

Antigen/MHC Class II/Ig Dimers for Study of Uveitogenic T Cells: IRBP p161–180 Presented by both IA and IE Molecules

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PURPOSE. Detection and modulation of effector T cells specific to immunodominant epitopes is a central issue in autoimmune diseases. Experimental autoimmune uveitis is a model for human autoimmune uveitis, induced in B10.RIII mice with interphotoreceptor retinoid binding protein or with its immunodominant epitope encoded by residues 161–180.

METHODS. The authors generated a dimer composed of p161–180 fused in frame to IA^r and mouse IgG1, and studied its effects on a CD4⁺ uveitogenic T-cell line specific to p161–180 and on a T-cell clone derived from that line.

RESULTS. Immunofluorescent staining of the T-cell line with the peptide/IA^r/Ig dimer revealed that about 90% of the cells bound the reagent, and 10% did not. The T-cell clone failed to bind the reagent. Consistent with this, the line proliferated when stimulated with the reagent plus anti-CD28, and the clone did not. Conversely, after being incubated with the reagent without CD28 cross-linking, the line showed decreased proliferation on subsequent stimulatory exposure to p161–180, whereas the clone was unaffected. Antigen-specific proliferation of splenocytes from B10.RIII mice primed with p161–180 was inhibited by anti-IA as well as anti-IE antibodies; proliferation of the T-cell line was inhibited strongly by anti-IA and poorly by anti-IE, and the clone showed the opposite pattern. Finally, the line, but not the clone, proliferated to p161–180 presented on a B-cell lymphoma expressing IA^r as its only restriction element.

CONCLUSIONS. Uveitogenic T cells can be detected as well as functionally modulated with their cognate peptide–class II reagent, suggesting the potential of such reagents for diagnostic and therapeutic use in uveitic disease; p161–180 can be presented by IA^r as well as IE^r major histocompatibility complex (MHC) class II molecules. The possibility that the same immunodominant fragment might be presented by more than one class II molecule should be taken into account when diagnostic or clinical use of peptide–MHC reagents is

considered. (*Invest Ophthalmol Vis Sci.* 2005;46:3769–3776)
DOI:10.1167/iovs.05-0187

Detection and modulation of autoreactive T cells that recognize self-antigens is an important goal in the diagnosis and therapy of autoimmune diseases. Identification and tracking of the autopathogenic T cells themselves, based on their unique antigen receptor, promises to provide critical information about disease development and progression, and possibly even help predict an individual's likelihood to develop disease. Chimeric molecules consisting of extracellular domains of major histocompatibility complex (MHC) class I and class II molecules combined with an antigenic peptide provided new tools for identification of T cells bearing the specific T-cell receptor (TCR) and modulation of their effector functions.¹ Class I MHC–Ag multimers have been used as a research and diagnostic tool for some time, but class II MHC-based reagents have lagged behind, and use of class II–self-Ag reagents has been particularly problematic.² Nevertheless, despite the problems inherent to self-antigens, of low frequency of Ag-specific cells and low binding affinity to the TCR, divalent and tetravalent chimeric molecules are beginning to be used successfully for tracking and modulation of pathogenic T cells in several autoimmune disease models, including autoimmune diabetes in the TCR transgenic BDC2.5 model^{3,4} and experimental autoimmune encephalomyelitis (EAE) induced with proteolipid protein.^{5,6}

Human cells can also be detected and modulated in this fashion. Thus, glutamic acid decarboxylase (GAD)-HLA-DR4 tetramers detect and modulate GAD-specific cells in type 1 diabetes,^{7,8} and dimeric HLA-DR2-IgG fusion protein with a bound peptide from myelin basic protein (MBP) can functionally activate human MBP-specific T cells.⁹ Importantly, in the absence of costimulation, TCR engagement by the chimeric molecule renders the T cells anergic to a subsequent stimulation by peptide-pulsed antigen-presenting cells (APCs).⁹

Experimental autoimmune uveitis (EAU) is an important animal model for human autoimmune uveitis. The EAU model in B10.RIII (H-2^r haplotype) mice is induced by immunization with interphotoreceptor retinoid binding protein (IRBP). A major pathogenic epitope of the IRBP molecule for B10.RIII mice is encoded within residues 161–180.¹⁰ Immunization with this peptide, or adoptive transfer of CD4⁺ T cells specific to p161–180, elicits EAU that is as severe as that induced by the whole IRBP molecule. The objective of the present study was to generate and characterize a soluble peptide–MHC class II chimeric molecule based on this immunodominant epitope, as a reagent for detection and modulation of uveitogenic T cells in this model. We chose to engineer the construct as a dimer on an IgG backbone, for ease of production and purification, and to link the peptide to the MHC element covalently, for maximal compound stability.

We report here that an engineered 161–180/IA^r/Ig dimer, produced in insect cells, binds to a uveitogenic 161–180-specific T-cell line, as determined by FACS analysis. This binding

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Supported by the Intramural Research Program of the National Institutes of Health, National Eye Institute, Bethesda, Maryland.

Submitted for publication February 11, 2005; revised May 26 and June 18, 2005; accepted August 22, 2005.

Disclosure: **Z. Karabekian**, None; **S.D. Lytton**, None; **P.B. Silver**, None; **Y.V. Sergeev**, None; **J.P. Schneck**, None; **R.R. Caspi**, None

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has functional consequences, which—depending on the presence of costimulation—can be either proliferation or anergy. Use of this reagent revealed that the pathogenic 161–180 fragment can be presented to specific T cells by IA^r as well as by IE^r molecules, which may help to explain its high pathogenicity. The ability to negatively modulate a T-cell line, which represents a mature effector-cell phenotype, suggests that this type of reagent has the potential to affect autopathogenic T cells in ongoing disease.

