse. The surface phenotypes of these clones were Thy-1.2^+, $CD4^+$, $CD8^-$, TCRαβ$^+$, and $CD3^+$. The clones were not cytolytic to FBL-3 tumor cells (data not shown).

Figure 2 shows that irradiated spleen cells were required together with FBL-3 tumor cells for the proliferation. The proliferative responses were blocked in the presence of nonpolymorphic anti-MHC class II MAbs even though tumor cells themselves did not express class II antigen.

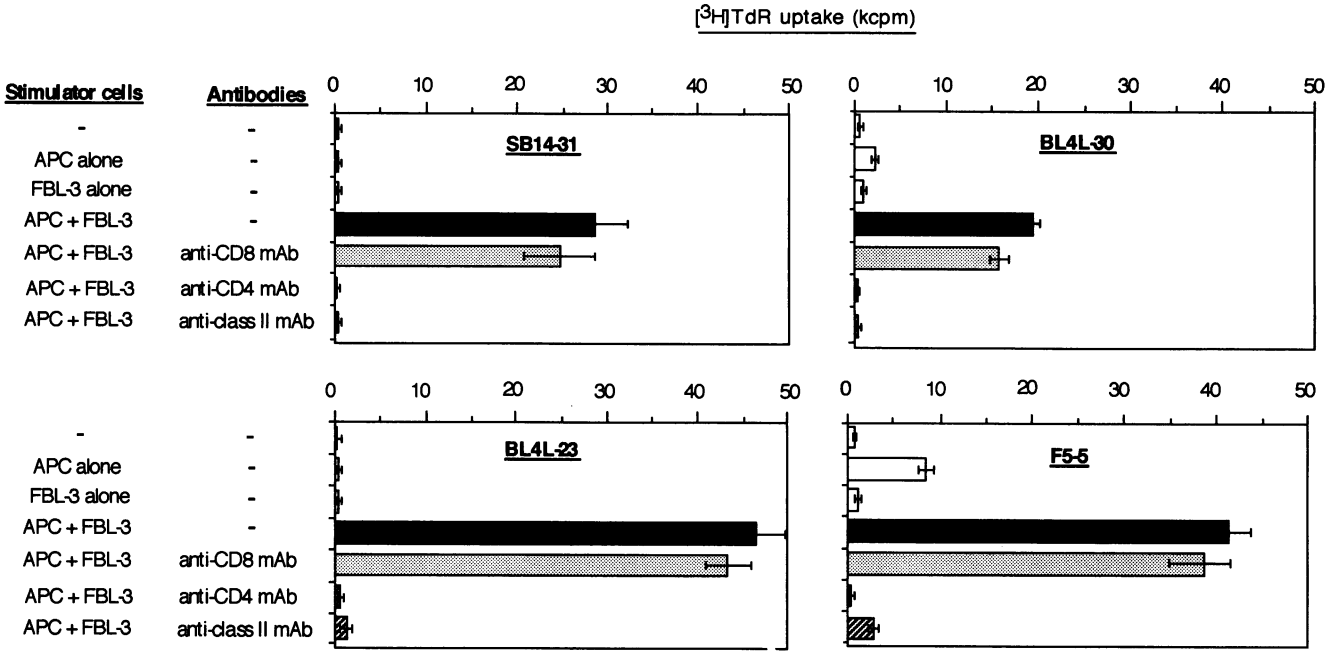

FIG. 2. Effects of anti-MHC class II and anti-CD4 MAbs on the proliferative responses of Th clones. Each Th clone (1×10^5 cells) was cultured for 2 days with 5×10^5 irradiated syngeneic spleen cells as APC and 1×10^4 irradiated FBL-3 tumor cells. Spleen cells from B6 mice were used for SB14-31, BL4L-23, and BL4L-30, and those from $CB6F_1$ mice were used for F5-5. Cells were cultured in the presence or absence of one of the following MAbs: anti-CD8 (anti-Lyt-2.2), anti-CD4 (GK1.5), and nonpolymorphic anti-MHC class II (M5/114.15), at 10 μg/ml. The [^{3}H]TdR was added for the last 4 h of culture. The results of three experiments were consistent with this representative figure.

