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Fine Specificity of the Myelin-Reactive T Cell Repertoire: Implications for TCR Antagonism in Autoimmunity[1]

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The use of altered peptide ligands (APL) to modulate T cell responses has been suggested as a means of treating T cell-mediated autoimmune disorders. We have assessed the therapeutic potential of TCR antagonist peptides in autoimmunity using murine experimental autoimmune encephalomyelitis (EAE) as a model. The Tg4 transgenic mouse expresses an MHC class II-restricted TCR specific for the immunodominant encephalitogenic epitope of myelin basic protein, Ac1–9 (acetylated N-terminal nonamer). We have used T cell lines derived from Tg4 mice to define the TCR contact residues within Ac1–9. APL with appropriate substitutions at the primary TCR contact residue were effective antagonists of Tg4 T cells. These antagonist APL, however, were found to induce EAE in susceptible, nontransgenic strains of mice. Underlying this, the Ac1–9-specific T cell repertoire of normal mice, rather than reflecting the Tg4 phenotype, showed considerable diversity in fine specificity and was able to respond to the Tg4 antagonist APL. Defining antagonist APL in vitro using T cell clones, therefore, was not a reliable approach for the identification of APL with EAE-suppressing potential in vivo. Our findings highlight the complexities of the autoreactive T cell repertoire and have major implications for the use of APL in autoimmune diseases. ***The Journal of Immunology,*** **1998, 161: 3357–3364.**

Recognition by $CD4^+$ T cells of processed peptide Ags in association with MHC class II molecules is central to the generation of an adaptive immune response. The TCR interacts with defined residues within the peptide, and appropriate TCR stimulation induces an intracellular signaling cascade leading ultimately to T cell activation, clonal expansion, and effector function. Studies in recent years have provided evidence that T cell stimulation using variant synthetic peptides with amino acid substitutions at residues interacting with the TCR (TCR contact residues) can have differential effects on T cell responsiveness (reviewed in Ref. 1). Such altered peptide ligands (APL)[3] have been shown to induce qualitatively different activation of T cell clones so that cytokine production or cytolytic activity is induced without the concomitant proliferation induced with the wild-type agonist peptide (2–4). Also, certain APL have been shown to inhibit the proliferation of T cell clones in response to full agonist stimulation when both agonist and APL are presented by the same APC. This inhibitory activity is not due to MHC blockade, is TCR specific, and has been termed TCR antagonism (5). The use of TCR antagonist peptides has been proposed as a potential therapy in T cell-mediated autoimmune disorders (6).

Murine experimental autoimmune encephalomyelitis (EAE) is an extensively studied model of multiple sclerosis. EAE can be induced by immunization with whole myelin, isolated myelin Ags such as myelin basic protein (MBP) and proteolipid protein (PLP), or synthetic peptides containing defined T cell epitopes from within these Ags (reviewed in Ref. 7). The disease is mediated by activation of $CD4^+$ T cells and can be transferred into naive recipients using T cell lines (TCL) displaying the Th1 profile of cytokine production (8). Previous studies of EAE, induced by using the immunodominant encephalitogenic T cell epitope $PLP_{139-151}$ in the SJL mouse, have identified TCR antagonist peptides capable of inhibiting EAE induced with the wild-type 139–151 peptide (9–11).

The immunodominant encephalitogenic T cell epitope of MBP recognized by mice of the H-2^u MHC haplotype is the acetylated N-terminal nonamer (Ac1–9) (12). T cell recognition of this epitope has been dissected thoroughly. Analysis of a panel of Ac1–9-specific T cell clones derived after various immunization and restimulation protocols revealed almost identical TCR variable gene usage. Of nine clones tested, eight expressed $V_\beta 8.2$ and all expressed a particular variant of $V_\alpha 4$ (13). These findings suggested that EAE induced using Ac1–9 would be well suited to manipulation using antagonist APL active on single, or closely related, TCRs. The Ac1–9-specific T cell hybridoma 1934.4, generated using one of these clones, has been used to identify residues within Ac1–9 that interact with the TCR (positions Gln^3 and Pro^6) and the I-A^u restriction element (positions Lys^4 and Arg^5) (14–16). A surprising finding was that Ac1–9 forms highly unstable complexes with I-A^u. Substitution of Lys^4 for several other amino acids, most notably Tyr, greatly enhances this interaction and produces variant peptides with affinities for I-A^u that are measurable in direct binding assays (16, 17). Several studies have reported the use of Ac1–9 position 4 analogues with increased MHC binding affinity for the induction of T cell tolerance and prevention of EAE (18–20). Use of alanine-substituted peptide analogues revealed a requirement for Gln^3 and Pro^6 for effective TCR engagement and activation of the 1934.4 hybridoma. Analysis of APL with all of

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[3] Abbreviations used in this paper: APL, altered peptide ligand(s); EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PLNC, primed lymph node cells; PLP, proteolipid protein; TCL, T cell line(s); Ac1–9, acetylated N-terminal nonamer.

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the possible substitutions at either position 3 or 6 revealed that position 6 must be a Pro to activate hybridoma 1934.4, whereas some flexibility was allowed at position 3 with APL having Met, His, Phe, or Tyr in place of the wild-type Gln acting as agonist peptides (i.e., positions 6 and 3 are "primary" and "secondary" TCR contact residues, respectively) (15). The TCR-α and -β genes of the 1934.4 hybridoma were cloned and used to generate the Tg4 TCR transgenic mouse (21). This mouse did not develop EAE spontaneously, but developed the disease after immunization with either the Ac1–9 peptide or whole myelin in CFA.