MATERIALS AND METHODS

Mice

Female B10.RIII mice (H-2^f) were purchased from Jackson Laboratories (Bar Harbor, ME) at 5 to 6 weeks of age. The mice were kept in a specific pathogen-free facility and used between 6 and 8 weeks of age. The use of laboratory animals conformed to institutional guidelines and to the provisions of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents and Cells

Human peptide 161–180 (sequence SGIPYIISYLHPGNTILHVD), encoding a major pathogenic T-cell epitope present in the first homologous repeat of the IRBP, was synthesized on a peptide synthesizer (Model 432A; Applied Biosystems, Foster City, CA) using Fmoc chemistry. Peptide 161–180 encodes a dominant pathogenic IRBP epitope for the H-2^r haplotype.¹⁰ B10.RIII splenocytes to serve as antigen-presenting cells were obtained from naïve mice. Splenocytes primed to p161–180 were obtained from mice immunized subcutaneously (in both thighs and base of tail) 2 weeks earlier with 25 µg p161–180 emulsified in 0.2 mL complete Freund's adjuvant supplemented to 2.5 mg/mL with *Mycobacterium tuberculosis* strain H37RA. The derivation of the uveitogenic Th1 line specific to p161–180 has been described earlier.¹⁰ This T-cell line reliably elicits EAU in naïve recipients on infusion of 0.5 million or more cells. The Th1-cell clone was derived from that line by limiting dilution cloning and is pathogenic on infusion of 3 million or more cells (Silver PB, unpublished data, 1996). Both the line and the clone were maintained by alternating cycles of stimulation with 1 to 2 µg/mL p161–180 in the presence of syngeneic APCs (splenocytes irradiated with 2500 rad) and expansion in IL-2-containing medium (100 U/mL) every 2 to 3 weeks. B-cell lymphoma transduced with IA^r was kindly donated by Edward Rosloniec (VA Medical Center, Memphis, TN).¹¹ Subclones, positive and negative sublines for expression of the IA^r molecule, were derived by single-cell cloning and used as APCs in the specified experiments. Monoclonal antibodies anti-IA^k (clone 10.2.16), anti-IA^q (clone KH118), anti-IA^p (clone 17.16.17), and anti-IE^k (clone 14-4-4S or clone 17.3.3) were purchased from Pharmingen (BD Pharmingen, San Diego, CA), and anti-IA public epitope (1:10 diluted Y-3P hybridoma supernatant)¹² was generously provided by Ronald Germain (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). All these anti-IA and anti-IE antibodies cross-react with their H-2^f class II equivalents.

FACS Analysis by Indirect Immunofluorescence for p161–180-Binding Cells

Rested p161–180-specific T cells (line or clone) were washed from IL-2-containing media, and 1 × 10⁶ cells per sample were incubated with 1 µg CD16/CD32 antibody (BD Pharmingen) for 10 minutes at 4°C to block nonspecific binding to the Fc receptor. The cells were then incubated with 3 µg purified dimer (161–180/IA/Ig) or irrelevant class I/Ig-restricted reagent (unloaded anti-H2-K^b-Ig¹³) in 100 µL FACS buffer (0.5% BSA/PBS and 0.05% sodium azide) for 1 hour at room temperature. Cells were washed twice in FACS buffer and stained with PE conjugated anti-mouse Ig antibody (BD Pharmingen) for 30 minutes on ice. Cells then were washed and suspended at 1 × 10⁶ cells/mL, and

10,000 live cells per sample were analyzed on a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ) using CellQuest software (BD Biosciences, San Jose, CA).

Genetic Construction of Soluble Class II/Ig Chimeric Dimers

The MHC class II IA^r-α and IA^r-β cDNAs were obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) of mRNA derived from splenocytes of B10.RIII mice with oligo dT primers (Clontech Inc, Palo Alto, CA). The truncated versions of MHC class II IA^r-α and -β genes were subsequently generated by PCR using primers designed to introduce a cloning site at the 5' (*Xba*I for α and *Eco*RI for β) and the 3' (*Hind*III for α and *Kpn*I for β) ends. The genes were truncated just before the transmembrane region (C-terminal amino acid Trp 182 for α and Arg 217 for β chains, respectively) and nucleotide sequences encoding the chimeric Ig protein linkers were introduced: G₃T was introduced at the junction between the 3' end of the IA^r-β and the mature mouse IgG1 heavy-chain constant regions and GSL at the 3' end of the IA^r-α and constant region of Igκ light chain.

The DNA sequence for the uveitogenic peptide 161–180 (SGIPYIISYLHPGNTILHVD) linked to a flexible spacer (G)₄S(G)₄S was inserted between the native β-chain leader and the IA^r-β1 domain (first external domain of the mature IA-β protein) by combination of an overlap extension PCR and cassette-cloning strategy.^{14,15}

The β-chain specific oligonucleotides used to construct 650-bp fragment *Eco*RI-LP-pep161–181 G₃T Ia^r were as follows: 5' sense primer *Eco*RI oligo 1, ggaatccatggctctg; 3' antisense primer oligo 2, cccgggtgcaggtaggagatgatgtacgggataccggaggagttccgcctcag; 3' antisense primer *Bam*HI oligo 3, cgggatccaccggatccaccacctccatcaacgtgcaggatggtgttaccgggtgcag; 3' antisense primer *Kpn*I oligo 4, gaat-tgcccttggggtagctctcccctccaccacagtgatggg; and 5' sense primer *Bam*HI oligo 5, ggtgatccgggtggagggggaagtgagggtggagggtctgaaagg-cattctgtggcc.