Accordingly, it was thought that spleen cells functioned as APC in this response. In addition, anti-CD4 MAb completely inhibited the proliferative responses (Fig. 2). The three $CD4^+$ T-cell clones of B6 mouse origin appeared to respond to FBL-3 tumor antigenic determinants bound to $I\text{-}A^b$ molecules, since the B6 mouse does not express class II MHC I-E antigens.

On the other hand, the $CD4^+$ clone F5-5 of $CB6F_1$ mouse origin required APC from the same F_1 mice, but parental B6 or BALB/c spleen cells could not induce its proliferation (Fig. 3). These results indicated that antigen-binding MHC class II molecules differed between F5-5 and the three other clones of B6 mouse origin and suggested that the epitope recognized by Th clone F5-5 bound to either hybrid I-A or I-E MHC class II molecules expressed on spleen cells of the F_1 mouse.

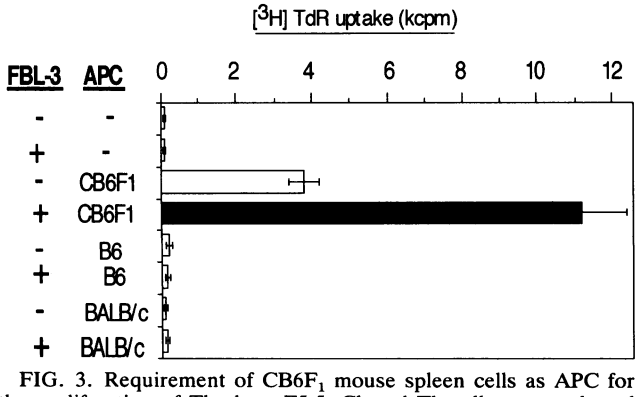

FIG. 3. Requirement of $CB6F_1$ mouse spleen cells as APC for the proliferation of Th clone F5-5. Cloned Th cells were cultured with $CB6F_1$, BALB/c, or B6 mouse spleen cells as APC in the presence or absence of FBL-3 tumor cells. [^{3}H]TdR uptake was measured as indicated in Fig. 2. Parental BALB/c or B6 spleen cells were unable to present antigen to F5-5. The results of repeated experiments were consistent with this representative figure.

TCR α- and β-chain gene usages of the four Th clones. To identify the expressed TCR genes of Th clones, the cDNAs for the α and β chains were cloned and analyzed by nucleotide sequencing (Fig. 4). VαJα and VβDJβ sequences were translated into protein sequences. Every gene segment except for the α chain of clone BL4L-23 was assigned to known Vα or Vβ subfamilies or to Jα or Jβ segments (22, 23, 51). The VαJα sequence of BL4L-23 retained amino acids conserved in Vα and Jα segments, but no equivalents have been identified to date. We termed these segments Vα4L-23 and Jα4L-23, respectively. Four Th clones showed TCR subsets composed of different gene segments: Vα4Jα22/Vβ1DJβ2.5 for SB14-31, Vα4L-23Jα4L-23/Vβ15DJβ2.1 for BL4L-23, Vα11Jα19/Vβ8.1DJβ2.6 for BL4L-30, and Vα4Jα 3DT/Vβ14DJβ1.2 for F5-5 (Fig. 4).

Localization of Th epitopes with rVVs expressing FV gene segments. When cultured with B2 cells infected with different rVVs, all four Th clones were stimulated in the presence of syngeneic spleen cells plus B2 cells expressing F-MuLV *env* (Fig. 5). In contrast, neither SFFV *env* nor F-MuLV *gag* gene products were stimulatory for any of these clones. The localization of the epitopes in the *env* gene products was further examined with rVV expressing whole *env* or deleted *env* clones (Table 1 and Fig. 1). Three Th clones of B6 mouse origin proliferated in response to the short *env* protein expressed by rVV MR3. Thus, the determinant recognized by these Th clones appeared to be localized in the N-terminal region of the gp70 *env* protein between the initiation site and the *Hin*cII site at position 6926. Clone SB14-31 and

