Identification of an Immunodominant Mouse Minor Histocompatibility Antigen (MiHA)

T Cell Response to a Single Dominant MiHA Causes Graft-versus-Host Disease

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Abstract

T cell responses to non-MHC antigens are targeted to a restricted number of immunodominant minor histocompatibility antigens whose identity remains elusive. Here we report isolation and sequencing of a novel immunodominant minor histocompatibility antigen presented by H-2D^b on the surface of C57BL/6 mouse cells. This nonapeptide (AAPDNRETF) shows strong biologic activity in cytotoxic T lymphocyte sensitization assays at concentrations as low as 10 pM. C3H.SW mice primed with AAPDNRETF in incomplete Freund's adjuvant generated a potent anti-C57BL/6 T cell-mediated cytotoxic activity, and T lymphocytes from AAPDNRETF-primed mice caused graft-versus-host disease when transplanted in irradiated C57BL/6 recipients. These results (*a*) provide molecular characterization of a mouse dominant minor histocompatibility antigen, (*b*) identify this peptide as a potential target of graft-versus-host disease and, (*c*) more importantly, demonstrate that a single dominant minor antigen can cause graft-versus-host disease. These findings open new avenues for the prevention of graft-versus-host disease and should further our understanding of the mechanisms of immunodominance in T cell responses to minor histocompatibility antigens. *(J. Clin. Invest.* 1996. 98:622–628.*)* Key words: amino acid sequence • antigenic determinant • CD8-positive T-lymphocytes • H-2 antigens • peptide

Introduction

MHC molecules are peptide binding proteins whose ligands are mostly derived from the partial proteolysis of endogenous self proteins distributed among various cellular compartments (1, 2). These peptide ligands provide a molecular definition of self that is critical for T and NK cell function. Indeed, class I- and class II-associated self peptides are involved in positive and negative selection of thymocytes, and the former also regulate susceptibility to NK cell-mediated lysis (3–6). Some of these self peptides, called minor histocompatibility antigens (MiHAs)[1], are polymorphic and can therefore trigger potent T cell responses between MHC-identical individuals (7). The most important immune reactions elicited by in vivo alloreactivity to MiHA are graft rejection, graft-versus-host disease (GVHD), and graft-versus-tumor effect (8). When it was realized that the number of MiHA differences between two strains of mice, such as C57BL/6 and BALB.B, is greater than 40, MiHAs were considered an insurmountable obstacle to successful transplantation (9, 10). However, analysis of cytotoxic T cell (CTL) responses in the BALB.B anti-C57BL/6 strain combination revealed that, in fact, anti-MiHA responses could be attributed to a very limited number of antigens, called immunodominant, while the vast majority of MiHAs are neglected and said to be dominated (11). The phenomenon of immunodominance is a hallmark of all anti-MiHA T cell responses, not only in vitro but also in vivo (12–14). Thus, we have recently shown that after bone marrow transplantation, anti-host T cell responses are oligoclonal in nature and targeted to a restricted number of dominant MiHAs (13). Two human MiHAs have recently been sequenced, but it is impossible to determine conclusively whether or not they are immunodominant as methodological problems prevent adequate evaluation of MiHA dominance in man (15, 16). In addition, the only three mouse MiHAs sequenced are clearly not dominant (17–19). To decipher the complex mechanisms of immunodominance, we studied the molecular structure of a dominant mouse MiHA. We report herein the molecular characterization of a dominant H-2D^b-associated MiHA, and demonstrate that T cell response to this single nonapeptide can cause GVHD.

Methods

Mice and cell lines. Male C57BL/6J and C3H.SW/SnJ mice, 8–20 wk old, were purchased from the Jackson Laboratory (Bar Harbor, ME). T2D^b and T2K^b (kindly provided by Dr. P. Cresswell, Yale University, New Haven, CT) were created by transfection of T2 cells with D^b and K^b class I genes, respectively (20). T2 is a human hybrid lymphoblastoid cell line with a large deletion in the MHC class II region, including genes for the TAP1/2 and LMP2/7 products.

Acid extraction and HPLC fractionation. MHC class I–associated peptides were extracted by acid elution from C57BL/6 spleen cells cultured for 3 d in the presence of Concanavalin A (Con A; Sigma

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1. *Abbreviations used in this paper:* GVHD, graft-versus-host disease; MiHA, minor histocompatibility antigen.