Overlapping sequences of oligonucleotides oligo 2 and oligo 3 are italicized. The oligo 2 was used at 10-fold less molar concentration than *Eco*RI oligo 1 and *Bam*HI oligo 3 with alternating cycles of low (6 × 40°C) and high (22 × 64°C) annealing temperatures as described previously.¹⁵ The IA^r-β-chain PCR products were inserted in frame into the TOPO-TA cloning vector (Invitrogen, Carlsbad, CA) using the fragment of oligos 1 to 3 into *Eco*RI and *Bam*HI sites and the fragment of oligos 4 to 5 into the *Bam*HI and *Kpn*I sites. The IA^r-α-chain specific oligonucleotides were as follows: 5' sense primer *Xba*I Ia^r-α, ccgctc-gagcgggaagacagcattgaggccgaccag; and 3' antisense primer *Hind*III Ia^r-α, taagcttcccagtggttcagaaccggctcc; italicized nucleotides encode the GSL for linkup to Igκ chain. To make the IA^r-α chain compatible for chimeric IgG, the internal *Hind*III site was obliterated in the TOPO-TA vector by Quick-Change site-directed mutagenesis kit using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) and 5' sense primer agtttg-gccaattgactgactttgaccccca and 3' antisense primer tcaaacgggttaactgatc-gaaactgggggt.

After subcloning of the IA^r-α into *Xba*I and *Hind*III sites of pSP72 (Promega Corporation, Madison, WI) (Fig. 1a) and IA^r-β into *Eco*RI and *Kpn*I sites of pSP73 (Promega) (Fig. 1b), the inserts of 500-bp IA^r-α-Igκ and 750-bp IA^r-β-IgG1, respectively, were excised from the chimeric Ig cassettes and assembled into the modified pAcUW51 baculovirus expression vector (pZlg) described previously.¹ The full-length sequences of chimeric genes in pZlg were obtained using IA^r-β- and IA^r-α-chain specific primers and confirmed to existing IA^r-β and IA^r-α sequences in gene bank. The chimeric gene constructs were tested in *in vitro* transcription-coupled reticulocyte lysates (TNT T7; Invitrogen) and encoded proteins of correct molecular mass were determined by analysis of ³⁵S-labeled proteins in SDS-PAGE autoradiograms (data not shown). Expression of the recombinant MHC class II/Ig chimeric protein in baculovirus infected Hi-Five insect cells was done as previously described.¹

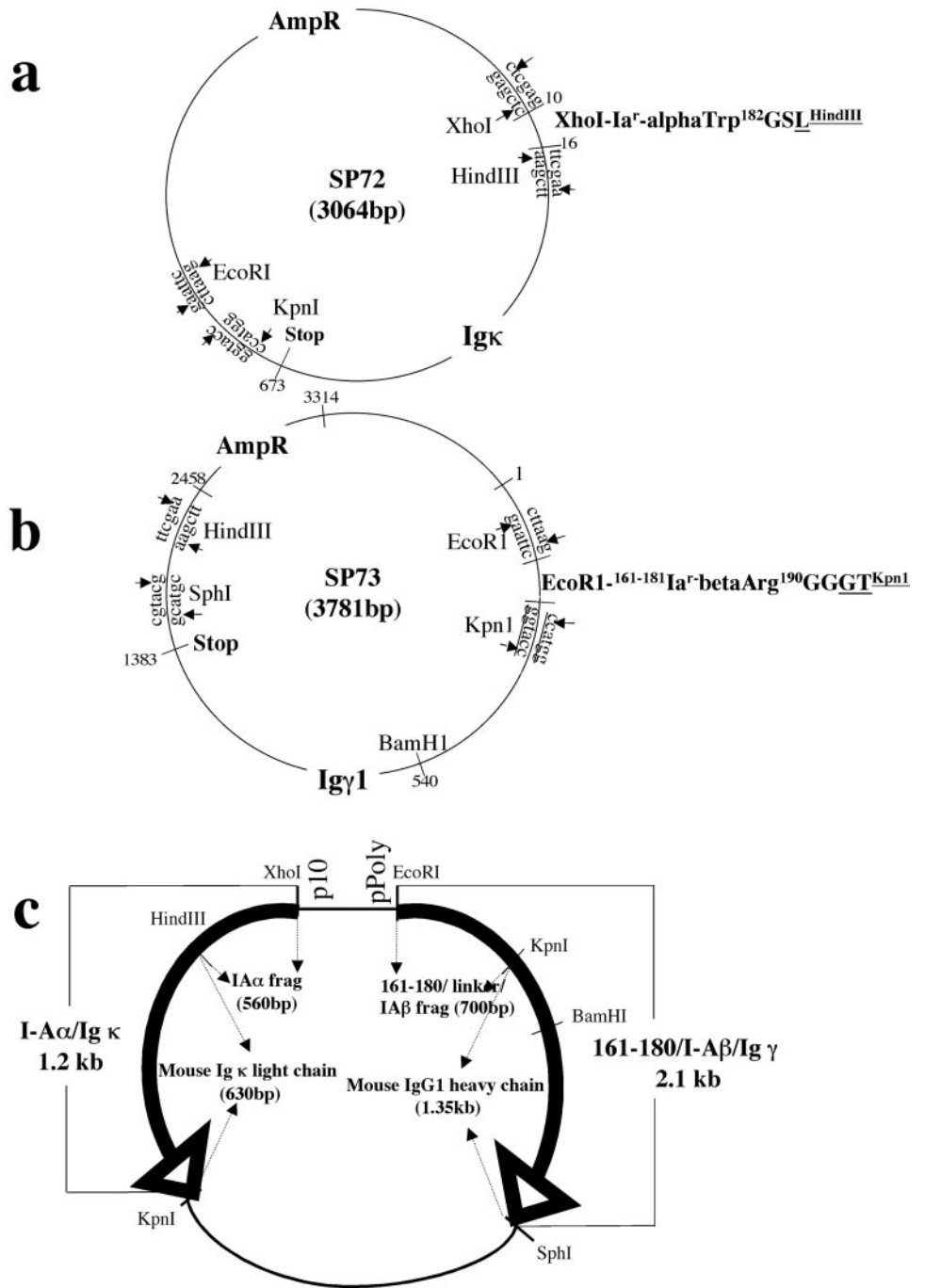


FIGURE 1. Schematic representation of genetic construction of 161-180/IA/IgG dimer. Details of the construction are described in the text. The sequences and orientation of the primers used for the cloning are indicated by arrows for (a) α and (b) β chains, respectively. (c) Both chains were sequenced and cloned in the final dual-expression vector pAcUW51.