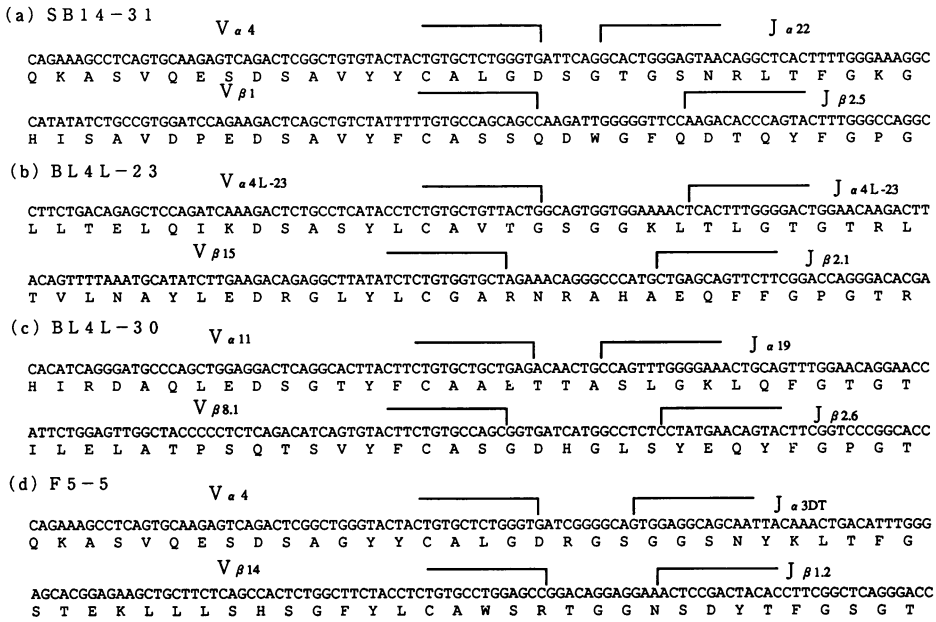

FIG. 4. Nucleotide sequences of TCR α and β cDNA from Th clones SB14-31 (a), BL4L-23 (b), BL4L-30 (c), and F5-5 (d). The V and J junctional regions are indicated. The assigned amino acid sequence is shown by the one-letter code.

BL4L-23 also showed a weak but significant reactivity to MR4 in which the middle portion of the *env* gene was deleted (Table 1). This suggested that all or part of the epitope recognized by these two clones was located upstream from the *Bst*EII site at position 6470 and that this epitope might be different from the one recognized by clone BL4L-30, which showed even less reactivity with rVV MR4. The determinant recognized by Th clone F5-5 of $CB6F_1$ origin appeared to be located between the second *Bst*EII and *Pst*I sites, which was included only in rVV P4-4 and MR1, since this Th clone lost reactivity to MR2 and MR3 (Table 1). Thus, the epitopes recognized by the four Th clones used in these experiments were distributed in at least two separate positions on the F-MuLV envelope protein.

Reactivity of T-cell clones to leukemia cells induced by FV and M-MuLV. F-MuLV shows sequence homology to both M-MuLV and Rauscher MuLV, and the antigenic determinants common to these three viruses are generally referred to as FMR antigen(s), a term originally defined serologically (5, 6, 15, 30, 34, 38). Since all the Th clones reacted only with the helper virus *env* gene products (Fig. 5), we attempted to compare the reactivity of these clones to tumor cells induced by FV or M-MuLV. Two clones, SB14-31 and BL4L-23, were strongly responsive to FV-induced tumor FT-5 and

