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Detection of autoreactive T cells in H-2^u mice using peptide–MHC multimers

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Abstract

Myelin basic protein (MBP)-specific T cells play a critical role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), a prototype for T cell-mediated autoimmunity. In PL/J and B10.PL mice (H-2^u haplotype), the immunodominant epitope of MBP is represented by an N-terminal nonameric peptide, MBP1–9. To date, the MBP1–9-specific T cell repertoire has not been analyzed in quantitative terms. In the present study we demonstrate, using MHC class II tetramers, that 15,000–70,000 self-antigen-specific T_h cells accumulate in the draining lymph nodes following immunization with spinal cord homogenate or MBP1–9. In contrast, MBP1–9-specific T cells are undetectable in unimmunized H-2^u mice and represent >60% of the CD4 cells in naive mice transgenic for a TCR specific for this epitope. The results suggest that the extremely low affinity of the N-terminal peptide for I-A^u does not limit the MBP1–9-specific T cells from expanding into a sizeable pool of autoreactive T cells. Therefore, the primary immune response to MBP1–9 does not differ quantitatively from previously reported CD4$^+$ T cell responses to foreign antigens.

Introduction

Murine experimental autoimmune encephalomyelitis (EAE) is believed to be a representative model of T cell-mediated autoimmune disease and, more specifically, of multiple sclerosis (reviewed in 1). In H-2^u mice, the immunodominant epitope of MBP is represented by an N-terminal peptide, MBP1–9 (2), that binds to its restricting element, I-A^u, with low affinity (3,4). This weak interaction has been suggested to result in the escape of autoreactive T cells from tolerance induction (3,5). Taken together with recent data indicating that MBP peptides with higher affinity for I-A^u induce negative selection of cognate T cells (6,7), this might explain the encephalitogenic potential of this N-terminal peptide. Despite the extensive use of this peptide to induce EAE, little is known about the frequency of MBP1–9-specific T cells in naive or immunized mice. In this respect, multimeric peptide:MHC complexes have recently proved to be valuable reagents for 'counting' and characterizing antigen-specific T cells, especially for class I-restricted responses (reviewed in 8). In contrast, there appears to be a paucity of data involving the application of such complexes for CD4 T cells (9–13). For mouse models of autoimmunity, this might be due to the relative instability of peptide:I-A complexes associated with some of the commonly used models (3,5,7,14,15). By combining the use of acidic/basic zippers to stabilize $\alpha\beta$ chain association together with the covalent tethering of antigenic peptide, we recently described the expression of a functional, recombinant I-A^u molecule complexed with the N-terminal 11mer of MBP (16). In the current study we have produced these recombinant molecules in multimeric form and assessed their ability to bind diverse encephalitogenic T cell populations. We have also analyzed the frequency of MBP1–9-specific T cells in transgenic mice expressing a MBP-specific TCR and in MBP1–9- or spinal cord homogenate (SCH)-immunized non-transgenic animals. Our results demonstrate that a very low number of MBP1–9-specific T cells in naive mice (below the detection limit of our method) is efficiently expanded by autoantigen immunization, despite the low affinity of MBP1–9 for I-A^u.

Methods

T cell hybridomas and transfectants

The 1934.4 T cell hybridoma (17) is specific for the N-terminal 11mer (or nonamer) of MBP bound to I-A^u. P1.D2 T cells were

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generated by transfecting the 1934.4 TCR genes into a TCR^-/$CD4^-$ cell line ($58\alpha^-\beta^-$) [as described elsewhere (18)]. The DO11.10 T cell hybridoma (19) is specific for residues 323–339 of ovalbumin presented by I-A^d, and was a generous gift from Drs J. Kappler and P. Marrack (University of Colorado Health Sciences Center, Denver, CO). These cells were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies), penicillin/streptomycin (100 U/ml) and 2 mM L-glutamine.