Chemicals Co., St. Louis, MO) at a concentration of 2 μg/ml (13, 21). Briefly, 5×10^9 Con A blasts were suspended in citrate-phosphate buffer (0.131 M citric acid/0.066 M Na_2HPO_4, pH 3.3) at a concentration of 4×10^6 cells/ml for 1 min at room temperature, and then centrifuged for 5 min. The acid-stripped peptide supernatant was harvested and loaded into a SEP-PAK C18 cartridge (Waters, Milford, MA); the bound material was eluted at 4°C with 80% acetonitrile/20%H_2O, then lyophilized and stored at −80°C. Peptides were redissolved in 0.1% TFA, and were separated on a SUPERPAC™ Pep-S C18 column (5 μm, 4 × 250 mm; Pharmacia, Uppsala, Sweden) using a Waters HPLC system. Solvent A was 0.1% TFA in H_2O, and solvent B was 0.1% TFA in acetonitrile. The gradient was 0–60% B from 5 to 50 min (1.33%/min), the flow rate 1 ml/min, and 1-min fractions were collected (system I). An aliquot from each fraction was assayed in CTL sensitization assays (see below) and the rest was lyophilized and stored at −80°C. This procedure was repeated ten times over a five-week period; acid eluates were thus harvested from a total of 5×10^{10} Con A blasts. Active fractions from the 10 runs were then pooled and subjected to a second reversed-phase HPLC, using a DELTA PAK C18 column (5 μm, 3.9 × 150 mm; Waters) with an increasing acetonitrile gradient of 0.5%/min in 0.13% heptafluorobutyric acid (0–60% over 120 min; flow rate, 1 ml/min; system II). Fractions (1 ml) were again tested in CTL sensitization assays, and the active fraction was rechromatographed twice on a Brownlee HPLC microbore C8 column (RP-300, 2.1 × 30 mm; Applied Biosystems, Foster City, CA) using an Applied Biosystems 130A Separation system. Solvent A was 0.1% TFA and solvent B was 70% acetonitrile containing 0.085% TFA. For fractionation III, solvent B was increased from 0 to 80% from 3 to 43 min (2%/min), with a flow rate of 0.2 ml/min. For fractionation IV, the concentration of solvent B was increased from 0 to 15% from 3 to 10 min, and then from 15 to 30% from 10 to 40 min. The active peak was subjected to microsequencing. System V was used to compare the retention time of selected synthetic peptides to that of the natural MiHAs: the solvents were the same as in system I but the gradient rate was shallower with a linear increase of solvent B from 0 to 25% over 100 min (1 ml/min, 1-min fractions).

Peptide sequencing and synthesis. HPLC fractions that contained specific CTL-sensitizing activity were sequenced at the Biotechnology Research Institute (Montreal, Quebec, Canada) using an automated Edman degradation (Model 470A; Applied Biosystems) with phenylthiohydantoin amino acid analyzer. Peptides were synthesized by standard solid-phase methods using Fmoc chemistry in a peptide synthesizer (Model MPS.396; Advanced Chemtech, St. Louis, MO) or with Multipin Peptide Synthesis system (Chiron Mimotopes, San Diego, CA). The homogeneity of AAPDNRETF used for in vivo priming was established by reversed-phase HPLC and the structure was verified by fast atom bombardment mass spectrometry and amino acid analysis.

Immunoprecipitation. Spleen Con A blasts from C57BL/6 mice were lysed in PBS containing 1% NP-40, 25 mM iodoacetamide, 0.04% NaN_3, 1 mM aprotinin and 1 mM PMSF for 30 min at 4°C with shaking. Cell lysates were cleared for 1 h with protein A–agarose (Sigma, St. Louis, MO), incubated for 1 h with anti-H-2K^b (AF6-88.5.3; ATCC) or anti-H-2D^b (28-14-8s; kindly provided by Dr. G.L. Waneck, Massachusetts General Hospital, Boston) hybridoma culture supernatants, and precipitated with protein A–agarose. After extensive washing, agarose-bound complexes were denatured with 0.1% TFA for 30 min at room temperature to release the peptides from H-2^b molecules. The peptide mixtures were then subjected to reversed-phase HPLC, as described above.

Peptide sensitization and CTL assays. Splenocytes from primed C3H.SW mice were restimulated by coculture with irradiated (2,500 rads) C57BL/6 cells in RPMI-1640 (GIBCO BRL, Burlington, Canada) supplemented to a final concentration of 10% FCS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine (GIBCO BRL), 10 mM sodium pyruvate (GIBCO BRL) and 50 μM 2-ME (Sigma) and incubated at 37°C in a humidified atmosphere of 5% CO_2. Effector cells were harvested five days later and incubated with 5×10^3 ^{51}Cr-labeled Con A–induced target blasts at an effector/target cell ratio of 100:1 in a volume of 200 μl. For sensitization experiments, HPLC-peptide fractions or synthetic peptides diluted in complete culture medium were preincubated with C3H.SW Con A blast targets for 90 min at 37°C before addition of effector cells. After 4 h at 37°C, 100 μl of supernatants were harvested and counted. The percentage of specific lysis was determined using standard methods (22). Results are reported as the mean of triplicate cultures. Spontaneous ^{51}Cr release for the various target cells ranged from 5 to 20%.