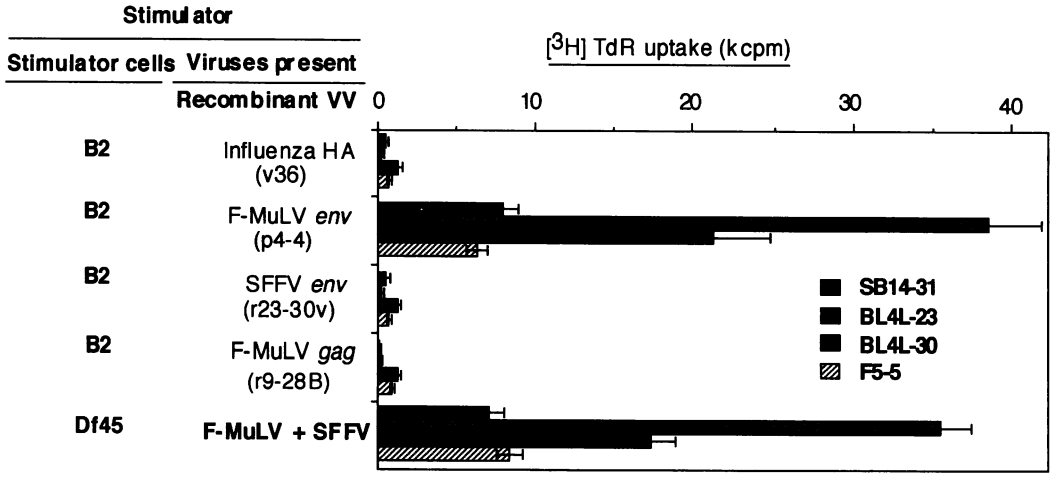

FIG. 5. Determination of the gene segment of F-MuLV containing the proliferation-stimulating determinants. [^{3}H]TdR incorporation by four Th clones was measured after stimulation with B2 cells infected with an rVV expressing either the *gag* or *env* sequence of F-MuLV or the SFFV *env* sequence plus irradiated syngeneic spleen cells as APC. rVV expressing influenza virus hemagglutinin (HA) was used as a negative control, and Df45 cells expressing both F-MuLV and SFFV genomes were used as a positive control. Cells were cultured as indicated in the legend to Fig. 2. Data shown here are representative of three separate experiments.

TABLE 1. Proliferative responses of Th clones stimulated by rVVs expressing intact F-MuLV *env* or deleted *env* clones[a]

Stimulation with:	[^{3}H]TdR uptake (cpm ± SD)			
	SB14-31	BL4L-23	BL4L-30	F5-5
Nothing	53 ± 18	304 ± 118	85 ± 44	62 ± 26
APC alone	197 ± 17	373 ± 54	307 ± 176	980 ± 115
APC plus: FBL-3	5,499 ± 2,153	32,701 ± 471	2,250 ± 89	2,748 ± 581
VV-influenza hemagglutinin	170 ± 25	480 ± 31	238 ± 47	266 ± 130
p4-4 (VV-F-MuLV *env*)	**44,113 ± 1,306**	**60,621 ± 3,118**	**17,574 ± 1,384**	**10,161 ± 282**
MR1 (*Bam*HI-*Pst*I)	**38,668 ± 972**	**42,918 ± 9,116**	**19,653 ± 2,964**	**11,603 ± 2,100**
MR2 (*Bam*HI-*Bst*EII)	**39,179 ± 2,203**	**35,697 ± 987**	**20,759 ± 938**	156 ± 16
MR3 (*Bam*HI-*Hin*cII)	**28,395 ± 3,558**	**20,029 ± 3,469**	**21,466 ± 3,365**	124 ± 29
MR4 (Δ*Bst*EII)	**3,616 ± 926**	**1,461 ± 289**	**540 ± 120**	365 ± 185

[a] Each Th clone was cultured for 2 days with the syngeneic spleen cells as APC together with either irradiated FBL-3 tumor cells or with B2 cells infected with an rVV expressing either the whole F-MuLV *env* or its shorter gene segments (MR1 to MR4). rVV expressing influenza virus hemagglutinin was used as a negative control. The assay was performed as indicated in the legend to Fig. 2. Significantly positive responses of each clone compared with the responses observed under stimulation with B2 cells infected with the rVV expressing influenza virus hemagglutinin are shown in boldface. Representative data from six repeated experiments are shown.