Mice, antigens, immunization protocols and generation of T cell lines

The Tg4 transgenic mouse expressing the 1934.4 TCR has been described previously (20). Tg4, B10.PL and B10.PL×SJL/J (H-$2^{u\times s}$) F_1 mice were bred under specific pathogen-free conditions at the School of Medical Sciences (University of Bristol, UK). SCH was prepared as previously described (21). MBP1–9 (AcASQKRPSQR) was synthesized on an Abimed AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany). Mice were immunized s.c. in one hind leg with a total of 100 μl antigen emulsified with complete Freund's adjuvant (CFA) supplemented with 4 mg/ml *Mycobacterium tuberculosis* strain H37RA (Difco, Detroit, MI). *M. tuberculosis* purified protein derivative (PPD) was obtained from the UK Central Veterinary Laboratory (Weybridge, UK). Each mouse received either 1 mg SCH as a source of myelin or 200 μg of MBP1–9 peptide. Ten days later, draining lymph nodes (inguinal and popliteal) were removed and used as a source of primed lymph node cells (PLNC) for flow cytometric analysis and generation of T cell lines. Generation of the Tg4.TCL and 4Kuxs.TCL cell lines has been described previously (22). Briefly, the Tg4.TCL was generated using splenocytes from an unimmunized Tg4 transgenic mouse by primary *in vitro* stimulation with MBP1–9 peptide. The polyclonal 4Kuxs.TCL was derived from H-$2^{u\times s}$ mice immunized with MBP as described above and then generated by *in vitro* stimulation of PLNC with MBP1–9. The 170.1 T cell clone is specific for MBP89–101 presented by I-A^s (S. M. Anderton, manuscript in preparation). For generation of short-term cell lines, PLNC from SCH or MBP1–9 immunized mice were maintained in X-VIVO 15™ serum-free medium (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-glutamine (Life Technologies) and 5×10^{-5} M 2-mercaptoethanol. Cells were re-stimulated with either 20 μM MBP1–9 or 5 μg/ml PPD. Three days later, viable cells were isolated using a Nycoprep 1.077 animal density gradient (Nycomed Pharma, Oslo, Norway) and expanded in IMDM (Life Technologies) supplemented with 2 mM L-glutamine (Life Technologies), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 5% FCS (Sigma) and 5% concanavalin A-activated rat spleen supernatant.

Generation of peptide:I-A^u tetramers

Several modifications were made to the original construct used previously for the expression of recombinant I-A^u molecules (16). The native leader sequences of α and β chains were replaced by a signal peptide sequence derived from honey bee melittin (23). A *Kpn*I site and a sequence encoding a six residue flexible linker (GSGSGS) were engineered between the honey bee melittin signal peptide and the β^u chain sequence. The *Kpn*I site was used to insert the codons corresponding to the antigenic peptide, rat MBP1–11 (ASQKRPSQRHG, note that the core epitope of MBP1–9 is identical to the murine counterpart) or a derivative of this peptide in which the lysine at position four was replaced by tyrosine ('4Y'). A glycine was inserted before the MBP sequence, as this residue can substitute for the N-terminal acetyl group required for T cell recognition (D. C. Wraith, unpublished observation). The α and β chains were truncated before the transmembrane region and sequences encoding acidic and basic zippers were appended as described (16). In the β chain construct the basic zipper was followed by a sequence encoding a biotinylation signal peptide similar to that described previously (24). Both α and β chain constructs were tagged with sequences encoding C-terminal polyhistidine tags, and were cloned as a *Bgl*II or *Bam*HI fragment respectively in the dual baculovirus expression vector pAcUW51 (PharMingen, San Diego, CA). The resulting plasmid was co-transfected with Baculogold DNA (PharMingen) into *Sf9* cells and high-titer recombinant virus stocks were generated and used to infect HIGH FIVE™ cells (Invitrogen, Carlsbad, CA). The 72 h supernatants were subjected to chromatography on a Y3P (25)–Protein A–Sepharose (Pharmacia, Piscataway, NJ) affinity column. Recombinant proteins were eluted with 100 mM Na_2CO_3/150 mM NaCl, pH 11.2 and immediately neutralized using 2 M Tris–HCl, pH 7.4. Following a second step of affinity purification on Ni-NTA^{2+}-agarose (Qiagen, Valencia, CA), proteins were dialyzed into 10 mM Tris–HCl, 50 mM NaCl, pH 7.4. Biotin ligase (Avidity, Denver, CO) and biotin were added to a final concentration of 10 μg/ml and 40 μM respectively, and after 24 h at room temperature the reaction mixture was extensively dialyzed into PBS to remove the free biotin. Tetrameric peptide:I-A^u complexes were prepared by adding phycoerythrin (PE)-labeled streptavidin (Biosource International, Camarillo, CA) or Extravidin (Sigma, St Louis, MO) to the purified proteins at a molar ratio of 1:4.