In this study, we have addressed the potential applicability of TCR antagonist peptides in the treatment of Ac1–9-induced EAE. Using substitutions at position 6 (the primary TCR contact residue) of Ac1–9, we were able to develop APL that antagonized the Ac1–9-specific response of a "clonal" TCL derived from the Tg4 mouse. Far from preventing the development of EAE, however, these APL were able to induce disease in susceptible, nontransgenic mice. The reason for this was apparent on analysis of the fine specificity of Ac1–9-reactive polyclonal TCL derived from normal mice. These TCL recognized Ac1–9 with position 3 as primary and position 6 as secondary TCR contact residues (i.e., the exact opposite of the Tg4 system). Thus, APL that acted as antagonists for the Tg4 TCR behaved as agonists for polyclonal Ac1–9-specific T cells and induced EAE when administered to normal mice. These findings dispute the previously held belief of a highly restricted, almost monoclonal, Ac1–9-reactive T cell repertoire and highlight the hazards inherent in the use of limited numbers of T cell clones to identify antagonist APL for therapeutic use in human autoimmune disorders.

Materials and Methods

Mice

The generation of the Tg4 TCR transgenic mouse has been described previously (21). The MBP TCR1 transgenic mouse, which expresses a different Ac1–9-specific TCR (22, 23), was kindly provided by Dr D. M. Zaller (Merck Research Laboratories, Rahway, NJ). Tg4, MBP TCR1, B10.PL (H-2^u), SJL (H-2^s), and B10.PL×SJL (H-2^{uxs}) F_1 mice were bred under specific pathogen-free conditions at the School of Biomedical Sciences, University of Bristol.

Ags and immunizations

The wild-type Ac1–9 peptide (AcASQKRPSQR) and panels of APL were synthesized as C-terminal amides using standard F-moc chemistry on an Abimed AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany). The APL had all possible amino acid substitutions at either position 3 or 6. A second panel of APL were synthesized with substitutions at position 3 or 6, as described above, which also contained a Lys > Tyr substitution at position 4. Previous experiments have found no variation in either I-A^u binding or the antigenic properties of C-terminal amide peptides compared with free acid peptides (D.C.W., unpublished observations). OVA $_{323–339}$ peptide (ISQAVHAAHAEINEAGR) was used as an I-A^u-binding control peptide. Spinal cord homogenate was prepared as described previously (18) as a source of whole myelin.

For production of primed lymph node cells (PLNC), mice were immunized s.c. with a total of 100 μl of Ag emulsified with CFA containing 4 mg/ml *Mycobacterium tuberculosis* strain H37RA (Difco, Detroit, MI) at the tail base and in one hind limb. Each mouse received 1 mg spinal cord homogenate. Ten days later, draining popliteal and inguinal lymph nodes were removed and used as a source of PLNC.

Generation of T cell lines

Ac1–9-specific TCL were derived from the Tg4, H-2^u, and H-2^{uxs} mice. The Tg4.TCL was generated using transgenic splenocytes from an unimmunized Tg4 mouse by primary in vitro stimulation with Ac1–9. Polyclonal TCL were derived from H-2^u mice (4Ku.TCL) or H-2^{uxs} mice (4Kuxs.TCL) immunized with spinal cord homogenate as described above. TCL were then generated by in vitro stimulation of PLNC with Ac1–9. Established TCL were maintained using a standard 14-day restimulation/expansion cycle. TCL were restimulated for 3 days with Ac1–9 in the presence of irradiated (30 Gy) syngeneic spleen APC (normal H-2^u APC were used for Tg4.TCL). T cell blasts were isolated using a NycoPrep 1.077 animal density gradient (Nycomed Pharma, Oslo, Norway) and expanded in culture medium supplemented with 5% Con A-activated rat spleen supernatant as a source of T cell growth factors.

Tissue culture medium

Iscove's modified Dulbecco's medium supplemented with 2 mM L-glutamine, 5×10^{-5}M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Paisley, U.K.) was used as the tissue culture medium. Medium without serum was used for washing lymphoid cells and TCL. For the 3-day restimulation period of TCL, medium was supplemented with 0.5% normal mouse serum. For all other culture conditions and assays, medium was supplemented with 5% FCS (Sigma, Poole, U.K.).

T cell proliferation assays

Analyses of response patterns of Ac1–9-specific T cell populations were performed in triplicate over 72 h using flat-bottom, 200-μl microtiter wells (Becton Dickinson, Mountain View, CA). Assays testing responses of naive Tg4 and MBP TCR1 transgenic mice used 3×10^5 splenocytes per well. Responses of TCL were tested using 2×10^4 TCL and 3×10^5 irradiated (30 Gy) syngeneic APC per well. Cultures were pulsed with 0.5 μCi [^{3}H]TdR (Amersham, Amersham, U.K.) for the final 18 h and TdR incorporation measured using a liquid scintillation beta counter (LKB Wallac, Turku, Finland). Results are expressed as mean cpm of triplicate cultures.

Tg4.TCL antagonism assays

APL were tested for TCR antagonist activity using a modification of previously described methods. The wild-type Ac1–9 peptide forms highly unstable complexes with I-A^u (16) and for this reason cannot be used in assays requiring peptide pulsing and washing of APC. Therefore, all assays of TCR antagonism used Ac1–9(4Y) analogues with high affinity for I-A^u. Irradiated (30 Gy) syngeneic spleen APC were first incubated at 5×10^6/ml for 2 h with or without a suboptimal dose (0.5 μM) of Ac1–9(4Y) and washed extensively. Pulsed APC were then incubated for a further 2 h in 96-well plates at 5×10^5/well in the presence of varying concentrations of Ac1–9(4Y) APL with all possible substitutions at position 6 before addition of Tg4.TCL at 2×10^4/well. Proliferation of Tg4.TCL was then measured, as described above, in a 72-h assay. Antagonism of Tg4.TCL responses by APL is expressed as percentage of inhibition of the response to agonist peptide in the absence of APL.

Induction and assessment of EAE

EAE was induced with a single s.c. injection of 100 μl of CFA emulsion containing 100 μg of individual synthetic peptides at the base of the tail. Mice also received 200 ng pertussis toxin (Speywood Pharmaceuticals, Maidenhead, U.K.) in 0.5 ml of PBS i.p. on the same day and 2 days later. Clinical signs of EAE were assessed using scores of 0 through 5: 0, no signs; 1, flaccid tail; 2, partial hind limb paralysis and/or impaired righting reflex; 3, total hind limb paralysis; 4, hind and forelimb paralysis; 5, moribund or dead.