M-MuLV-induced tumor MBL-2, but were weakly responsive to FBL-3 (Fig. 6). The other two clones, BL4L-30 and F5-5, showed a totally different pattern of reactivity to these tumor cells: they reacted strongly to FT-5, less strongly to FBL-3, but not significantly to MBL-2. Thus, clones SB14-31 and BL4L-23 appeared to recognize the same antigenic epitope based on both reactivity with rVV (Table 1) as well as cross-reactivity with F- and M-MuLV-induced tumor lines (Fig. 6). In contrast, clone BL4L-30 appeared to recognize a different epitope in the N-terminal half of the envelope protein based on its lack of cross-reactivity with the M-MuLV-induced tumor (Fig. 6). Clone F5-5 showed a similar lack of cross-reactivity with M-MuLV, but this clone recognized a third epitope in the C-terminal portion of the envelope protein based on its pattern of reactivity with the rVV shown in Table 1.

Determination of Th-cell epitopes with synthetic peptides. To define more precisely the epitopes recognized by these clones, we searched for potential epitopes encoded within the F-MuLV *env* gene by detecting periodic sequence patterns of hydrophobicity specific to the α and 3_{10} helices (26). The hydrophobicity value for each amino acid type derived by Fauchere and Pliska (11) was used. Patterns were detected by least square fitting a sinusoid to all possible fragments of 11 residues from a target protein. This method was applied to both FV and M-MuLV envelope proteins (21, 43). We first listed all individual 11-residue fragments with strong specific patterns from the two proteins. From this list, we selected fragments common to the two envelope proteins and rank ordered them according to the strength of sequence patterns. The top two sequences were selected, and 9 more residues were added to each of the 11-residue fragments by referring to the sequence of the two proteins to make the fragment 20 residues long. Each of the 20-residue fragments was synthesized according to the rank order, and proliferation was assayed. Th clones SB14-31 and BL4L-23 were cultured in the presence of various concentrations of each peptide, and proliferation was examined. Only the peptide representing F-MuLV $env_{122-141}$ (DEPLTSLTPRCNTAW NRLKL) stimulated proliferation over a wide range of concentrations (Fig. 7). This peptide did not stimulate Th clone BL4L-30 at the same dose (data not shown). Thus,

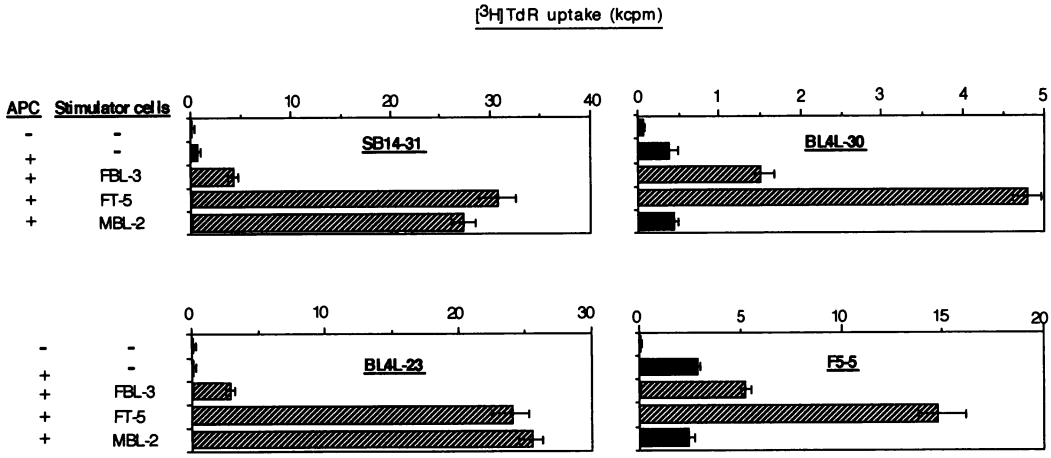

FIG. 6. Reactivities of Th clones to F- and M-MuLV-induced tumors. Each Th clone was stimulated with various tumor cells in the presence of syngeneic spleen cells as described in the legend to Fig. 2. Tumor cells used were as follows: FV-induced tumors, FBL-3 and FT-5; M-MuLV-induced tumor, MBL-2. Experiments were repeated four times. Data shown are from a representative experiment.