Tetramer staining

Cells were incubated with tetramers for 3 h at 20°C or for 30 min at 37°C. An antibody specific for the CD3ε chain (145-2C11; PharMingen) was added (at 10 μg/ml) together with the tetramers since preliminary experiments showed that this improved tetramer staining. A similar observation was recently reported by Kotzin *et al.* (13). Cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). In some experiments cells were co-stained with an antibody against CD4 (FITC-conjugated GK1.5 or H129.19; PharMingen) and 7-amino actinomycin D (7-AAD; Sigma) was added (at 1 μg/ml) to allow exclusion of dead cells. TCR levels were determined using an FITC-labeled anti-C_β antibody (H57-597; PharMingen). Percentages of tetramer-positive cells were expressed relative to the total number of $CD4^+$ T cells.

T cell stimulation assays using recombinant peptide:I-A^u molecules

T cell hybridomas and transfectants (10^5/well) were stimulated at 37°C for 24 h with different concentrations of recombinant molecules pre-coated onto 96-well plates. IL-2 levels in the

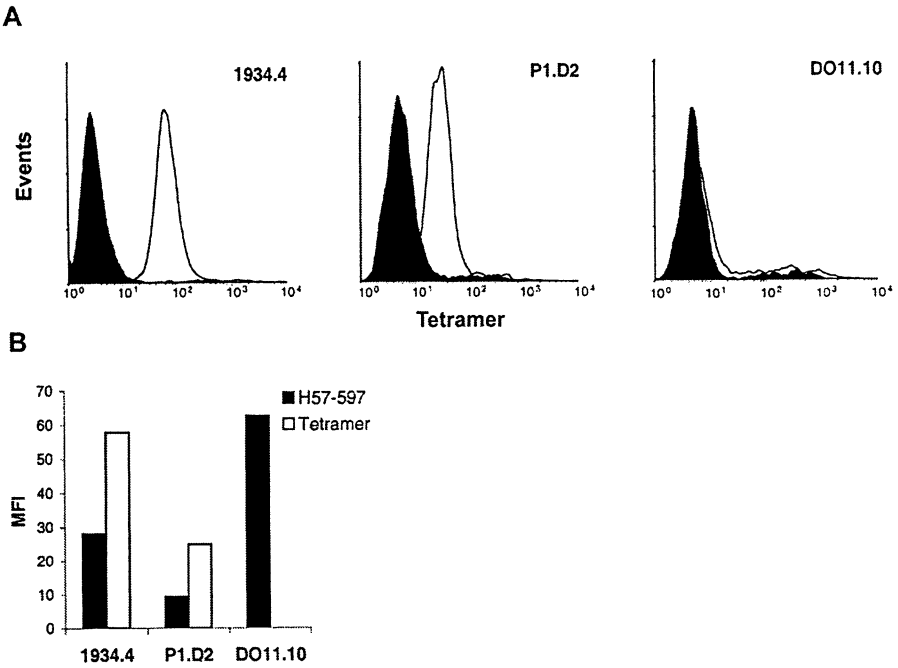

Fig. 1. Characterization of peptide:I-A^u tetramers. (A) Tetramer staining of 1934.4 T cell hybridoma, P1.D2 transfectants and DO11.10 T cell hybridoma. Shaded curves correspond to the conjugate background. (B) Staining of 1934.4 T cells (CD4$^+$) and P1D2 transfectants (CD4$^-$) with anti-Cβ (H57-597 labeled with FITC) or fluorescent tetrameric I-A^u[4Y] complexes expressed as mean fluorescence intensities.

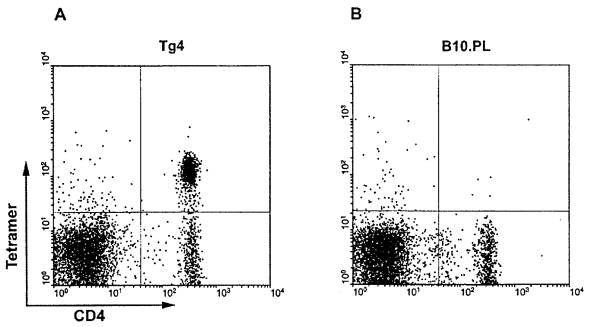

Fig. 2. Flow cytometric analysis of splenocytes from Tg4 transgenic mice (A) and from naive non-transgenic littermates (B). Cells were stained with PE-labeled tetramers, FITC-labeled anti-CD4 and 7-AAD. Data are representative of at least two independent experiments.