Immunization. Effector cells were generated from splenocytes of C3H.SW mice primed either by a single intraperitoneal injection of 2×10^7 C57BL/6 spleen cells or by two subcutaneous injections (at an interval of 7 d) of various amounts of synthetic peptides in incomplete Freund's adjuvant (Sigma).

Cell transplantation. Mice were transplanted as described previously (22). Briefly, recipient mice received 950 rads total body irradiation from a ^{60}Co source at a dose rate of 128 rads/min on day 0, the day of transplant. Bone marrow cells were obtained from the tibiae and femurs collected from donor mice. Bone marrow cells (10^7) mixed with spleen cells (5×10^7) were given as a single intravenous injection, via the tail vein, in a volume of 0.5 ml of serum-free RPMI-1640 medium.

Immunofluorescence staining and FACS® analysis. Direct immunofluorescence staining was performed with FITC-, PE- and CyCHROME™-conjugated monoclonal antibodies as previously described (13). After staining, cells were analyzed for surface fluorescence on an FACScan® flow cytometer (Becton Dickinson, San Jose, CA) using the LYSYS II program (Becton Dickinson).

Results

Fractionation of acid eluate from C57BL/6 cells and from affinity-purified class I molecules. Fractionation of acid eluates from C57BL/6 Con A blasts by reversed phase-HPLC (RP-HPLC) using system I (see Methods), consistently yielded five peaks that sensitized C3H.SW targets to lysis by C3H.SW anti-C57BL/6 CTLs (Fig. 1 *A*). This is consistent with previous reports showing that the number of peptide epitopes recognized by CTLs generated against "multiple" MiHAs is limited (23, 24). These MiHAs have been termed as "immunodominant" (8, 11). Titration experiments showed that two peaks, corresponding to fractions 25–28 and fractions 33–35, could be considered as "major peaks" because MiHA activity was detected up to a 1,000-fold dilution; activity of the three other peaks (fractions 29, 36, and 39) was lost at 10-fold dilution (data not shown). Testing of peptides extracted from affinity-purified class I molecules showed that MiHAs recovered in fractions 25–28 and in fractions 33–35 were associated exclusively with H-2D^b and H-2K^b, respectively (Fig. 1 *A*). The three minor peaks were not recovered in eluates from affinity-purified class I molecules, possibly because their concentration or sensitizing activity was much (≥ 100 times) lower than those of the two major peaks. Indeed, we estimated by using dilution assays that the recovery of MiHAs was 10 times lower when starting with purified MHC molecules rather than whole cells (data not shown). Thus, the amounts recovered for the minor peaks were below the detection limits of our assays.

To obtain sufficient material for sequencing, peptides were eluted from 5×10^{10} C57BL/6 Con A blasts. When material recovered in the first peak (D^b-associated; fractions 26–28, system I) was further separated using system No. II, MiHA activity was recovered in two fractions (no 51 and 58, Fig. 1 *B*). These fractions were rechromatographed twice on a C8 column, using

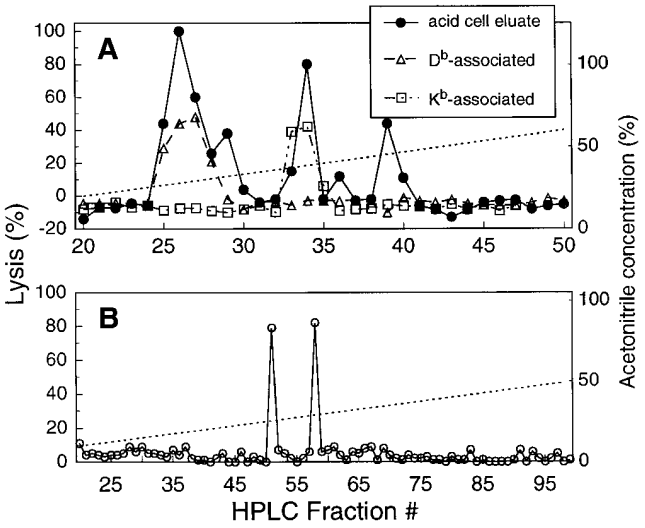

Figure 1. RP-HPLC separation of dominant MiHAs recognized by C3H.SW anti-C57BL/6 CTLs. (*A*) Peptides contained in acid eluates from C57BL/6 cells and from affinity-purified C57BL/6 class I molecules were separated by RP-HPLC on an analytical C18 Superpac Pep-S column using system I. Each fraction, containing the peptide material extracted from 3×10^8 C57BL/6 Con A blasts, was preincubated with C3H.SW Con A blasts for 90 min and tested for recognition by C3H.SW anti-C57BL/6 CTLs. (*B*) Fractions 26–28 were pooled, rechromatographed on a C18 Delta Pak column using system II, and tested in a CTL sensitization assay. Effector/target ratio was 100:1.