Purification of I-A^u and binding assay

I-A^u class II molecules were purified from the I-A^u-expressing B cell line PL8 as described previously (14). Briefly, cells were lysed using 1 M triethanolamine-buffered saline/0.5% Nonidet P-40 containing 100 mM PMSF on ice for 30 min. I-A^u was affinity purified from cell lysates using the I-A^u-binding mAb OX6 coupled to a CNBr-activated Sepharose 4B column (Sigma). Purity of the I-A^u preparation was assessed using SDS-PAGE.

Binding assays were performed as described previously (24). Initially, assays were performed using 10 nM and 100 nM purified I-A^u protein, with a range of concentrations of biotinylated Ac1–9(4Y), to determine the peptide concentration giving 50% maximum binding to MHC. Competition assays involved incubation of 10 nM I-A^u protein and 100 nM Ac1–9(4Y) with a dose range of nonbiotinylated APL as competitor peptides. Reactions were conducted in 50 μl final volume of PBS, pH 5.5, 100 mM KH_2PO_4, 0.02% dodecyl-β-D-maltoside, 1 mM PMSF, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin in 96-well polypropylene plates for 48 h at 37°C. Reaction mixtures were neutralized with 50 μl of 50 mM Tris, pH 8.0, containing 0.02% dodecyl-β-D-maltoside, subsequently transferred to 96-well polystyrene plates previously coated overnight with 5 μg/ml anti-I-A^u mAb in PBS, and blocked with 5% newborn calf serum at room temperature for 4 h. These plates were then incubated overnight at 4°C. Plates were then washed three times with PBS/0.05% Tween 20, and 100

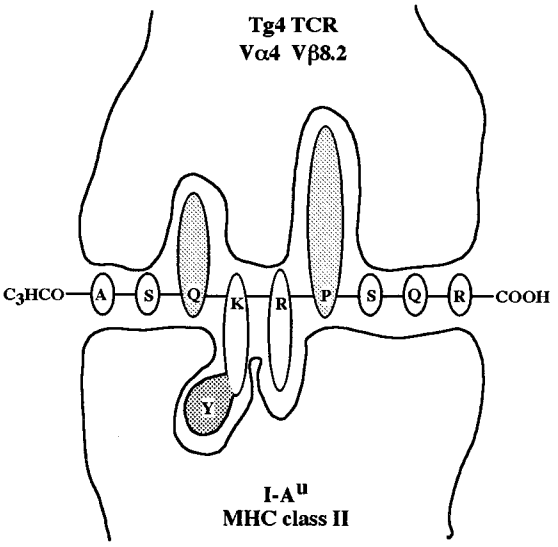

FIGURE 1. Schematic representation of interaction of Ac1–9 with I-A^u and the Tg4 TCR. The I-A^u peptide-binding cleft interacts with position Lys^4 and Arg^5 of Ac1–9. The stability of this interaction is extremely poor but can be enhanced greatly by substitution of Lys^4 by Tyr. Positions Pro^6 and Gln^3 act as primary and secondary contact residues, respectively, for the Tg4 TCR. Any substitution at position 6 ablates T cell activation, whereas APL with Met, His, Phe, or Tyr at position 3 act as weak agonists. This information is derived from previous studies (14–17).

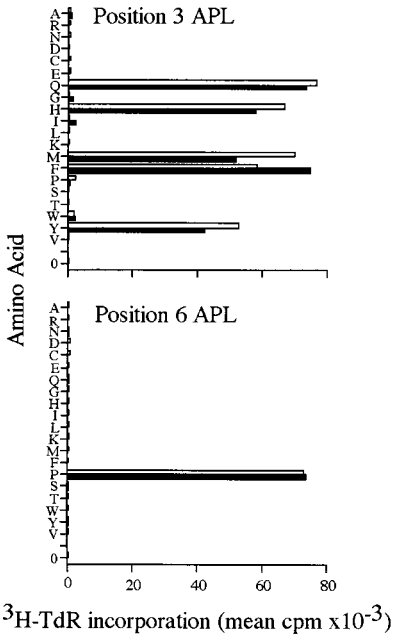

FIGURE 2. Fine specificity of Tg4 T cells determined using Ac1–9 position 3 and position 6 APL. Naive Tg4 splenocytes (open bars) and the Tg4. TCL (filled bars) were tested for proliferative responses to Ac1–9 APL with all possible substitutions at position 3 or position 6. Relatively high Ag concentrations were used (20 μM shown here), and proliferation is expressed as mean cpm of triplicate cultures. These experiments were repeated with consistent results.

μl of Europium-labeled streptavidin (LKB Wallac) was added to each well at a 1:500 dilution in DELFIA assay buffer (LKB Wallac). Plates were incubated for 4 h at 4°C and washed 3 times with PBS-0.05% Tween 20. Enhancement solution (200 μl/well) was then added and the plates shaken for 5 min. Fluorescence was measured using a Victor time-resolved fluorometer (LKB Wallac).

Results

Interaction of Ac1–9 with the Tg4 transgenic TCR

Previous studies in our laboratory have defined Gln^3 and Pro^6 within Ac1–9 as TCR contact residues required for stimulation of the 1934.4 hybridoma (Fig. 1) (15). Our initial experiments examined the requirements for stimulation of Tg4 transgenic T cells at positions 3 and 6 using Ac1–9 analogue peptides with every possible amino acid substitution at either of these positions. Splenic T cells from unimmunized Tg4 mice displayed primary proliferative responses in vitro when cultured with APL in which the native Gln at position 3 was replaced by either Met, His, Phe, or Tyr, but not APL with other substitutions at this position. In contrast, no substitution of the native Pro at position 6 was allowed (Fig. 2). The Tg4.TCL, derived by primary in vitro stimulation of Tg4 splenocytes with Ac1–9, showed an identical response pattern to that of Tg4 splenocytes (Fig. 2). These results confirm that the Tg4 mouse expresses T cells recognizing positions 6 and 3 as primary and secondary TCR contact residues, respectively, a fine specificity identical to that displayed by the 1934.4 hybridoma from which the TCR transgenes were derived (16).