supernatants were determined by cytokine capture ELISA using JES6-1A12 and JES6-5H4 antibodies (PharMingen) followed by Extravidin–horseradish peroxidase (Sigma). For stimulation of T cell lines (10^5/well) and PLNC (3×10^5/well), cells were incubated at 37°C for 72 h with different concentrations of recombinant peptide:I-A^u complexes. For the final 18 h, 0.5 μCi of [^{3}H]thymidine (Amersham, Little Chalfont, UK) was added to each well. Thymidine incorporation was measured using a liquid scintillation β-counter (LKB Wallac, Turku, Finland). Results are expressed as mean c.p.m. of triplicate cultures.

Results

Generation and characterization of the I-A^u tetramers

The N-terminal epitope of MBP binds to the I-A^u restriction element with low affinity (3,4) generating complexes with a half-life of ~15 min (26). Molecular modeling combined with site-directed mutagenesis has recently led to the conclusion that MBP1–9 does not occupy the N-terminal region of the I-A^u binding cleft and the position 4 lysine interacts with the hydrophobic P6 pocket of I-A^u (27). The affinity of this peptide for I-A^u can be increased ~1500 times by replacing the hydrophilic lysine with tyrosine (4K → 4Y) (3,28). The available data (3,27) indicate that, although this substitution results in shifts of dose–response curves towards lower antigen doses, it does not affect the qualitative nature of T cell recognition. The stability of the peptide:MHC complex is critical for the generation of functional tetramers and therefore two I-A^u constructs were initially generated: I-A^u[4K] in which I-A^u is complexed with the wild-type peptide and I-A^u[4Y] comprising the 4Y analog bound to I-A^u. Covalent linkage of the antigenic peptide to the N-terminus of the β chain might be expected to compensate for the low affinity of the wild-type peptide. However, the I-A^u[4Y] complexes were ~100-fold more active in stimulating 1934.4 T cell hybridoma cells than the I-A^u[4K] complexes (data not shown). Furthermore, the expression yields were significantly lower for the I-A^u[4K] molecules. This apparent instability of the I-A^u[4K] complexes prompted us to use only the tetramers incorporating the high-affinity 4Y analog in further studies.

The specificity of the I-A^u[4Y] tetramers was initially ana-

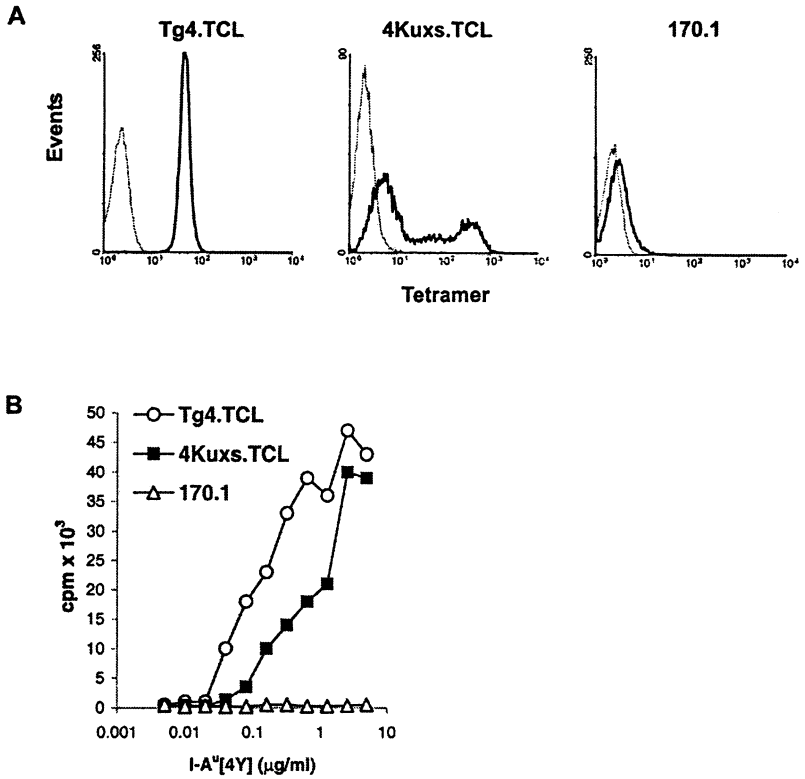

Fig. 3. I-A^u[4Y] tetramers stain T cell lines representative of the encephalitogenic repertoire (A) and tetramer binding correlates with the proliferative response of the cells to the recombinant I-A^u[4Y] molecules (B). Thin lines correspond to the conjugate background and thicker lines to tetramer stained cells. Proliferation of T cells in response to recombinant I-A^u[4Y] is represented as mean c.p.m. of triplicate cultures following background subtraction. Data are representative of three independent experiments.