systems III and IV. After each separation, fractions were tested individually for MiHA activity. Only material derived from fraction 58 contained sufficient quantity for sequencing by Edman degradation. The HPLC fraction used for sequencing contained more than one peptide, but none was longer than nine amino acids. Amino acids found at each position in this fraction are listed in Table I. Based on these data, numerous sequences were possible. However, it has been shown that H-2D^b ligands are usually nonapeptides, whose motif is relatively well defined and mainly characterized by the invariable presence of an asparagine (N) at position 5. By taking into account the peptide motif of H-2D^b, as reviewed by Rammensee (25), we deduced that four nonamer sequences were most likely compatible with sequences of H-2D^b ligands (Table I).

Evaluation of candidate synthetic peptides. We tested the ability of 15 synthetic peptides to sensitize C3H.SW cells to lysis by C3H.SW anti-C57BL/6 CTLs (Fig. 2 *A*). The peptides were synthesized by starting with the MiHA motif deduced from Table I and by substituting other amino acid residues observed at positions 1, 2, 3, 4, 6, and 7 in the sequenced material. Only threonine (T) and phenylalanine (F) had been found at positions 8 and 9, respectively, and asparagine (N) is always present at position 5 in H-2D^b ligands (Table I). Four nonapeptides showed significant biologic activity at 0.1 nM concentration (Fig. 2 *A*). These four peptides had non-polar hydrophobic amino acids at positions 1–3, and shared the DNRETF COOH-terminal motif. The nonapeptide AAPDNRETF had higher activity than the other three when tested at 0.01 nM in CTL sensitization assay. The concentration of AAPDNRETF required to sensitize target cells was similar to that of the most potent D^b-associated epitopes (27). Interestingly, lysis of target cells decreased at peptide concentrations greater than 10^{-9} M (Fig. 2 *C*).

The fact that AAPDNRETF showed the highest activity suggests, but does not prove, that it corresponds to the naturally presented MiHA. It could be a heteroclitic peptide, i.e., a synthetic peptide that is more active than the natural one. The peptide whose sequence corresponds to that of the natural MiHA should be recognized only in association with H-2D^b (see Fig. 1 *A*), and should have the same retention time on RP-HPLC as the natural MiHA. Thus, to ascertain the identity of the natural MiHA, the four active nonapeptides (Fig. 2 *A*) were chromatographed with system V, designed to maximize peptide resolution (28). With this system, the natural H-2D^b-associated MiHAs can be separated into four major peaks (Fig. 2 *E*). When natural MiHAs and synthetic peptides were tested in CTL assays, AAPDNRETF and GAPDNRETF activity was found in fractions 66–67, identical to one of the MiHAs (Fig. 2 *E*). On the other hand, the retention time of AGPDNRETF or AAMDNRETF did not correspond to that of the natural MiHAs. Furthermore, the fact that GAPDNRETF and AAPDNRETF (Fig. 2 *D*) could sensitize T2D^b but not T2K^b cells to lysis by C3H.SW anti-C57BL/6 CTLs shows

Table I. Amino Acid Compostiton of a D^b-associated Dominant MiHA

Position	1	2	3	4	5	6	7	8	9
Sequence data*	A,G E,D	A,Q G	T,P,N M,D	S,D	K,N M	R,V	E,R	T	F
H-2D^b preferred amino acid residues‡	A,N,I F,P,S T,V	M,A Q,D	I,L,P V,G	K,E,Q V,D,T	N§	L,F,A Y,T,V M,E,Q H,I,K P,S	D,E,Q V,T,Y	F,H,K S,Y	M,I
Most likely MiHA sequence	A	A/Q	P	D	N	V/(R)‖	E	T¶	F¶

*Single-letter amino acid code. ‡Based on reference 25. §The only invariant H-2D^b anchor residue. ‖Although R is not a preferred residue, this position is practically unrestricted. ¶Although T and F are not recognized as preferred residues, their presence at positions 8 and 9 has already been reported in some H-2D^b-associated epitopes (25), e.g., a subdominant CTL epitope expressed on human papillomavirus type 16-transformed C57BL/6 cells (26).