Protein Production and Purification

The 161-180/IA/Ig dimeric molecule was produced in a baculovirus insect-cell expression system. Hi-Five cells were grown in HyQ SFX-Insect (HyClone, South Logan, UT) or Express Five SFM (Gibco, Carlsbad, CA) media and were infected in the exponential stage of growth at the concentration of 1.5×10^6 cells/mL. Cells were inoculated with a multiple of infection of 10 in a total volume of 1 L in the presence of leupeptin (0.5 μg/mL). Infected cells were incubated at 27°C with constant rotation (1200 rpm) for 5 to 6 days until 70 to 80% of the cells were dead. The culture supernatant was centrifuged (1000g for 30 minutes), filtered (0.4-μm filter; Millipore, Billerica, MA), and then dialyzed for 24 hours at 4°C in PBS and 24 hours in binding buffer (3M NaCl, pH 8–9) supplemented with leupeptin (0.5 mg/mL). To achieve optimal binding of mouse IgG1 Fc portion to protein A, the salt and

acidity of dialysate was adjusted to 3M NaCl, pH 8 to 9. The 161-180/IA/Ig dimeric molecule was affinity purified by passage of 700 mL of dialyzed supernatant over a 5-mL HiTrap rProtein A FF column (Amersham Biosciences, Piscataway, NJ). The bound material was washed and eluted according to manufacturer’s instructions. The column eluate was centrifuged and concentrated on Centriprep YM-10 membrane (Millipore). Protein quality was determined by Western blotting (see below), and the protein concentration was determined by the BSA protein assay (BioRad, Hercules, CA).

Western Blot Analysis

The quality of purified dimer was tested by Western blot analysis. Three micrograms of purified dimer (161-180/IA/Ig) was denatured (or not) by boiling for 5 minutes in SDS and β-mercaptoethanol (2-ME)

containing buffer, and SDS-PAGE was performed in a 12% gel (Gradipore, New York, NY) according to manufacturer's instructions. Samples were electrophoresed for 60 minutes at 150 constant V, then electrotransferred to a nylon membrane, and blocked in 5% nonfat dry milk (BioRad) in Tween-Tris-buffered saline overnight at 4°C. Membranes were incubated with two different primary antibodies, goat anti-mouse IgG or anti-IA^b biotin, for 1 hour and detected with either anti-mouse IgG-HRP or Streptavidin-HRP, respectively. The blots were developed using the ECL Plus kit (Amersham Biosciences).

Lymphocyte Proliferation Assay

For antigen-specific lymphocyte proliferation, 2.5×10^5 line or clone T cells specific for 161-180 peptide were seeded in triplicate into round-bottom 96-well plates and stimulated with 1 μg/mL 161-180 peptide, in the presence of irradiated (3000 rad) B10.RIII splenocytes (2.5×10^5 per well) as APCs in a total volume of 200 μL. Alternatively, irradiated B-cell hybridoma (10,000 rad, 5×10^4 cells per well), positive or negative for expression of H-2^f IA molecule, were used as APCs in the presence of anti-CD28 antibodies (clone 37.51, 100 μg/mL) (Harlan Laboratories, Indianapolis, IL) as a co-stimulation signal. In experiments where 161-180-specific T cells were simulated with 161-180/IA/IgG dimer, anti-CD28 antibodies were added at final concentration of 25 μg/mL. In some experiments, IRBP-primed B10.RIII lymph node cells (pool of 5 mice, 5×10^5 cells per 0.2-mL well) stimulated with the specified dose of p161-180 were used. After 48 hours of culture, [³H]thymidine (1 μCi/well) was added for 18 hours. The cultures were harvested on a PhD harvester (Cambridge Technology, Cambridge, MA) and counted by liquid scintillation (Perkin Elmer, Shelton, CN). In some experiments, anti-class II monoclonal antibodies (described in Reagents and Cells, above) were used to block MHC-dependent T-cell proliferation at 20 μg/mL final concentrations.

Induction of T-Cell Anergy

T-cell anergy was induced by treatment of T cells (161-180-specific T-cell line and T-cell clone) with soluble 161-180/IA/Ig dimer (20 μg/mL). T cells were cultured with these molecules in the presence of IL-7 (5 ng/mL, to maintain maximal viability) without IL-2 for 4 days in 24-well plates. They were then washed and counted, and their ability to proliferate to p161-180 under stimulatory conditions was compared to that of parallel T cells maintained during that time in expansion medium. The T-cell proliferation assay was performed as described above using irradiated splenocyte as APCs and 0.1 to 10 μg 161-180 peptide/mL.

RESULTS

Construction of Soluble IA^f-Ig Molecules with Covalently Bound Autopathogenic Peptide