lyzed using MBP1–9-specific T cell hybridomas and transfectants. Tetramers stained the 1934.4 T cell hybridomas and BW5147-derived transfectants expressing the 1934.4 TCR (Fig. 1A) and the level of staining was proportional to the TCR levels. Background levels of staining were observed for the I-A^d-restricted DO11.10 hybridoma cells. Comparison of tetramer staining of CD4$^+$ 1934.4 hybridoma cells and the 1934.4 TCR transfectants P1.D2 (CD4$^-$) indicated that, following normalization for TCR expression levels, tetramer binding is not affected by the absence of CD4 (Fig. 1B). This is consistent with the observations of others (9,29) indicating a lack of influence of CD4 on binding of multivalent peptide:MHC complexes.

Analysis of transgenic mice expressing a MBP-specific TCR

The Tg4 mice (20) express a transgenic TCR (1934.4) specific for MBP1–9 and utilizing V_α4.2 and V_β8.2. Of the CD4$^+$ splenic population, ~60% of cells stained with the tetramer (Fig. 2A). In contrast, analysis of splenocytes derived from non-transgenic littermates failed to detect any tetramer-positive cells (Fig. 2B) indicating that the number of MBP1–9-specific cells in naive mice is extremely low and providing further support for tetramer specificity. Since the Tg4 mice are not on a Rag$^{-/-}$ nor C_α knockout background, the tetramer-negative CD4 population in these mice most likely represents cells for which deficient allelic exclusion results in expression of endogenous α chains.

I-A^u[4Y] tetramers stain T cell lines representative of the MBP1–9-specific repertoire

Although tetramers clearly stain 1934.4 Tg T cells, recent studies in H-2^u mice have indicated that these cells represent only a subpopulation of the entire encephalitogenic repertoire (22). In addition, it is conceivable that the modifications required for the generation of MBP1-11:I-A^u tetramers (4K → 4Y substitution, replacement of the N-terminal acetyl group of MBP with glycine and anchoring the peptide to the N-terminus of the β chain via a flexible linker) might affect binding to some MBP1–9-specific T cells. We addressed these aspects by using tetramers to stain T cell lines previously shown to encompass the MBP1–9-specific repertoire in H-2^u mice (22). The Tg4.TCL, derived by primary *in vitro* stimulation of Tg4 splenocytes with MBP1–9, recognizes position 6 of MBP1–9 as the primary TCR contact and position 3 as a secondary contact. In contrast, the polyclonal 4Kuxs.TCL, derived from wild type H-2$^{u\times s}$ mice immunized with myelin and generated by *in vitro* stimulation with MBP1–9, shows a reversed hierarchy of TCR contacts with position 3 as the primary contact (22). Tetramer staining of the Tg4.TCL (Fig. 3A) revealed a uniform population of tetramer-positive cells whereas the 4Kuxs.TCL shows a more complex pattern of tetramer staining with three discrete populations reflecting the polyclonal nature of this T cell line. A similar observation was previously reported in a different system (12) and correlated with a wide range of receptor affinities. The 170.1 T cell