Identification of Ac1–9 position 6 APL that act as TCR antagonists

Our next step was to identify APL that behaved as TCR antagonists for the Tg4.TCL in vitro. Antagonism experiments require the pulsing of APC with agonist peptide before the addition of APL to the culture system (5). The wild-type Ac1–9 peptide displays immeasurably low affinity for the I-A^u restriction element producing highly unstable complexes which dissociate within minutes (16). This makes pulsing APC with the wild-type peptide impossible. However, replacement of Lys at position 4 of Ac1–9 with Tyr produces an analogue peptide with greatly enhanced affinity for I-A^u resulting in stable peptide-MHC complexes (Fig. 1) (16, 17). For antagonism experiments, we therefore used Ac1–9(4Y) as agonist peptide and double-substitution APL with Tyr at position 4 and all possible substitutions at position 6, the primary TCR contact residue.

Analysis of the effects of position 6 APL on proliferative responses of Tg4.TCL to Ac1–9(4Y) revealed that most substitutions produced peptides with no inhibitory effects. We refer to these inactive peptides as null peptides. However, APL with either Gly, Ile, Leu, Thr, or Val were effective inhibitors of Tg4.TCL responses (Fig. 3). Peptides with Thr, Val, or Gly at position 6 were consistently the most potent antagonists. It is interesting to note that four of these substitutions (Thr, Val, Leu, and Ile) are amino acids with similar properties, having small nonpolar or polar uncharged side chains. This similarity may be important in conferring antagonist activity in this system, as has been reported previously (25).

The affinity of active peptides for I-A^u was measured by a direct binding assay, to exclude simple MHC blockade as an explanation for the inhibitory activity of our antagonist peptides. None of the antagonist peptides displayed increased binding affinity compared with Ac1–9(4Y) or control null peptides, which could account for the inhibitory activity (Table I). Also, the 323–339 peptide of OVA, which binds I-A^u with an affinity similar to that of Ac1–9(4Y) (Table I and Ref. 17) was unable to inhibit responses to Ac1–9(4Y) (Fig. 3). In some experiments, the responses to Ac1–9(4Y) could be partially blocked using position 6 APL with the wild-type Lys^4 (i.e., low MHC affinity), providing further evidence against MHC blockade (data not shown).

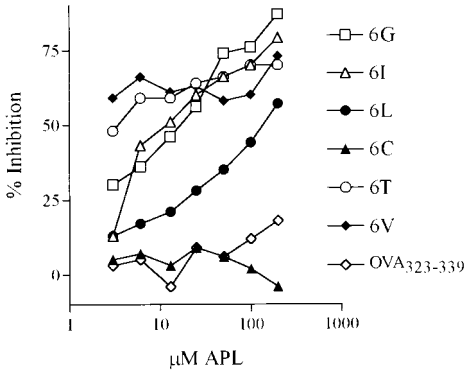

FIGURE 3. Ac1–9 position 6 APL can act as antagonists for the Tg4 TCR. Ac1–9 (4Y, 6X) APL were tested for inhibitory effects on proliferation of Tg4.TCL in response to a suboptimal dose of Ac1–9(4Y) (0.5 μM). All position 6 APL were tested. Shown here are the five APL showing inhibitory effects and the null peptide Cys^6 as an example of the ineffectiveness of all other APL tested. The 323–339 peptide of OVA was used as an I-A^u-binding control peptide. The response of Tg4.TCL to Ac1–9(4Y) in the absence of APL was 49,326 cpm (mean of triplicate cultures). This experiment is representative of two additional experiments with similar results.

APL that antagonize Tg4 TCL in vitro can induce EAE in vivo

Identification of APL capable of antagonizing the encephalitogenic Tg4 TCR in vitro led us to assess their therapeutic potential in EAE. We chose to test the three most potent antagonist peptides: Gly^6, Thr^6, and Val^6. The ultimate aim was to test these peptides for inhibition of EAE induced with the wild-type Ac1–9. Initially, mice were immunized with the antagonist peptides alone to exclude any in vivo encephalitogenic activity. Surprisingly, all three of the TCR antagonist peptides were able to induce EAE on their own when administered using an EAE inducing protocol (Table II). Groups of Tg4 transgenic mice or normal H-2^u or H-2^{uxs} mice were immunized with Ac1–9(4Y) or the Ac1–9(4Y,6X) APL. As expected, the transgenic Tg4 mice developed EAE only after immunization with Ac1–9 containing the native Pro^6 and not when the antagonist peptides were used. However, both the normal H-2^u and H-2^{uxs} mice developed EAE not only when Ac1–9 was used but also after immunization with the Gly^6, Thr^6, and Val^6 APL. In H-2^{uxs} mice, EAE induced with the APL was comparable to that induced with Ac1–9 in terms of both incidence and severity of disease. In H-2^u mice, the Gly^6 and Val^6 APL induced a higher incidence of disease than did Ac1–9. APL with either Gly, Thr, or Val at position 6 and wild-type Lys at position 4 also induced EAE in normal mice. Tg4 mice immunized with APL in CFA did not develop EAE. Therefore, the possibility that Tg4 T cells could be activated in vivo by APL presented by APC exposed to inflammatory stimuli can be excluded.

Table I. *Binding affinities of Ac1–9 analogue peptides for I-A^{ua}*

Peptide	IC$_{50}$ (nM)	Relative Binding Affinity
Ac1–9(4K)	>100,000	<0.00001
Ac1–9(4Y)	567	1
Ac1–9(4Y,6C)	1,688	0.333
Ac1–9(4Y,6G)	3,680	0.15
Ac1–9(4Y,6T)	3,750	0.15
Ac1–9(4Y,6V)	2,958	0.19
OVA$_{323–339}$	1,136	0.5

[a] The I-A^u-binding affinities of Ac1–9, Ac1–9(4Y), and position 6 APL, previously identified as antagonists of the Tg4 TCL, were compared using a competitive binding assay. As controls, the null Cys^6 APL and the OVA$_{323–339}$ peptide were also tested.