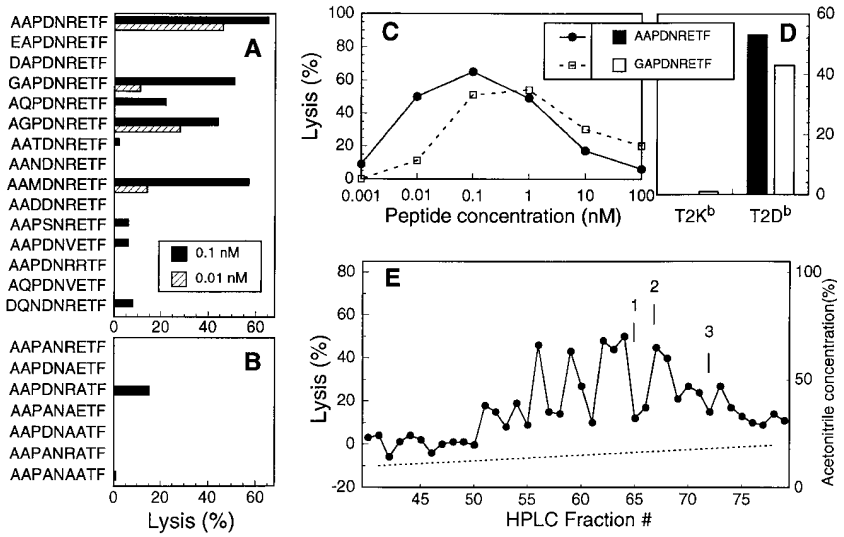

Figure 2. Recognition of various peptides by C3H.SW anti-C57BL/6 CTLs. (*A*) 15 peptides were tested in a CTL sensitization assay. The peptides were synthesized by starting with the MiHA motif deduced in Table I and by substituting other amino acid residues observed at positions 1, 2, 3, 4, 6, and 7 in the sequenced material. (*B*) Recognition of AAPDNRETF variant peptides with alanine substitutions. These peptides were tested at the same time as those of A. (*C*) Dose/response curve of AAPDNRETF and GAPDNRETF in CTL sensitization assay. (*D*) After incubation with AAPDNRETF and GAPDNRETF (0.1 nM), $T2D^b$ but not $T2K^b$ cells were killed by C3H.SW anti-C57BL/6 CTLs. No lysis was observed when $T2D^b$ were not coated with peptides (data not shown). (*E*) Acid peptide eluate from C57BL/6 cells and four synthetic peptides were subjected to RP-HPLC using system V, and individual fractions were tested in CTL sensitization assay. The line drawing represents the elution profile of the natural MiHAs. Fractions containing the activity of the synthetic peptides are indicated: 1, AGPDNRETF; 2, AAPDNRETF and GAPDNRETF; 3, AAMDNRETF. Effector/target ratio was 100:1. Target cells were C3H.SW Con A blasts, except in *D*.

that they are recognized only in association with $H\text{-}2D^b$. Thus, these two nonapeptides have all the characteristics of a natural MiHA. On a molar basis, AAPDNRETF nonapeptide shows higher activity in CTL sensitization assays than GAPDNRETF (Fig. 2), but this finding would not be sufficient to exclude the possibility that glycine may be present at position 1 in the natural MiHA.

The three-dimensional structure of $H\text{-}2D^b$/peptide complexes shows that residues at positions 4, 6, and 7 are those that are most solvent accessible and probably constitute the principal T cell receptor contact residues (29). We therefore substituted an alanine residue at each of these positions in AAPDNRETF and observed that each substitution abrogated the activity of the peptide (Fig. 2 *B*). This suggests that the three charged residues at positions 4, 6, and 7 in AAPDNRETF have a determining influence on the formation of the T cell epitope recognized by C3H.SW anti-C57BL/6 CTLs.

In vivo priming with AAPDNRETF generates potent CTL activity. Standard conditions to generate CTLs against MiHAs include in vivo priming with MiHA-incompatible cells followed by in vitro culture with the same type of cells (8). When C3H.SW mice were primed with two subcutaneous injections of AAPDNRETF in incomplete Freund's adjuvant, they generated strong CTL responses, comparable to those obtained after priming with C57BL/6 cells (Fig. 3). Effectors killed both C3H.SW cells coated with synthetic AAPDNRETF peptide and unmanipulated C57BL/6 targets. Mice primed with GAPDNRETF generated less potent CTLs that killed C57BL6 targets but not GAPDNRETF-coated C3H.SW targets (Fig. 3). CTLs from mice primed with either C57BL/6 cells or AAPDNRETF recognized the same D^b-associated HPLC fractions (data not shown). No cytotoxic activity was generated in a control group injected with adjuvant only. Therefore, injection with AAPDNRETF is effective for in vivo priming against a dominant C57BL/6 MiHA presented by the $H\text{-}2D^b$ class I molecule. GAPDNRETF also showed some activity that was, however, inferior to that of AAPDNRETF.