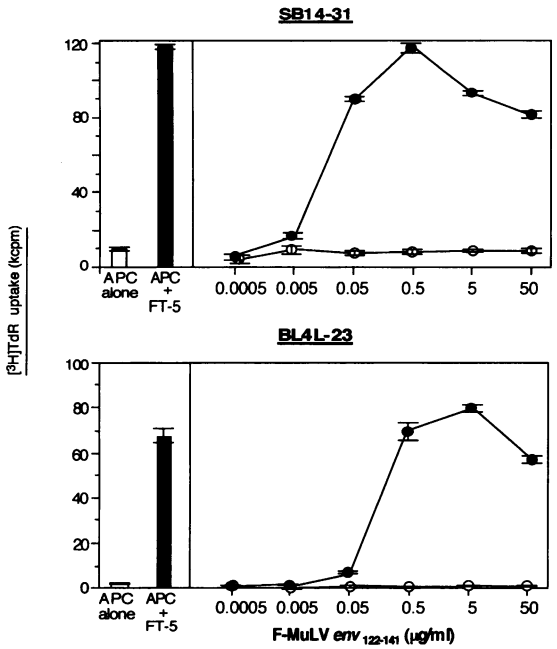

FIG. 7. Proliferative responses of two Th clones to stimulation with the synthetic peptide corresponding to F-MuLV $env_{122-141}$ (●). A synthetic peptide representing F-MuLV $env_{281-299}$ was used as a negative control (○). Each Th clone was cultured with irradiated B6 spleen cells as APC in the presence of the synthetic peptide at the indicated concentration. The proliferative responses of each Th clone stimulated by FT-5 tumor cells are shown in the left side of the figures by vertical columns as a positive control. [^{3}H]TdR was added for the last 4 h of 2-day cultures. Data shown are from a representative of five repeated experiments.

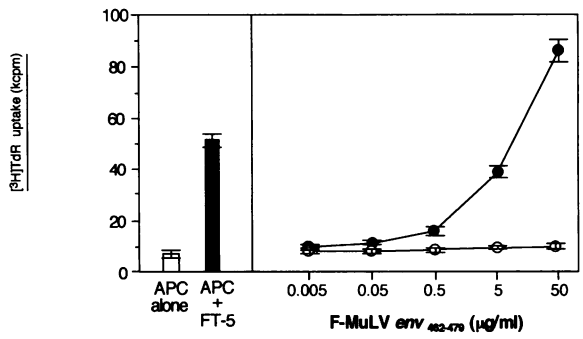

FIG. 8. Proliferative response of Th clone F5-5 stimulated with the synthetic peptide representing F-MuLV $env_{462-479}$ (●). Th clone F5-5 was cultured with the irradiated $CB6F_1$ spleen cells as APC in the presence of the synthetic peptide at the concentration indicated. Another synthetic peptide representing F-MuLV $env_{518-535}$ (○) did not show stimulatory activity. The assay was performed as described in the legend to Fig. 7. The results of three repeated experiments were consistent with this representative figure.

F-MuLV $env_{122-141}$ was not the epitope recognized by BL4L-30. The F-MuLV $env_{122-141}$ sequence differed from that of M-MuLV by only one amino acid at N-terminal position 122 (Asp to Glu) (1). This was consistent with the similar reactivity patterns of SB14-31 and BL4L-23 to the FV and M-MuLV-induced tumor cells.