Table II. *Ac1–9 position 6 APL that act as antagonists in vitro can induce EAE in normal susceptible strains of mice*[a]

Mouse Strain	EAE Incidence (mean maximal score) Ac1–9 analogue peptides			
Expt. 1	4Y,6P	4Y,6G	4Y,6T	4Y,6V
Tg4	4/5 (4.2)	0/5	0/5	0/5
B10.PL	2/5 (1.6)	4/5 (1.6)	2/5 (1)	5/5 (2)
B10.PL×SJL	4/5 (2.4)	5/5 (1.8)	3/5 (1.8)	5/5 (2)
Expt. 2	4K,6P	4K,6G	4K,6T	4K,6V
B10.PL×SJL	6/6 (2)	3/6 (0.8)	6/6 (2.2)	6/6 2.5)

[a] Ac1–9 APL were compared with wild-type Ac1–9 for the ability to induce EAE in Tg4 transgenic mice or mice of the EAE-susceptible B10.PL and B10.PL×SJL strains. Each mouse received 100 μg of the indicated peptide. In Expt. 1, APL with Tyr at position 4 were used, whereas Lys^4 APL were used in Expt. 2. Results are shown as incidence of EAE onset with the mean of the maximum score reached by each mouse in a given group shown in parentheses.

Previous reports have described APL that behave as agonist/antagonists, i.e., APL that act as agonists at high concentrations but antagonists at lower concentrations (6, 26). This may have accounted for our findings, especially when using the Tyr^4 APL with high affinity for I-A^u. Increased avidity of the TCR-peptide-MHC interaction due to the presence of Tyr^4 may have conferred agonist activity not present in APL with the same position 6 substitution but with the wild-type Lys at position 4. Such APL may be able to induce EAE in vivo. To test this possibility, we assessed the agonist potential of position 6 antagonist APL on the Tg4.TCL. These experiments showed that a high dose of the Val^6,Tyr^4 APL was able to stimulate Tg4.TCL suboptimally, while the Val^6,Lys^4 APL, and all other 6X,Tyr^4 APL, remained inactive (data not shown). The Val^6,Tyr^4 APL was stimulatory only at saturating doses (above 50 μg/ml), whereas the Pro^6,Lys^4 peptide was active at concentrations below 1 ng/ml. The Tg4.TCL cannot, therefore, be stimulated maximally by recognition of the Val^6 APL. This stimulatory activity was also seen when Val^6,Tyr^4 was tested on the 1934.4 hybridoma (data not shown). Increasing MHC affinity, therefore, can convert an inactive APL into a weak agonist/antagonist in vitro. However, we do not believe the EAE induced with the Val^6 APL was a result of increased biologic activity on Tg4-like T cells in vivo. Such a mechanism would predict that Tg4 mice would develop EAE, but only the normal mice were affected (Table II). Also, the Gly^6 and Thr^6 APL, which induced EAE in normal mice, showed no stimulatory activity on Tg4.TCL when the Tyr^6 substitution was introduced. Moreover, position 6 APL with the wild-type Lys at position 4 also induced EAE in normal mice (Table II, Expt. 2).

These experiments demonstrate that use of clonal T cell populations in vitro can define antagonist APL for the TCR tested, but that the same APL can induce pathology when administered to the intact animal.

Polyclonal Ac1–9-specific T cell populations display Ag fine specificity that is remarkably different from the Tg4 phenotype

Thus far, we have demonstrated that analogue peptides defined as TCR antagonists, using clonal T cell populations in vitro, can have pathogenic effects in vivo when given to normal susceptible mice. To explain these findings, we reasoned that the immune repertoire in vivo must contain potentially autoaggressive T cells capable of being activated by immunization with our defined TCR antagonist APL. To address this possibility, we analyzed the fine specificity

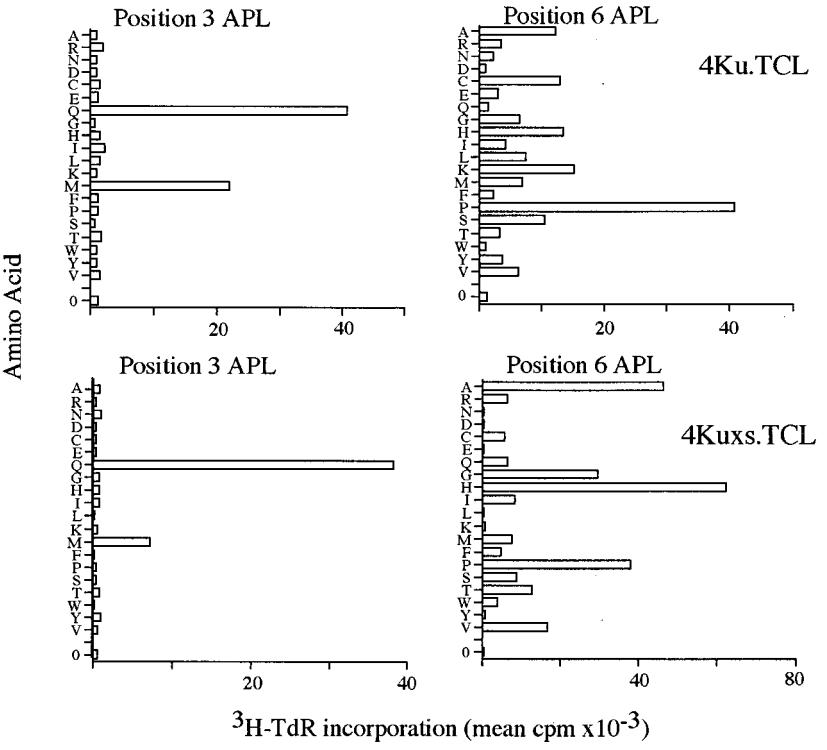

FIGURE 4. Fine specificity of polyclonal Ac1–9-specific TCL differs drastically from that of the Tg4 TCR. The 4Ku.TCL and 4Kuxs.TCL were derived from H-2^u and H-2^{uxs} mice, respectively. Both TCL were tested for proliferative responses to Ac1–9 APL with all possible substitutions at position 3 or position 6 (20 μM). These experiments were repeated three times with consistent results.