T lymphocytes primed with AAPDNRETF cause GVHD in irradiated recipients. After allogeneic bone marrow transplantation across minor histocompatibility barriers, GVHD is observed in only some donor/recipient combinations and is triggered by immunodominant MiHAs whose identity remains elusive (12, 13, 22). We previously reported that C57BL/6 recipients of an unmanipulated C3H.SW transplant present no signs of GVHD (22). To assess if T lymphocytes primed with AAPDNRETF could cause GVHD, we transplanted irradi-

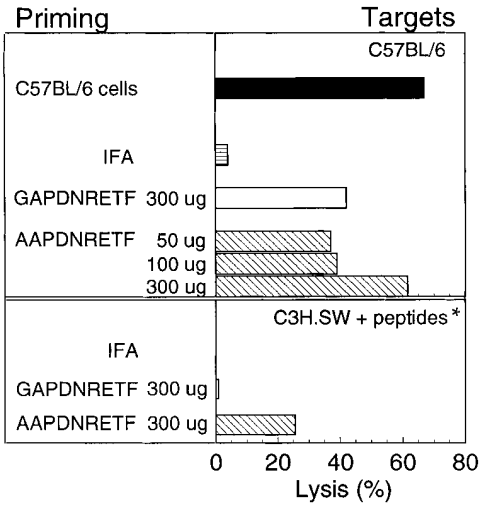

Figure 3. In vivo priming with AAPDNRETF generates potent CTL activity. C3H.SW mice were primed either with C57BL/6 cells (2×10^7 cells i.p. on day -14), peptides in incomplete Freund's adjuvant (50 to 300 μg s.c. on days -17 and -10), or adjuvant alone (on days -17 and -10). On day 0, their spleen cells were restimulated in vitro with C57BL/6 cells, and tested in CTL assays 5 d later against C57BL/6 Con A blasts, and C3H.SW Con A blasts coated with 1 nM AAPDNRETF or GAPDNRETF (*C3H.SW Con A blasts were coated with the same peptide as the one used for CTL priming). Effectors primed with C57BL/6 cells or peptides did not kill uncoated C3H.SW Con A blasts (specific lysis ≤ 5%, $n = 3$). Effector/target ratio was 100:1. IFA = incomplete Freund's adjuvant. These results are representative for three separate experiments.

ated C57BL/6 recipients with an inoculum composed of 10^7 bone marrow cells + 5×10^7 spleen cells from C3H.SW mice primed with C57BL/6 cells (group 1), AAPDNRETF in incomplete Freund's adjuvant (group 2), or adjuvant alone (group 3). For the three groups, the numbers of Thy1.2$^+$/CD4$^+$ and Thy1.2$^+$/CD8$^+$ cells in donor spleen were similar to those found in untreated C3H.SW controls (Table II). Mice in group 3 ($n = 5$) showed no signs of GVHD, but all mice in group 1 (6/6), and 6/7 mice in group 2 developed cutaneous GVHD that evolved in the following sequence: hair loss → erythematous lesions → skin ulcerations (Fig. 4 *A*). Affected skin areas showed changes characteristic of GVHD, i.e., mononuclear cell infiltrates in the superficial dermis and epidermis, and epidermal as well as follicular dyskeratosis (data not shown). Skin lesions appeared around day 25 and progressively worsened up to day 60. Lesions were similar in groups 1 and 2 up to day 40, but eventually became more severe in group 2, as these mice lived longer (see below). The GVHD in group 2 was not limited to the skin. When compared to group 3, these recipients presented significant thymic hypoplasia, another hallmark of GVHD (Fig. 4 *C*). On day 60±2, recipients from AAPDNRETF primed donors showed a 50% reduction in the number of mature single positive thymocytes (TCR $\alpha\beta^{high}$/CD4$^+$ or CD8$^+$), double positive thymocytes, and total thymocytes (Thy1.2$^+$). Thymic cells were not evaluated in group 1 as only two moribund recipients were alive on day 60. While the severity of skin GVHD was similar in groups 1 and 2, the overall severity of GVHD was greater in group 1. Indeed GVHD was rapidly lethal in 67% of mice in this group but in none of group 2 (Fig. 4 *B*). It should be remembered that group 1 donors had been primed against all D^b- and K^b-associated MiHAs, while priming in group 2 involved only one MiHA, AAPDNRETF.

Discussion

While immunodominant MiHAs are probably the only one that are important in immune reactions, their identity remains elusive and it has not been possible previously to analyze the immunogenicity of a single immunodominant MiHA. A major finding of our study is characterization of a novel H-2D^b-associated MiHA which allowed us to define in molecular terms an immunodominant MiHA and to evaluate in vivo and in vitro T cell responses to this antigen. The results presented in Fig. 2 show that two nonapeptides, AAPDNRETF and GAPDNRETF, whose structure was deduced following microsequencing of extensively purified peptide material eluted from

Table II. T Cell Subsets in Donor (C3H.SW) Spleen Cells

Treatment	Thy1.2$^+$CD4$^+$ cells		Thy1.2$^+$CD8$^+$ cells	
	%	*n* ($\times 10^6$)	%	*n* ($\times 10^6$)
Untreated	15.3±0.7	13.8±0.9	4.1±0.6	3.7±0.1
IFA on days −17 and −10	14.2±1.2	13.6±3.3	4.6±1.6	4.1±0.4
AAPDNRETF/IFA on days −17 and −10	14±1.7	13.5±1.1	3.6±0.8	3.4±0.6
C57BL/6 cells	14.5±0.9	13.7±1	4±1.2	3.6±0.4

Mean±SD, three mice per group.