The major pathogenic fragment of IRBP for B10.RIII mice (H-2^f) is encoded by residues 161 to 180 of IRBP. From previous data generated in our laboratory (Rizzo and Caspi, unpublished data, 1999) we had evidence that anti-IA antibodies cross-reactive with IA^f (there are no antibodies generated to the actual IA^f molecule) inhibited antigen-driven proliferation of B10.RIII T cells to p161-180 to a variable extent. This suggested that IA^f could be the restricting element of p161-180. To generate a stable chimeric molecule that will bind specifically to the 161-180 T-cell receptor, we used the approach described previously¹ and modified the background cloning plasmid (see Materials and Methods). In principle, the cognate peptide was covalently linked to the β chain of the IA^f molecule and the constant region of the mouse IgG1 molecule. The α chain of the dimer was designed to consist of the α chain of IA^f fused to the constant region of κ light chain of mouse Ig. The genes encoding the IA^f-β and -α chains were obtained by RT-PCR from mRNA of B10.RIII spleen cells, and the sequence

coding for 161-180 peptide was introduced upstream of the IA^f-β cDNA sequence. This fragment was then ligated into SP73 vector in frame with mouse IgG1 heavy-chain Fc-portion coding sequence. IA^f-α-chain/IgG-κ chimera was generated by cloning of IA^f-α cDNA sequence into SP72 in frame with mouse IgG1 light-chain Fc-portion coding sequence. The molecular model of the construct is shown in Supplementary Figure S1, available online at <http://www.iovs.org/cgi/content/full/46/10/3769/DC1>. Both sequences were confirmed by sequencing analysis and, for IA^f-α and -β chains, were excised from their subcloning vectors and cloned into pZIg¹ (Fig. 1) baculovirus dual-expression vector under p10 or polyhedron promoters, respectively. The quality of the purified reagent was confirmed by Western blot analysis with anti-mouse Ig antibody and with cross-reactive anti-IA antibody, which showed a single band.

Indirect Immunofluorescent Staining of 161-180-Specific T Cells with 161-180/IA/IgG Dimer

To test whether the reagent we produced would bind to 161-180-specific T-cell receptors, a highly uveitogenic T-cell line specific to p161-180 was reacted with the 161-180/IA^f/Ig dimer (1 million cells with 3 μg reagent), stained with anti-Ig-FITC, and analyzed by flow cytometry. Two distinct staining patterns emerged: approximately 90% of the cells stained strongly with the reagent (two positively staining subpopulations were apparent), and up to 10% of the cells did not appear to bind the reagent (Fig. 2a). A clone that had been derived from that line by single-cell cloning and was likewise maintained in culture by periodic stimulation with p161-180 failed to bind the reagent, similarly to the minority of the line cells (Fig 2b). Because the nonstaining population had persisted in the line for many passages and had apparently given rise to the (also nonstaining) clone, these cells must have a TCR specific to p161-180, even though they failed to bind the peptide presented on IA^f. This raised the possibility that the negative population would recognize p161-180 on IE^f, which is the other class II restricting element in B10.RIII mice.

Functional Effects of 161-180/IA/IgG Dimer on p161-180-Specific T Cells

We next examined whether binding of the 161-180/IA/IgG dimer to the TCR of p161-180-specific T cells would have functional consequences in terms of inducing either stimulation or anergy. We first incubated the T-cell line and clone with the reagent in the presence of anti-CD28 Ab to provide co-stimulation. In keeping with the staining results, the T-cell line proliferated significantly when stimulated with the dimer, whereas the clone did not proliferate above background levels (Fig. 3). This indicated that binding of the reagent to the TCR was able to provide signal 1, which, in conjunction with the signal 2 provided by cross-linking of CD28, led to productive stimulation of the T-cell line.

We next incubated the T-cell line and clone with the reagent in the absence of anti-CD28 antibodies. Because T cells require signal 1 and signal 2 for productive activation, and because exposure to signal 1 alone often results in anergy, we expected that exposure of 161-180-specific T cells to the reagent in the absence of co-stimulation should inhibit their ability to respond to p161-180 under stimulatory conditions. Indeed, after the 161-180-specific line was incubated for 4 days with the 161-180/IA/IgG dimer, its ability to proliferate when subsequently stimulated with p161-180 on splenic APCs was reduced almost threefold compared to control cells that were not exposed to the reagent. Again in keeping with the reagent binding data, the clone did not demonstrate any changes in proliferative ability under these conditions (Fig. 4a and 4b).

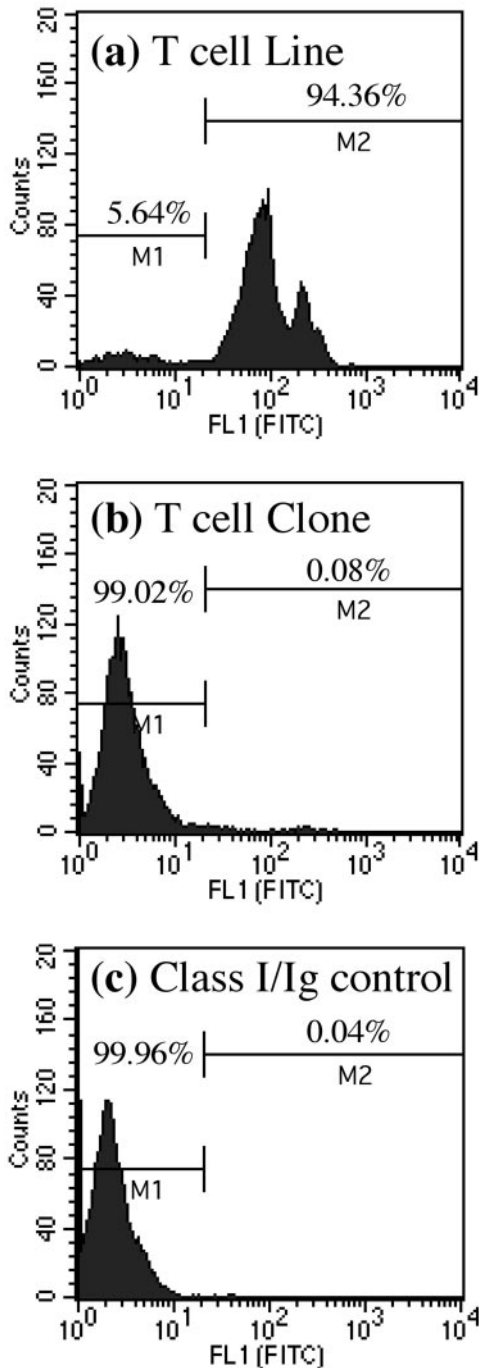


FIGURE 2. 161-180-Specific T cells stain differently with 161-180/IA/IgG dimer. One million rested T cells were incubated with 3 μ g purified dimer or irrelevant control class I/Ig molecule for 1 hour at room temperature. FITC conjugated anti-mouse Ig antibody (BD Pharmingen, San Diego, CA) was used as a secondary antibody at the final concentration of 1 μ g/million cells for 30 minutes. FACS analysis: (a) T-cell line; (b) T-cell clone; (c) T-cell line with control irrelevant class I dimer.