of polyclonal T cell populations from nontransgenic mice. We generated Ac1–9-specific, I-A^u-restricted polyclonal TCL from H-2^u mice (4Ku.TCL) or H-2^{uxs} mice (4Kuxs.TCL) after immunization with whole myelin. The requirements for activation of each of these TCL were tested using the Ac1–9 APL with all possible substitutions at positions 3 or 6 (Fig. 4). The response patterns of these TCL differed drastically from those found with the Tg4.TCL (compare Figs. 2 and 4). First, using position 3 APL, only Met^3 behaved as a weak agonist. In the polyclonal system, therefore, recognition of position 3 displays less flexibility than is the case using the Tg4 TCR. At position 6 (which must be a Pro in the Tg4 system), however, the opposite was true, with both 4Ku.TCL and 4Kuxs.TCL responding to the majority of APL tested. Thus, the polyclonal system used position 3 as the primary and position 6 as the secondary TCR contact residue, the exact opposite of the pattern found with the Tg4 TCR. Responses to all three of the position 6 APL that induced EAE were evident, although, with the exception of the response of 4Kuxs.TCL to 6Gly, at relatively low levels compared with the wild-type Ac1–9. The 4Kuxs.TCL also responded strongly to Ala^6 and His^6, with all of the other APL that induced responses falling into the weak agonist category. Thus, position 6 APL, defined as antagonists using the Tg4 system, acted as agonists for the polyclonal T cell repertoire.

We showed formally that the position 6 APL, identified as antagonists using the Tg4 system, were ineffective on polyclonal TCL by using the antagonism assay with the 4Kuxs.TCL (Fig. 5). In 48-h assays, addition of position 6 APL to a suboptimal dose of Ac1–9(4Y) resulted in elevated proliferative responses in this TCL. Indeed, APL behaved as agonists in the absence of Ac1–9(4Y). In cultures lasting 72 h, the presence of APL resulted in lower proliferative responses due to the enhanced kinetics of stimulation seen after 48 h (data not shown). Figure 5 shows results obtained using position 6 APL, previously shown to act as antagonists of the Tg4.TCL. We also tested all other position 6 APL on the 4Kuxs.TCL, and none displayed antagonist activity.

The discrepancy in fine specificity between the Tg4 TCR and the polyclonal TCL was confirmed by analysis of PLNC taken from H-2^u and H-2^{uxs} mice 10 days after immunization with whole myelin. Of the position 3 APL, only Met^3 induced significant proliferation, whereas several position 6 APL activated these PLNC populations (data not shown). These findings using polyclonal Ac1–9-specific T cell populations demonstrate that the potential Ac1–9-reactive T cell repertoire in vivo is broader than previous reports have suggested.

To examine the Ac1–9 T cell repertoire further, we analyzed the response pattern of the distinct MBP TCR1 transgenic mouse. Splenic T cells from this transgenic mouse responded to 5 position

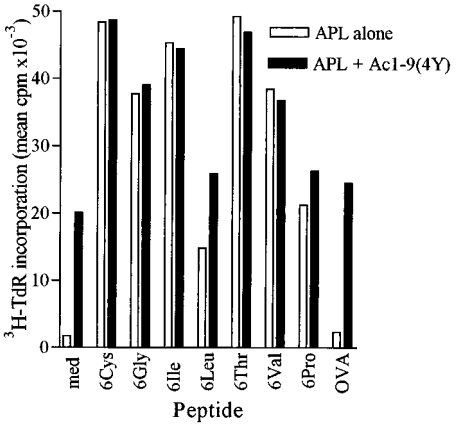

FIGURE 5. Ac1–9 position 6 APL that antagonize the Tg4 TCR fail to antagonize polyclonal Ac1–9-specific T cells. The response pattern of 4Kuxs.TCL to Ac1–9(4Y, 6X) APL was analyzed in terms of responses to APL alone or the ability of APL to influence responses to a suboptimal dose of Ac1–9(4Y) (0.5 μM). APL were added to cultures at 50 μM and assayed after 48 h. The 323–339 peptide of OVA was used as a control.

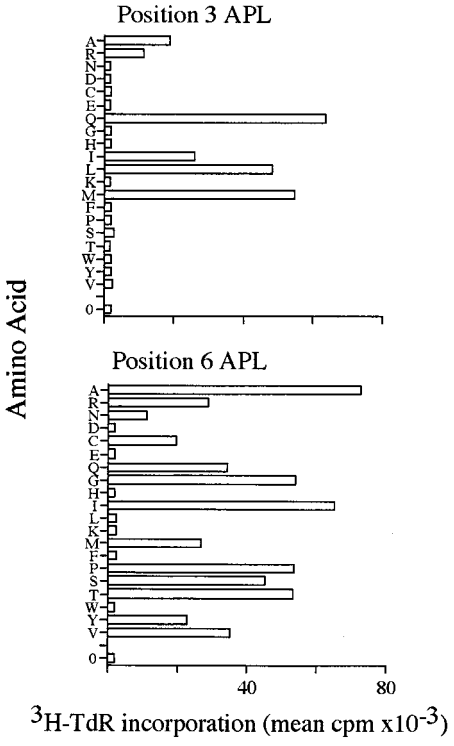

FIGURE 6. The MBP TCR1 transgenic TCR shows remarkable flexibility for Ac1–9 recognition at positions 3 and 6. Naive splenocytes from MBP TCR1 transgenic mice were tested for proliferative responses to Ac1–9 APL with substitutions at position 3 or 6 (50 μM). This experiment was repeated twice with consistent results.