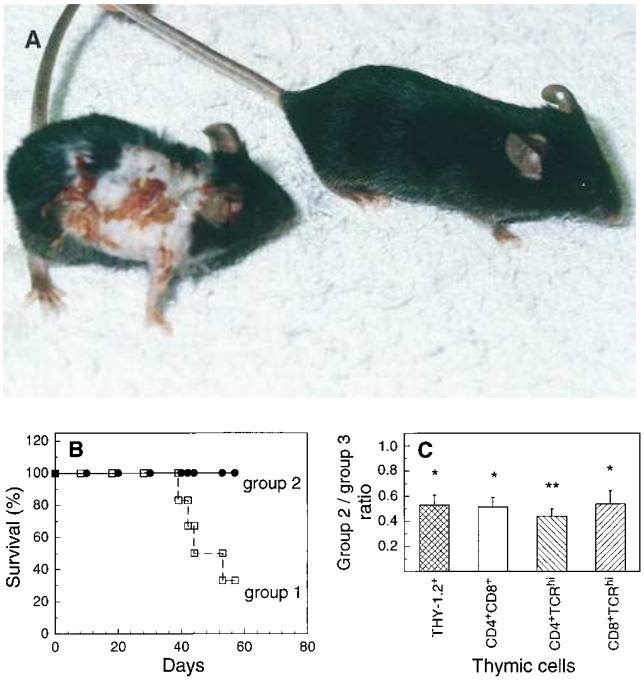

Figure 4. T lymphocytes primed with AAPDNRETF cause GVHD in irradiated recipients. C3H.SW mice were primed with C57BL/6 splenocytes (group 1), AAPDNRETF 300 μg in adjuvant (group 2), or adjuvant alone (group 3) under the same conditions as in Fig. 3. On day 0, irradiated (950 rads) C57BL/6 recipients received 10^7 bone marrow cells + 5×10^7 spleen cells from primed donors. The mean numbers ($\times 10^6$) of Thy1.2$^+$CD4$^+$ and Thy1.2$^+$CD8$^+$ cells in donor spleen cell suspensions were 2/7.2, 1.8/7, and 2.3/7.1 for groups 1, 2, and 3, respectively. (*A*) Severe ulcerative skin lesions on day 55 in a recipient from group 2 (*left*). One recipient from group 3 is shown for comparison (*right*). (*B*) Survival of mice in group 1 ($n = 7$) and 2 ($n = 6$). (*C*) Absolute numbers of thymic cells bearing various cell surface antigens were assessed in mice from group 2 and group 3 on day 60±3. One mouse per group was tested in three consecutive experiments and ratios (numbers in group 2/numbers in group 3) were calculated for the three pairs of mice. Results are expressed as the mean ratio±SEM. $*P < 0.025$, $**P < 0.01$ (one-sample hypothesis, one-tailed *t* test). These results are representative for two separate experiments. IFA, incomplete Freund's adjuvant.

C57BL/6 Con A blasts, possess all the characteristics of an H-2D^b-associated dominant MiHA: (*a*) they have the same retention time as the natural MiHA on RP-HPLC, (*b*) they are recognized only in association with H-2D^b, (*c*) they show very high activity in CTL sensitization assays involving C3H.SW anti-C57BL/6 CTLs, and (*d*) in vivo priming, particularly with AAPDNRETF, generates specific anti-C57BL/6 CTL activity. Although we cannot exclude, we consider unlikely the possibility that glycine is present at position 1 in the natural MiHA because, while many D^b-associated peptides have an alanine at this position, a review of the literature (see reference 25) and a search in the MHCPEP database (version 1.1; Walter and Eliza Hall Institute of Medical Research) revealed no D^b ligand with glycine at position 1. It is also noteworthy that AAPDNRETF was more effective than GAPDNRETF in both in vivo priming and CTL sensitization assays. Therefore, we suggest that AAPDNRETF is the most likely sequence of the MiHA. Formal identification of the natural MiHA will require identification and sequencing of its source protein or

coding gene, and molecular definition of the allelic difference between C3H.SW and C57BL/6 mice. We are currently pursuing this issue by screening a C57BL/6-derived cDNA library with degenerate oligonucleotides in order to clone the MiHA cDNA. A search in EMBL database (February 20, 1996) revealed no perfect match, neither for AAPDNRETF nor for any of the peptides listed in Fig. 2.