Determination of the MHC Class II Restriction Element Presenting 161-180 Peptide of IRBP

The results above suggested the possibility that p161-180 can be presented by IA^r as well as by IE^r molecules, which are both expressed by B10.RIII mice. To study the restriction requirements for p161-180 presentation more directly, we took two

complementary approaches: inhibition of presentation by anti-IA and anti IE monoclonal antibodies, and use of modified APCs expressing IA^r as their only restriction element.

For the first approach, B10.RIII mice were immunized with p161-180, and cells from their draining lymph nodes were harvested 2 weeks later and were stimulated in culture with p161-180 in presence or absence of blocking anti-class II antibodies. Because anti-IA^r or -IE^r antibodies are not available, we used antibodies to IA^k and IE^k that are known to cross-react with their H-2^r equivalents. The results showed that proliferation of freshly explanted 161-180 primed cells was blocked both by anti-IA^k and -IE^k antibodies (Fig. 5a). Only anti-IA antibodies were able to markedly block proliferation of the T-cell line, whereas only anti-IE antibodies could block proliferation of the T-cell clone to p161-180 presented on syngeneic splenocyte APCs (Fig. 5b and 5c). Antibodies to IA^p, IA^q, and a public IA epitope (Y3P) had the same effects as anti-IA^k (data not shown). Notably, none of these anti-class II antibodies interfered with phytohemagglutinin-induced stimulation, which is not dependent on class II presentation, supporting the notion that their inhibitory effects are indeed due to functional blocking of IA and IE elements, rather than to some nonspecific toxicity of the antibody preparations (not shown).

For the second approach, the 161-180-specific T-cell line and T-cell clone were stimulated with p161-180 peptide presented by B cells from a lymphoma line transduced with IA^r, which express this molecule as the only H-2^r restriction element. Control cells were IA^r-negative lymphoma cells. Under these conditions the T-cell line showed a good proliferative response to p161-180, whereas the clone failed to proliferate, supporting the conclusion that IE^r is the restricting element for that clone (Fig. 6).

In the aggregate, the results presented above strongly support the notion that p161-180 is presented by both IA^r and IE^r molecules, possibly helping to explain the strong immunodominance of this peptide.

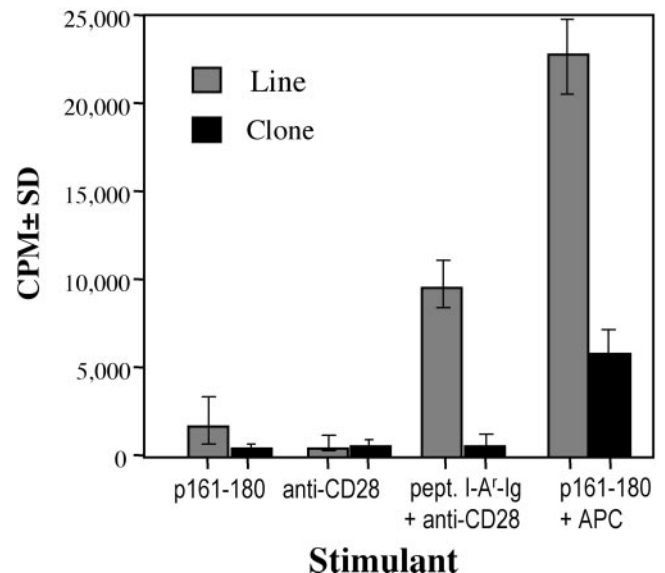


FIGURE 3. Proliferation of 161-180-specific T cells to the 161-180/IA/IgG dimer correlates with their binding pattern. The T-cell line and clone were stimulated with 161-180/IA/IgG dimer in the presence of anti-CD28 antibody (5 μ g/well) to provide for the missing co-stimulatory signal. Positive control was peptide 161-180 (1 μ g/mL) presented on syngeneic APCs (irradiated splenocytes). Triplicate cultures of 2.5×10^5 cells per well of each cell type were incubated with 30 μ g purified 161-180/IA/IgG dimer for 48 hours, then pulsed with H³ thymidine.