3 APL and 11 position 6 APL, thus showing a fine specificity intermediate between the Tg4 and polyclonal patterns (Fig. 6). These splenocytes responded to Met^3, but none of the other position 3 APL recognized by the Tg4 TCR. The position 6 response patterns of all T cell populations tested can be compared in Table III. MBP TCR1 T cells differed from the polyclonal TCL in responses to these APL; 5 APL stimulated polyclonal but not MBP TCR1 T cells, while Asn^6 and Tyr^6 stimulated MBP TCR1 T cells but not the polyclonal TCL. Also, the MBP TCR1 T cells responded strongly to APL with Arg, Ile, Thr, or Val, which stimulated the polyclonal TCL only weakly. The response pattern of the MBP TCR1 T cells was confirmed using a TCL derived from these mice and also the 172.10 hybridoma, from which the TCR transgenes were derived to generate the mouse (data not shown). This response pattern suggests that T cells with fine specificity displayed by MBP TCR1 constitute a subset of the polyclonal population.

Discussion

The discovery that certain APL can exert TCR antagonistic effects on T cell clones in vitro prompted considerable interest in their in vivo potential as therapeutic agents for human autoimmune disorders. EAE models induced with defined peptide Ags allow direct analysis of APL based on epitopes known to be relevant in pathogenesis. Early studies identified TCR antagonists as effective inhibitors of EAE (9, 10). Our findings show that, while this approach is clearly achievable in rodent models that are dissected extensively, even in this situation the previously unappreciated complexities of the T cell repertoire in vivo pose considerable obstacles to development of effective, safe therapeutic approaches. To transfer this approach to the human autoimmune disorders, in most of which the understanding of pathogenesis is limited, represents a very considerable challenge.

Table III. *Comparison of recognition of Ac1–9 position 6 APL by different Ac1–9-specific T cell populations*[a]

Residue at Position 6	Ac1–9-Specific T Cell Population		
	Tg4.TCL	Polyclonal TCL	MBP TCR1 T cells
Ala	−	+++	+++
Arg	−	+	++
Asn	−	−	+
Asp	−	−	−
Cys	−	+	+
Glu	−	−	−
Gln	−	+	++
Gly	Antagonist	+++	+++
His	−	+++	−
Ile	Antagonist	+	+++
Leu	Antagonist	+	−
Lys	−	+	−
Met	−	+	+
Phe	−	+	−
Pro	Agonist	+++	+++
Ser	−	+	+++
Thr	Antagonist	+	+++
Trp	−	+	−
Tyr	−	−	++
Val	Agonist/antagonist[b]	+	++

[a] The response patterns of Tg4 T cells, polyclonal TCL, and MBP TCR1 transgenic T cells to position 6 Ac1–9 APL are shown. In each column, + and − represent the presence or absence of agonist activity, respectively. The position 6 APL with Tyr at position 4, which acted as antagonists of the Tg4.TCL, are also shown.

[b] The Val^6 APL with Tyr at position 4 behaved as an antagonist of Tg4.TCL at low concentrations, but was stimulatory at high concentrations. The Val^6, Lys^4 APL did not stimulate Tg4.TCL.

The Tg4 TCR was derived from an encephalitogenic T cell clone; Tg4 mice develop severe EAE when immunized with Ac1–9 (Table II and Ref. 21). The Tg4 system, therefore, allows analysis of a TCR that is relevant to disease. The Tg4 TCR can be stimulated by Ac1–9 Met^3, His^3, Tyr^3, or Phe^3 APL. Of these, Met^3 is the most potent stimulator of the Tg4 TCR. Our findings that only Met^3 was consistently able to stimulate polyclonal TCL and PLNC populations suggests that the Tg4 phenotype represents a minor proportion of the polyclonal situation. This also explains why in a previous study that defined the stimulatory position 3 APL for the 1934.4 hybridoma, only immunization with Met^3 was able to induce EAE in $H\text{-}2^u$ mice (15). The PJR-25 T cell clone from which the Tg4 TCR was derived was representative of a panel of T cell clones generated after differing immunization and stimulation protocols. All of these clones showed identical response patterns when tested with Ala-substituted analogues of Ac1–9 (13). While this previous study did not undertake the in-depth study of fine specificity used here, it did use an Ala^6 analogue, which was stimulatory for our polyclonal TCL. Therefore, any T cell clones in the previous study with specificities similar to our polyclonal TCL should have been stimulated by Ala^6. As none were, we must conclude that the previous study, for whatever reason, consistently generated T cell clones representing a fraction of the total Ac1–9-specific T cell repertoire present in a mouse. This underlines the difficulties involved in the use of T cell clones, highly selected in vitro, to determine the flexibility of T cell recognition of a given antigenic epitope.

An intriguing question is what the recognition of so many position 6 APL by our polyclonal TCL tells us about the importance of this residue to the T cell repertoire? Does this broad pattern of responsiveness imply that most Ac1–9-specific T cells recognize

most position 6 APL, or are there several subpopulations of T cells each recognizing a limited number of APL? We believe that the latter is probably the case because the response pattern of the MBP TCR1 transgenic T cells appears to represent a subset of T cells within the polyclonal population. Interestingly, the MBP TCR1 T cells responded to Gly^6, Thr^6, and Val^6, the three APL that induced EAE in normal mice. The MBP TCR1 Tg mice develop EAE spontaneously when maintained in conventional, but not germfree conditions (22), whereas the Tg4 strain develops EAE only after active immunization (21). It is interesting to speculate that this difference is a reflection of the relative fine specificities of the TCR used by each strain. The MBP TCR1 TCR can be stimulated by several APL at both positions 3 and 6 and therefore has a greater chance of being stimulated by cross-reactive Ags from commensal microbes than does the Tg4 TCR, which displays a much more limited flexibility in Ag recognition. At this stage, it is not clear whether any other residues in Ac1–9 are critical for recognition by the MBP TCR1 TCR, although Ala-substituted analogues have revealed that only Ala^3 and Ala^5 (a MHC contact residue) fail to stimulate MBP TCR1 T cells (data not shown).