Immunodominance is the most important feature of T cell response to MiHAs. Knowledge of the structure of a dominant MiHA will be most helpful to understand this phenomenon in molecular terms. Thus, we recently observed that AAPDNRETF has a very high affinity for H-2D^b (unpublished observations). Furthermore, the structure of AAPDNRETF shows an interesting characteristic that could possibly contribute to its immunodominant status. This nonapeptide contains six uncharged residues and three residues with basic or acidic side chains. The latter are found at positions 4, 6, and 7, which are precisely those that are most solvent accessible and constitute the principal T cell receptor contact residues (29). In addition, substitution with alanine at any of these sites abrogated the activity of AAPDNRETF in CTL sensitization assays (Fig. 2 *B*). As charge interaction between residues on the peptide and the T cell receptor are of great significance in T cell response (30), the presence of three residues that can contribute to the formation of salt bridges raises the possibility of strong affinity interactions between AAPDNRETF/D^b complexes and specific T cells.

For many class I–associated peptides, a simple immunization in incomplete Freund's adjuvant cannot trigger CTL responses (31). To our knowledge, this has never been attempted with MiHA peptides. In our study, priming with AAPDNRETF generated potent specific CTLs, suggesting that it triggered class I-restricted CD8$^+$ CTLs which are CD4-independent, as reported for some optimal class I-restricted peptides (31). Alternatively, this nonapeptide could bind to both class I and class II molecules, an apparently rare phenomenon described for an HIV peptide (32).

GVHD is certainly the most important immunopathologic reaction triggered by MiHAs in clinical medicine (33). Recent studies have provided evidence that only a limited number of MiHAs could cause GVHD (12, 13, 22). Their identity, however, remains elusive. Using donor/recipient strains incompatible at either the H-Y, H-4, H-7, or H-9 loci, Korngold failed to induce not only clinical GVHD, but even subclinical pathology after transfer of T cells from either normal or presensitized donors (34). CTLs recognizing some host MiHAs, such as H-Y or HA-2, have been isolated from recipient's blood after human bone marrow transplantation. However, these findings do not necessarily imply a role for these MiHAs in GVHD, because host reactive anti-MiHAs CTLs were found with similar frequencies in patients with or without GVHD (35). Goulmy et al. have recently reported that a mismatch of HA-1 MiHA, whose molecular structure is unknown, was associated with an increased rate of GVHD in adult recipients of bone marrow transplants (36). Thus, HA-1 is heretofore the only MiHA that has been implicated in GVHD. Our results, presented on Fig. 4, conclusively demonstrate that T lymphocytes targeted to a single immunodominant MiHA can indeed cause GVHD. These findings lend experimental support to the notion of Loveland and Simpson that MiHAs are "neither weak nor minor (9)." However, it remains to be determined whether all dominant MiHAs could trigger GVHD.

Anti-MiHAs GVHD preferentially involves selected tissues and organs such as the skin, liver, intestine, and the thymus. In both man and mouse, the tissue specificity of GVHD lesions varies among different donor-recipient pairs. It is tempting to speculate whether this is related to the fact that some MiHAs have a restricted tissue distribution (37). It will now be possible to study the relation between the tissue expression of a target MiHA, AAPDNRETF, and the pathologic tissue lesions found in recipients suffering from GVHD induced by donors primed against this peptide. This will require a quantitation of AAPDNRETF epitopes expressed by various cell lineages coupled with detailed pathology studies on GVHD target organs.

The demonstration that a single dominant MiHA can cause GVHD should encourage further attempts to sequence dominant MiHAs. Molecular definition of these antigens could be of great significance in transplantation and cancer immunotherapy. For instance, in allogeneic bone marrow transplantation, generation of donor anti-host T cells targeted to dominant MiHAs might represent the most effective strategy to harness and amplify the crucial graft-versus-leukemia effect (14). Furthermore, as it is relatively easy to induce unresponsiveness to short peptides (38), tolerization of donor T cells to recipient's dominant MiHAs might be the most effective and specific strategy to prevent GVHD. In this regard, it is interesting to note that lysis of target cells sensitized with AAPDNRETF decreased at peptide concentrations greater than 10^{-9} M (Fig. 2 *C*). Such a marked CTL prozone effect has recently been observed with a peptide from the human immunodeficiency virus-1 envelope protein gp 160 (39). In the latter case, detailed analyses showed that inhibition of CTL-mediated lysis resulted from a transient anergy initiated by the binding of the antigenic peptide to MHC molecules on the CTL's own surface followed by engagement of the TCR (39). In vivo administration of peptides in large amounts could possibly entail not only functional inactivation but also deletion of reactive T cells by causing activation-induced cell death or exhaustion (38); clearly, further studies are required to evaluate the possible importance of these mechanisms for tolerization to MiHA peptides.

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