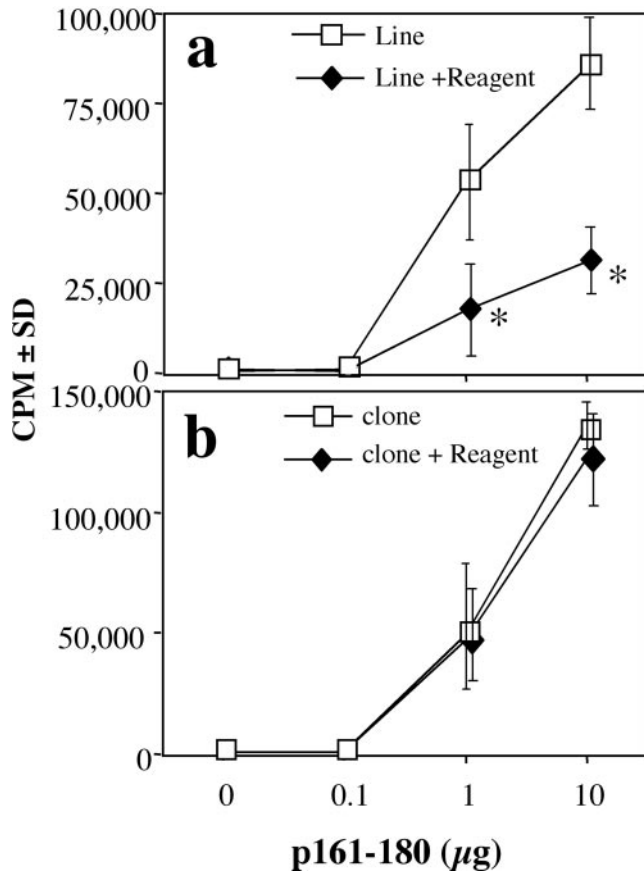


FIGURE 4. Interaction of 161-180/IA/IgG dimer with 161-180-specific T-cell line can induce anergy. 161-180-specific T cells were incubated with 161-180/IA/IgG dimer for 4 days at the 20- $\mu\text{g}/\text{mL}$ final concentration in the absence of other co-stimulatory molecules. On day 5, cells were washed and triplicate cultures of each cell type were stimulated with 0.1, 1, or 10 $\mu\text{g}/\text{mL}$ 161-180 peptide presented by syngeneic B10.RIII splenocytes. Proliferative responses of T-cell line (a) and T-cell clone (b) are shown. *Significant reduction in proliferation compared with control ($P < 0.05$, *t*-test).

DISCUSSION

Various types of antigen-MHC class II chimeric molecules have been used in the past decade as a powerful tool to detect Ag-specific T cells. Many different fusion protein designs, such as single-chain,¹⁶ dimers,^{1,17} tetramers,¹⁸ or higher-valency multimers,¹⁹ were developed and tested for T-cell identification and effector function modulation. In addition, for different scaffolds these chimeric molecules can be either synthesized as "empty" MHC molecules and loaded in vitro, which allows flexibility to introduce multiple epitopes,²⁰ or expressed with covalently bound cognate peptide that stabilizes the molecule.^{14,21} However, in the case of autoantigens, T cells with the highest affinity to the self-antigens are eliminated by negative selection during thymic maturation. Therefore, we decided to make a construct with covalently bound immunodominant peptide, to offset the possibility that 161-180-specific T cells may bear low-affinity TCRs²² and to maximize the stability of the synthesized fusion protein. This strategy proved successful and resulted in a reagent that specifically binds to p161-180-specific uveitogenic T cells. Furthermore, it has helped reveal that this antigenic fragment may be presented in the context not only of IA but also of IE molecules. Thus, the chimeric molecule described in this report represents a novel tool for

the study of IA^r-restricted T cells specific for the major immunodominant uveitogenic epitope of IRBP, peptide 161-180.

Utilization of 161-180/IA/IgG dimer for FACS staining of the 161-180-specific T-cell line detected an interesting pattern (Fig. 2). There were two distinct IA^r-restricted populations that both stained positively. T-cell lines over time tend to become oligoclonal as the cells best adapted to culture conditions undergo selection. Thus, these may be two major clonotypes that have taken over, one with a higher and the other with a

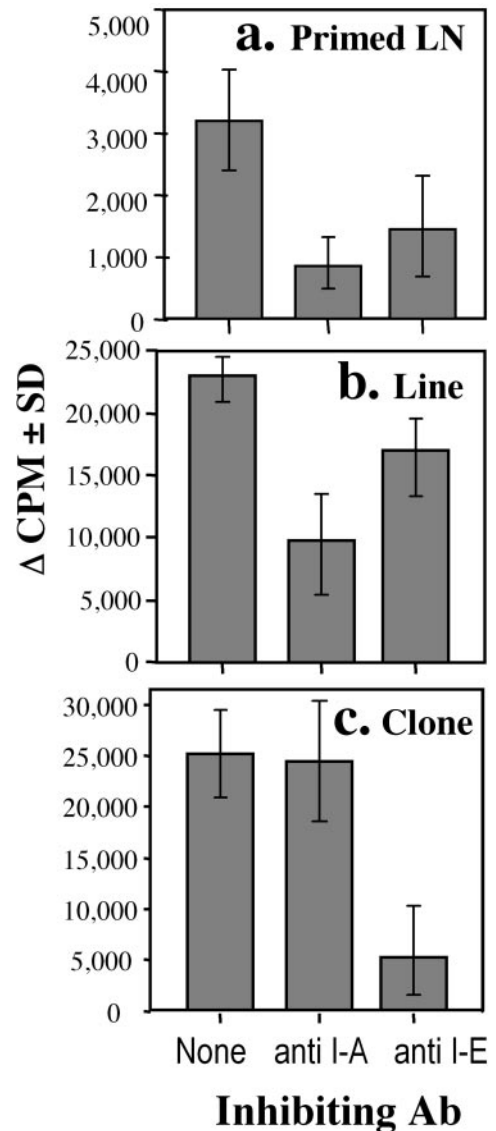


FIGURE 5. Anti-MHC class II antibodies cross-reactive with IA^r and IE^r block proliferation of primed B10.RIII lymph node cells and 161-180-specific T cells. (a) B10.RIII mice were immunized with 10 μg 161-180 peptide in complete Freund's adjuvant. Two weeks after immunization, lymph node cells were harvested and 5×10^5 cells were stimulated in vitro with the peptide (0.3 μM) in the presence or absence of anti-IA^k or anti-IE^k, which cross-react with their H-2^r equivalents. (b) 161-180-specific T-cell line (2.5×10^5 cells per well) was stimulated with the peptide (1 $\mu\text{g}/\text{mL}$) presented by 2.5×10^5 cells per well syngeneic splenocytes in the presence or absence of the indicated antibodies. (c) T-cell clone, cultured as described for the line. Shown are cpm after subtraction of background (500–2000 cpm). Responses to phytohemagglutinin (1 $\mu\text{g}/\text{mL}$) were approximately 24,500, 28,000, and 24,000 cpm for the lymph node, line cells, and clone cells, respectively, and did not change by >10% up or down in the presence of the antibodies.