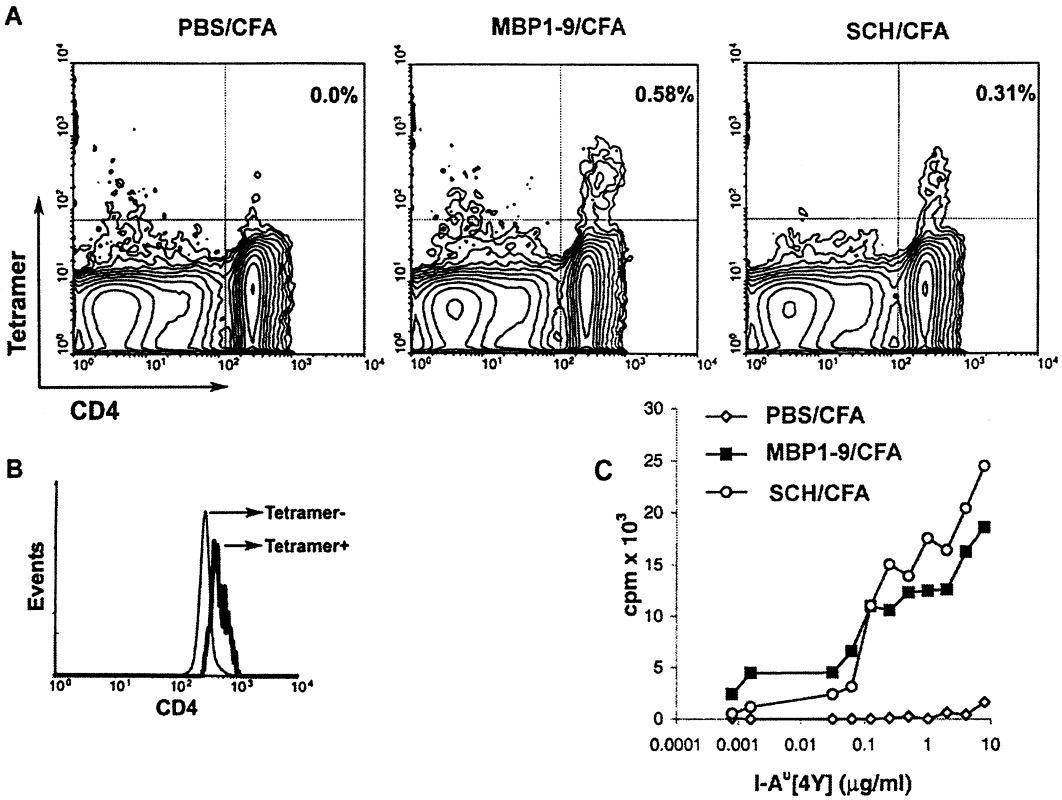

Fig. 4. Tetramer-positive CD4^{hi} cells can be detected only after immunization of H-2$^{u\times s}$ mice with MBP1–9 peptide or with SCH. (A) PLNC were stained with FITC-labeled H129.19 (anti-CD4), PE-labeled tetrameric I-A^u[4Y] (or Extravidin–PE in duplicate samples) and 7-AAD. In total, 5×10^5 events were acquired corresponding to live cells (7-AAD$^-$). Data (representative of three independent experiments) were analyzed with the software WinMDI 2.8 and the plots display CD4 and tetramer staining. The numbers in the upper right corner represent the percentages of tetramer-positive cells calculated relative to the CD4 population following subtraction of the conjugate background. (B) Tetramer-positive cells have up-regulated CD4 expression. (C) Proliferation of PLNC from mice immunized with MBP1–9, SCH or PBS in response to recombinant I-A^u[4Y].

clone specific for MBP89–101 peptide presented by I-A^s stained at background levels. Analysis of the proliferative responses of the tested cell lines to plate bound recombinant I-A^u[4Y] indicated a good correlation between responsiveness and tetramer binding (Fig. 3B).

Quantitation of autoreactive T cells following immunization of non-transgenic mice

Since the very low precursor frequency of MBP1–9-specific T cells precludes their direct detection in naive animals we decided to analyze the expansion of these cells during a primary immune response. For this, B10.PL×SJL/J (H-2$^{u\times s}$) mice were immunized s.c. with either MBP1–9 or whole MBP (in SCH). Both antigens were emulsified in CFA and control mice received PBS in the same adjuvant. PLNC from each group were stained with anti-CD4, tetramers (or Extravidin–PE in duplicate samples) and 7-AAD (to exclude dead cells which bind tetramer non-specifically; data not shown), and analyzed by flow cytometry (Fig. 4A). In contrast to PLNC from PBS/CFA immunized mice that, similar to naive animals, show no tetramer-positive cells, in MBP1–9- and SCH-immunized mice distinct populations of tetramer-positive cells are clearly visible. In three separate immunization experiments the percentage of tetramer-positive cells in the CD4 T cell population ranged from 0.14 to 0.31% for myelin and 0.28 to 0.58% for MBP1–9 primed cells. This would correspond to a population of 15,000–70,000 MBP1–9-specific T cells present in the draining lymph nodes of self-antigen immunized mice. Consistently, the number of antigen-specific cells was ~2-fold higher in mice primed with peptide than in mice immunized with whole myelin. A possible reason for this is that the amount of antigen presented to the T cells is lower for SCH immunization than for MBP1–9. Although direct testing of this hypothesis is made difficult by obvious technical limitations, this interpretation would be consistent with the effects of peptide antigen dose on the induction/expansion of antigen-specific T cells in another system (12). Tetramer-positive cells have also up-regulated expression of the co-receptor, CD4 (Fig. 4B). This extends earlier observations using other antigen systems in which CD4 up-regulation was demonstrated to be a marker for recently activated T cells (30). As demonstrated for the MBP1–9-specific T cell lines (Fig. 3B), there is a good correlation between tetramer binding and proliferative responses induced by the recombinant peptide:I-A^u molecules (Fig. 4C).