Previous studies have identified APL capable of modulating EAE induced in SJL mice using the immunodominant $PLP_{139-151}$ epitope (9–11, 27). APL with appropriate substitutions at the primary TCR contact, residue 144, could antagonize some, but not all, of a panel of 139–151-reactive T cell clones. Mice that received these peptides mixed with native 139–151 developed less severe EAE than mice receiving wild-type 139–151 alone (9). A further study revealed that a panel of 139–151-reactive T cell clones, although using diverse TCR V, J, and D gene segments, showed highly focused Ag fine specificity, all recognizing residue 144 as the primary TCR contact residue. An APL altered at residues 144 and 147 (a secondary TCR contact residue) also produced effective inhibition of EAE in coimmunization experiments (10). Further analysis of 139–151 responses led to APL-induced inhibition of EAE that correlated with stimulation of a Th2 cytokine production profile (27). Similarly, APL of the immunodominant MBP (87–99) epitope in SJL mice have been reported to modulate EAE and induce Ag-specific production of IL-4 (28). Also, an APL of 87–99 that behaved in vitro as a "super agonist" could induce apoptosis of autoreactive T cells in vivo (29). APL based on known myasthenogenic peptides of the acetylcholine receptor have been shown to inhibit T cell priming with the wild-type peptides in mice and proliferation of PBL from myasthenia gravis patients (30, 31). While these results have provided evidence that TCR antagonists may be immunosuppressive tools in vivo, it is not clear whether the in vivo effects reflect the capacity to antagonize responses directly. One drawback with several of these studies is that the APL were administered, at least in some experiments, in soluble form in PBS or emulsified in IFA (9, 28, 30). A large body of evidence shows that administration of wild-type peptides in this form is a very effective means of inducing T cell unresponsiveness capable of modulating EAE (18–20, 32). Unfortunately, several studies using APL have failed to include controls using the wild-type peptides, and where these have been included, the wild-type peptide proved equally or more effective in inhibiting disease than the APL (9, 28). Therefore, mechanisms subsequent to APL administration cannot be confirmed as TCR antagonism, and the mode of application may be of greatest relevance.

An APL of $PLP_{139-151}$ known to act as a TCR antagonist in vitro has recently been reported as priming for a regulatory T cell activity in vivo that corresponded with a Th2 phenotype (11). It may be that immune intervention to induce autoreactive T cells with regulatory potential may prove a more attractive therapeutic approach than simple TCR antagonism. Antagonism, by definition, is only effective against T cells capable of interacting with the APL used, whereas activation of a suppressive T cell population can potentially down-regulate the pathogenic effects of T cells recognizing distinct autoantigens at the site of autoimmune attack. Activation of such "bystander suppression" has been demonstrated to be involved in mucosal tolerance after administration of soluble protein or peptide Ags (reviewed in Refs. 33 and 34). On this point, APL have been used to alter cytokine profiles of human T cell clones specific for MBP_{85-99}, resulting in de novo TGF-β production (35) or down-regulation of IFN-γ production (36). A theoretical problem remains with such an approach, based on the complexity of the autoimmune repertoire as described in this paper. In our system, a given APL can act as agonist and antagonist on different T cells specific for the same epitope. It follows that APL that induce production of suppressive cytokines in T cell clones in vitro may potentially activate T cells with different fine specificities in vivo to produce cytokines such as IFN-γ and TNF, which may exacerbate the disease process. On this point, human T cell clones generated using MBP_{85-99} APL have been shown to exhibit enhanced production of IFN-γ in response to wild-type MBP_{85-99} (36). Furthermore, murine Th2 clones specific for $PLP_{139-151}$ can be stimulated by a "superagonist" APL to produce IFN-γ and TNF-α (37). It is worth noting here that in our studies we have found no separation of T cell proliferation from cytokine production when using APL (data not shown).

Our antagonist experiments required the use of Ac1–9(4Y) peptides. Alterations at sites adjacent to defined TCR contacts can have subtle influences on TCR recognition (25). To negate this possibility, we defined the fine specificity of our T cell populations using wild-type Lys^4 APL. We have no definitive evidence that the Tyr^4 substitution can influence TCR recognition in a qualitative manner (rather than the quantitative stabilization of the interaction with TCR). Also, the Tyr^4 substitution was not responsible for the encephalitogenic activity seen using APL, because Lys^4 APL also induced EAE in normal mice (Table II, Expt. 2). Recent reports suggest the possibility of an antigenic peptide binding an I-A molecule in more than one register (38). This does not appear to be the case for Ac1–9, because site-directed mutagenesis studies of the Ac1–9/I-A^u interaction indicate binding in a single register (39). Thus, the complexity in fine specificity for Ac1–9 reported here is most likely a function of TCR diversity rather than of the generation of multiple peptide/MHC complexes.

Our findings that APL behaving as TCR antagonists on clonal T cell populations in vitro can have disease-inducing potential in vivo have major implications for the potential applicability of APL in human autoimmune disorders. The development of such a strategy would require generation of panels of clonal and/or polyclonal TCL from patients, identification of TCR antagonist APL in vitro, and administration of these APL in vivo. This is, in essence, the approach used in this study in a mouse model previously believed to involve oligoclonal, if not monoclonal, reactivity to autoantigen in terms of fine specificity. Using this highly selected system, we observed a surprising degree of complexity and flexibility in recognition of Ac1–9 in inbred mouse strains. There is no reason to assume that the T cell repertoire will be any less complex in the outbred human population, with T cell populations specific for a single epitope showing diverse requirements at TCR contact residues. As such, this compounds the essential problem, namely that a given APL while antagonizing one T cell may be an effective agonist for other T cells. Moreover, there is no way of determining in the human situation the relative importance of a given T cell to pathogenesis. We are, therefore, left with the dilemma that an APL, identified as an effective antagonist in vitro, could activate

other unidentified autoaggressive T cell clones in vivo with disastrous consequences for the patient.

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