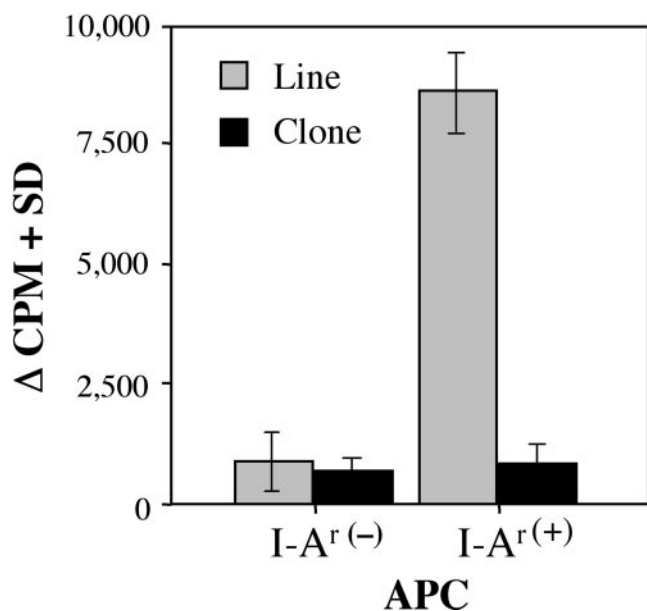


FIGURE 6. Only the 161–180-specific T-cell line proliferates to antigen presented on IA^r-expressing B cells. 161–180-Specific long-term and short-term T-cell lines (2.5×10^5 cells per well) and T-cell clone were stimulated with 161–180 peptide presented by IA^r-positive or -negative B-cell lymphoma (2×10^4 cells per well). Anti-CD28 (5 μ g/well) antibody was added to the cultures to supply missing co-stimulatory signal. Shown are counts after subtraction of background (500 to 700 cpm).

lower binding affinity. If this is indeed the case, it suggests that the reagent can be used as a semiquantitative measure of receptor affinity and/or density. The binding was stable and the same pattern of staining was detected after 2 hours incubation at room temperature (data not shown). In addition, a negative population of cells was detected, which we believe to be IE^r restricted, because these cells could not have persisted in the line for many passages without being able to respond and proliferate to antigen; proliferation of the line to Ag was inhibited (albeit weakly) by anti-IE Abs; and the T-cell clone, which is derived from the line, appears IE restricted. The conclusion of IE restriction of the clone is supported by its inability to proliferate in response to stimulation with 161–180/IA/IgG dimer (Fig. 3), as well as failure to be anergized by this reagent (Fig. 4). Moreover, the T-cell clone lacked the ability to proliferate to B-cell APCs that express IA^r as their only relevant class II molecule, although it did proliferate in the presence of splenic APCs, which express both restriction elements. Finally, only anti-IE antibodies were able to block proliferation of the clone to p161–180 (Fig. 5). We are currently working on generation of a 161–180/IE/IgG dimer, so that we will have reagents able to bind and modulate both the IA- and the IE-restricted 161–180-specific T-cell populations.

The novel reagent described here should be very useful to follow and manipulate autoreactive 161–180-specific T cells in the B10.RIII mouse EAU model, which serves as an important model for understanding human uveitis. We are aware that good binding of the reagent to the T-cell line may be a best-case scenario, because cells with the highest TCR may have been selected over time, and many of the 161–180-specific cells in vivo may have a lower affinity/avidity. In this case, the avidity of the reagent can be increased by further multimerizing the dimer. One of these strategies has been to produce tetrameric reagents by multimerization of biotinylated monomers with streptavidin.⁶ Although this approach has been by far the most popular, higher-order multimers to generate class II-peptide

reagents with more avidity are also possible, such as multimerization on a virus capsid, or on agarose beads, or generation of aggregates.²³

MHC class II multimers are already being used for diagnostics in human disease.^{7,24,25} Although these molecules have not yet been used for in vivo immunomodulation in humans, they have been applied in vivo in animals,⁴ and have been shown to modulate human cells in vitro.^{8,26} The retinal antigens relevant to autoimmune uveitis in humans are not yet conclusively defined. It is believed, however, that retinal arrestin (i.e., retinal soluble antigen [S-Ag]) is one of the proteins involved, because many uveitis patients have responses to retinal arrestin.^{27,28} In a recent double-blind placebo-controlled oral tolerance trial, patients were fed retinal S-Ag and the treatment appeared to show efficacy.²⁹ Notably, we recently showed that one of the S-Ag epitopes recognized by human uveitis patients is also recognized as an immunodominant epitope by HLA-DR3 transgenic mice and elicits typical uveitis when presented on human class II molecules³⁰ (Karabekian Z, et al., unpublished data, 2004). This supports the notion that retinal antigen-specific cells are involved in human uveitis, and points to the utility of retinal antigen-specific reagents to identify and track these T cells. Our present data, showing that binding of the peptide/IA^r/Ig dimer to its specific TCR has functional consequences on the uveitogenic T cell, including what appears to be induction of anergy, suggest that in the future such reagents might be useful to modulate T cells in disease.

Although both IE^r and IA^r are presenting p161–180, we do not know if those are two different epitopes, possibly partly overlapping, or the same sequence binding at a different register. The ability to be presented by more than one MHC molecule might help to explain the highly pathogenic nature of this peptide, since it is able to be presented to a broad population of T cells. We speculate that this might also be the case with the immunodominant peptide *n* (281–300), which represents a promiscuous epitope of S-Ag. Humans typically have several class II molecules as well as different allotypes that are able to present self-antigens by different MHC class II molecules³¹; therefore, when considering use of peptide-MHC class II reagents diagnostically or clinically, the possibility must be taken into account that the same immunodominant fragment might be presented by more than one MHC molecule.

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