Finally, we analyzed the expansion of antigen-specific cells following *in vitro* stimulation with MBP1–9. The PLNC from MBP1–9 and SCH immunized animals were stimulated with

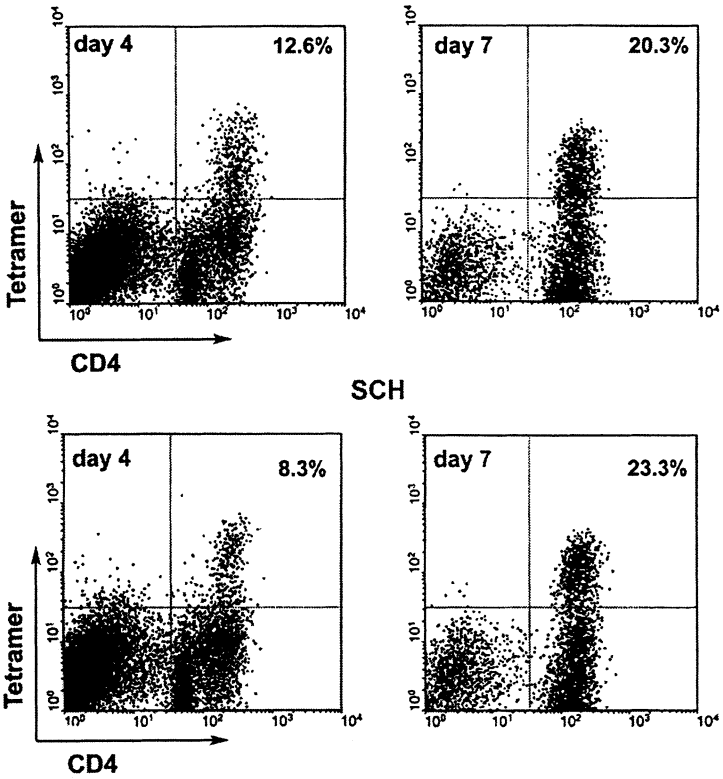

Fig. 5. Tetramer analysis of PLNC from MBP1–9 or SCH immunized mice expanded in culture by stimulation with MBP1–9. Cells were analyzed by flow cytometry 4 or 7 days after isolation from the draining lymph nodes of immunized animals (see Methods). Data (representative of three independent experiments) were analyzed and plotted as in Fig. 4(A).

MBP1–9 or, as a control, with PPD (since many of the primed cells are specific for Mycobacterium tuberculosis derived peptides) and analyzed for tetramer binding after 4 and 7 days of *in vitro* culture. The number of tetramer-positive cells reached ~10% of the CD4 cells after 4 days of *in vitro* MBP1–9 stimulation and by day 7 had increased to ~20% (Fig. 5). In contrast, PPD stimulation failed to increase the number of tetramer-positive cells (data not shown). Interestingly, although fewer cells are induced by SCH immunization (Fig. 4A), these lymphocytes are efficiently expanded *in vitro* to culminate in similar, or even higher, percentages to those seen for peptide-immunized mice (Fig. 5). Possible reasons for the apparent *in vitro* proliferative advantage of cells induced by immunization with whole protein over cells induced by peptide immunization are currently being investigated.