Recently, it has been reported that several endogenous viral peptides are bound to MHC class II molecules isolated from the B-cell lymphoma LB27.4(H-$2^{b/d}$) (41). One of the predicted amino acid sequences for antigenic determinants recognized by F5-5 was found to overlap with an endogenous viral peptide eluted from I-$E^{b/d}$ molecules, that is, AKV $env_{454-469}$ (SPSYVYHQFERRAKYK) (41). Therefore, the corresponding peptide F-MuLV $env_{462-479}$ was synthesized. This synthetic peptide stimulated the proliferation of Th clone F5-5 in a dose-dependent manner (Fig. 8). Therefore, it was likely that the determinant was bound to hybrid I-$E^{b/d}$ MHC class II molecules. Binding of this peptide to I-$E^{b/d}$ was confirmed by showing that the proliferative response of Th clone F5-5 to the peptide was inhibited in the presence of an anti-Eα MAb 14-4-4S (Fig. 9). F-MuLV $env_{462-479}$ represented HPPSYVYSQFEKSYRHKR and differed from the corresponding M-MuLV sequence ($env_{452-469}$, HSPSYVY GLFERSNRHKR) in five amino acids, as expected from the lack of reactivity of this Th clone to M-MuLV-induced tumor MBL-2 (Fig. 6).

DISCUSSION

In this study, we demonstrated two separate Th determinants located in both the N-terminal and C-terminal ends of the F-MuLV gp70 envelope molecule. The N-terminal determinant represented by the synthetic peptide F-MuLV $env_{122-141}$ was recognized by two Th clones in the context of I-A^b, while the C-terminal determinant was recognized in the context of hybrid I-$E^{b/d}$ by a single Th clone, F5-5. The fourth Th clone, BL4L-30, established from an immune B6 mouse, was similar to the two Th clones that recognized the N-terminal epitope in its reactivity to the rVV expressing a portion of F-MuLV *env* gene and in its being restricted by I-A^b. However, BL4L-30 was not significantly stimulated with an M-MuLV-induced tumor, MBL-2, in proliferative responses, while the two other Th clones showed reactivities to MBL-2 comparable in magnitudes to their reactivities to an FV-induced tumor cell line, FT-5. In addition, BL4L-30 was not reactive to the synthetic peptide F-MuLV $env_{122-141}$. Therefore, it is highly likely that BL4L-30 recognizes another undefined epitope located in the N-terminal portion of the gp70 molecule.

The portions encoding the two antigenic determinants identified in the present study with synthetic peptides were included in the truncated *env* gene expressed by rVV MR4 (Fig. 1). However, reactivities of the Th clones to the cells infected with rVV MR4 were weaker than their responses to the cells infected with rVV MR1, MR2, or MR3 (Table 1). When stimulated with B2 cells infected with rVV MR4, significant proliferative responses ($P < 0.02$ compared with the responses to the cells infected with VV-influenza virus hemagglutinin) were observed with clones SB14-31 and BL4L-23 in five of six repeated experiments, while proliferative responses of marginal significance ($P < 0.05$) were observed with clone BL4L-30 in three of six experiments. F5-5 showed marginal proliferative responses ($P < 0.05$) to rVV MR4-infected cells in only two of six repeated experiments. This weaker stimulation of Th clones with cells infected with rVV MR4 might be due to a lower expression level of the truncated envelope protein in infected cells or due to inefficient or inappropriate processing of the antigenic peptides from the modified envelope protein in APC. Further studies on biosynthesis, proteolytic processing, and intracellular transport of the intact and truncated gp70 molecules would be necessary to understand fully the reasons for inefficient stimulation of Th clones with rVV MR4.