Discussion

In the current study we have analyzed the frequency of MBP1–9-specific T cells in naive and autoantigen-immunized mice. For this we have generated MHC class II tetramers comprising I-A^u complexed with the immunodominant epitope of MBP. The ability of these multimeric reagents to capture a diverse repertoire of MBP1–9-specific T cells has been characterized using representative T cell lines. Although the very low precursor frequencies of MBP1–9-specific T cells precluded their detection in naive mice, we could reliably detect the expansion of these cells during the primary immune response to MBP. In contrast to naive non-transgenic mice, a large population of tetramer-positive cells (~60% of CD4 T cells) was readily detectable in mice transgenic for a MBP1–9-specific TCR (20).

How do our observations of self-antigen-driven expansion compare with previous reports concerning the frequency of foreign antigen-specific CD4 T cells in mice? In a previous study, McHeyzer-Williams and Davis (31) estimated the frequency of antigen (cytochrome *c*)-specific CD4 T cells to be 1 in 10^5 in naive animals and demonstrated that in the course of the primary immune response this number increased to 50,000 in the draining lymph nodes. Our data for the primary immune response to MBP1–9 indicate numbers of antigen-specific T cells (15,000–70,000) in a similar range. These numbers are also close to what has been reported by others for the responses to an I-A^b-restricted peptide and to hen egg lysozyme (12,32). Taken together with the observation that MBP1–9-specific cells are undetectable in unimmunized mice, this suggests that a large expansion of these cells occurs during the primary response. In this context, studies in mice transgenic for different MBP1–9-specific TCRs (20,33,34) indicate that MBP1–9-specific T cells escape central tolerance induction despite thymic expression of MBP (35) due to the low affinity of this epitope for I-A^u. More recent studies in non-transgenic mice (7) have shown that thymic expression of MBP does not have a negative influence on the MBP1–9-specific T cell repertoire. Therefore, the lack of negative selection would be predicted to result in precursor

frequencies of MBP1–9-specific T cells similar to those of T lymphocytes specific for foreign antigens (12,31), if positive selection is equally efficient in both situations. It will be of interest to expand the use of tetrameric peptide–MHC class II complexes to analyze autoreactive T cell frequencies/affinities and phenotypes in other models of autoimmunity where the mechanism for escape from tolerance has not as yet been elucidated. Such quantitative studies could also be used to test models in which partial tolerance is believed to result from deletion of high-avidity T cells whilst allowing low-avidity cells to escape to the periphery (6).

In contrast to MBP1–9:I-A^u-specific T cells where *in vitro* proliferative responses of unimmunized H-2^u mice to cognate antigen are below the level of detection, recent data (36) has indicated that T cells from unimmunized SJL/J mice respond to proteolipid protein (PLP) 139–151 bound to I-A^s. This PLP epitope is not expressed by thymic PLP isoforms, allowing PLP139–151-specific T cells to escape to the periphery (36,37). Following emergence into the periphery, these cells appear to be of the memory phenotype ($CD44^{hi}$) due to further expansion by an undefined cross-reactive antigen. The undetectable levels of MBP1–9-specific T cells in naive H-2^u mice suggest that they have not undergone peripheral expansion prior to antigenic challenge and, thus, the lack of response to MBP1–9 of lymphocytes from unimmunized mice is probably due to very low precursor frequencies.

An unexpected observation of the current study is that the low affinity of MBP1–9 for I-A^u apparently does not affect its ability to induce the expansion of very few specific T cells into a sizeable autoreactive pool. This suggests that following immunization with this peptide, a sustained proliferation of high-avidity T cells (that can respond to low antigen density) probably takes place. Evidence to support this has recently been obtained (S. M. Anderton, unpublished observations). In contrast, in systems using foreign antigens where immunodominance correlates with stable peptide:MHC complex formation (38–40) different ratios of high- to low-avidity T cells might be present.

In summary, the current study describes a quantitative analysis of the MBP1–9-specific T cells. The analysis indicates that in quantitative terms the autoreactive response in this model does not differ markedly from responses to foreign antigens and this is consistent with studies indicating that MBP1–9-specific cells escape central tolerance induction. Further experiments will be directed towards using the MBP1–9:I-A^u tetramers to analyze the dynamics and affinities of autoantigen-specific T cells prior to EAE onset and during active disease.

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Abbreviations

7-AAD	7-amino actinomycin D
CFA	complete Freund's adjuvant
EAE	experimental autoimmune encephalomyelitis
MBP	myelin basic protein
PE	phycoerythrin
PLNC	primed lymph node cells
PLP	proteolipid protein
PPD	purified protein derivative
SCH	spinal cord homogenate

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