It is of interest to know the TCR repertoire of Th cells

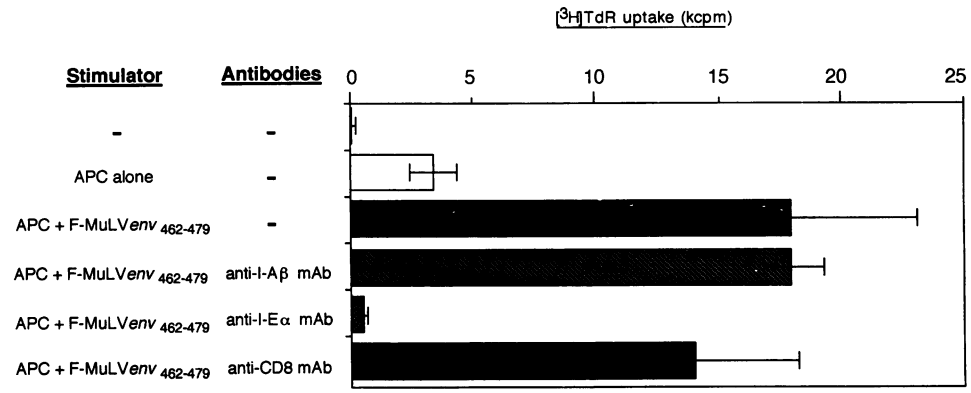

FIG. 9. Proliferative response of Th clone F5-5 stimulated with the synthetic peptide was inhibited by anti-I-Eα MAb 14-4-4S, but not by anti-I-Aβ MAb 25-9-17S. Th clone F5-5 was cultured with the irradiated $CB6F_1$ spleen cells as APC in the presence of the synthetic peptide (50 μg/ml) alone or together with the MAb in its ascitic form (final dilution, 1:1,000). Experiments were repeated twice and showed consistent results.

reactive to the determinants on F-MuLV envelope protein, because in CTL responses to FBL-3 tumor cells, a dominant population expressing a TCR encoded by a unique combination of α- and β-chain genes was induced (29). In this study, Th clones SB14-31 and BL4L-23 recognized the same epitope, but expressed TCRs of different α- and β-chain composition and showed quantitatively different reactivities to the synthetic peptide antigen. The peptide concentrations giving maximal responses differed by 10-fold in the two clones. This might reflect differences in binding affinities of the two TCRs expressed on the two clones.

The C-terminal epitope F-MuLV $env_{467-479}$ recognized by Th clone F5-5 is homologous in its primary structure to and shares the position in *env* genes with the reported sequence of an endogenous viral *env* gene product bound to and eluted from the hybrid MHC class II molecule $I\text{-}E^{b/d}$. This hybrid class II molecule is expressed on $H\text{-}2^{d/b}$ hybrid cells by complementation of $E_\beta{}^b$ of B6 strain with $E_\alpha{}^d$ of the $I\text{-}E^+$ BALB/c strain (41). The low but consistent autoreactivity of this particular clone, F5-5 (Fig. 3), might be explained by cross-reactivity to the endogeneus retroviral envelope antigen expressed in the F_1 mice.

Role of Th clones specific to the antigenic determinants identified in this study on F-MuLV envelope protein in protective immunity against FV infection and regression of FV-induced tumor cells in vivo are presently unknown. We have shown that the majority of F-MuLV envelope-specific Th cells in $H\text{-}2^{a/b}$ mice are restricted by $I\text{-}A^b$, and a small population of Th cells in the same hybrid mice recognize the envelope antigen in the context of $I\text{-}E^{k/b}$ (30). These results are consistent with the present observation that three F-MuLV envelope-specific Th clones established from $H\text{-}2^b$ B6 mice are restricted by $I\text{-}A^b$ and another clone from a hybrid $H\text{-}2^{d/b}$ mouse is restricted by $I\text{-}E^{d/b}$. *H-2* recombinant and mutant mice lacking the expression of $I\text{-}A^b$ molecules were not effectively protected against FV infection by immunization with the rVV expressing the *env* gene of F-MuLV (10) and were significantly more susceptible to FV infection than congenic or coisogenic counterparts that expressed $I\text{-}A^b$ (33). In addition, depletion of $CD4^+$ Th cells from homozygous $H\text{-}2^b$ mice by administration of an MAb rendered the otherwise resistant strain of mice significantly more susceptible to FV-induced leukemia (39). Thus, MHC class II-restricted $CD4^+$ Th cells seem to play an essential role in protective immunity against FV infection and in spontaneous regression of leukemic splenomegaly.

Immunization of mice with the synthetic peptides representing the Th epitopes identified in the F-MuLV envelope protein would enable us to induce Th clones of the same epitope specificity. This approach may contribute to understanding the mechanisms underlying not only the antitumor responses but also the protective immunity against FV infection by epitope-specific, noncytolytic T cells.

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