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Autoreactive CD8$^+$ T-cell responses to human myelin protein-derived peptides

(autoimmunity/major histocompatibility complex antigen-binding peptides)

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ABSTRACT **Identification of the targets of autoreactive T cells is important for understanding the pathogenesis of many autoimmune diseases. In multiple sclerosis, myelin proteins are thought to be the targets of autoreactive T-cell responses. To date only major histocompatibility complex class II-restricted CD4$^+$ T-cell responses to myelin proteins have been investigated. In the present study, the ability of self peptides derived from human myelin proteins to induce autoreactive CD8$^+$ T-cell responses has been assessed. Peptide sequences from human myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein have been identified that bind to and form stable complexes with HLA-A2. MBP 110–118, PLP 80–88, MAG 287–295, MAG 509–517, and MAG 556–564 were all able to induce peptide-specific HLA-A2-restricted CD8$^+$ cytotoxic T-lymphocyte (CTL) responses *in vitro* in HLA-A2$^+$ individuals. CTLs specific for MBP 110–118 and MAG 556–564 could recognize endogenously processed antigens presented by HLA-A2. CTL clones reactive to MBP 110–118 and MAG 556–564 produced tumor necrosis factor α and a subset of these clones also produced interferon γ. These results demonstrate that (*i*) self peptides derived from human myelin proteins can induce autoreactive CD8$^+$ CTLs and (*ii*) these CD8$^+$ T cells produce cytokines thought to be important in mediating demyelinating disease. These studies provide an experimental approach for the assessment of CD8$^+$ T-cell responses in such autoimmune diseases.**

Multiple sclerosis (MS) is a disease of the human central nervous system (CNS) that is believed to be a consequence of autoimmune mechanisms (1, 2). The pathogenesis of MS involves demyelination of axons and is, in part, mediated by an inflammatory response in which CD4$^+$ and CD8$^+$ T cells and macrophages infiltrate white matter (1, 2). In the studies to date, T-cell reactivity to myelin proteins has been analyzed by stimulating mononuclear lymphocyte populations with the isolated myelin proteins myelin basic protein (MBP) (3–8), proteolipid protein (PLP) (4, 9–11), myelin-associated glycoprotein (MAG) (4, 12), and myelin oligodendrocyte glycoprotein (MOG) (13, 14) or with relatively large synthetic peptides derived from these myelin protein sequences. Such T-cell responses have almost always been mediated by CD4$^+$ T cells.

At least three lines of evidence indicate that major histocompatibility complex (MHC) class I-restricted CD8$^+$ T-cell responses also may be involved in the pathogenesis of demyelinating disorders. (*i*) As mentioned above, CD8$^+$ T cells are found in CNS lesions in MS patients (15, 16). (*ii*) CD8$^+$ T cells recognize peptides presented almost exclusively by MHC class I molecules (17, 18), and adult oligodendrocytes constitutively express MHC class I molecules but do not express class II MHC molecules, even when stimulated by interferon γ (IFN-γ) (19). Thus, if adult oligodendrocytes are targets for T-cell reactivity, they will most likely be recognized by MHC class I-restricted CD8$^+$ T cells but not by class II-restricted CD4$^+$ T cells. (*iii*) In an animal model of demyelinating disease, experimental allergic encephalomyelitis (EAE), CD8$^+$ T cells have been shown to have an immunoregulatory effect on the course of the disease (20, 21). Despite these implications that autoreactive CD8$^+$ T-cell responses may play an important role in MS, a successful experimental approach to this question has not yet been reported.

To investigate the possible role(s) of CD8$^+$ T-cell responses to myelin proteins in the pathogenesis of demyelinating disorders, we have taken advantage of recent advances in the ability to predict peptide epitopes presented by the HLA class I molecule, HLA-A2 (22). A computer-based algorithm has been devised that predicts the stability of HLA-A2–peptide complexes by quantitating positive and negative effects on binding by each amino acid within a nonamer peptide (22). This algorithm has been used to predict HLA-A2 binding nonamer peptides from the myelin proteins MBP, PLP, MAG, and MOG. In this report we have identified HLA-A2-restricted epitopes derived from these proteins that are capable of inducing CD8$^+$ cytotoxic T-lymphocyte (CTL) responses *in vitro*.

MATERIALS AND METHODS

MS Patients and Normal Individuals. Ten patients between the ages of 34 and 55 years with clinically definite MS were studied. All patients were of Northern European descent and had a chronic progressive course. None of the patients had been on immunosuppressive therapy within 3 months prior to lymphocytopheresis. Informed consent was obtained from each patient and studies were reviewed and approved by the Institutional Review Board, National Institutes of Neurological Diseases and Stroke, National Institutes of Health. Normal individuals were recruited at the National Institutes of Health Department of Transfusion Medicine from local residents and laboratory workers. Peripheral blood lymphocytes (PBLs) were obtained by batch leukapheresis from normal individuals and from MS patients. At the time of PBL

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Abbreviations: β_2m, β_2-microglobulin; CNS, central nervous system; CTL, cytotoxic T lymphocyte; EAE, experimental allergic encephalomyelitis; EDSS, expanded disability status scale; IFN-γ, interferon γ; MS, multiple sclerosis; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; PBL, peripheral blood lymphocyte; PLP, proteolipid protein; TNF-α, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin.

collection, MS patient MS#1 (HLA-A2,26,B38,51,Cw2,DR2) was a 38-year-old female with chronic progressive MS of 6-years duration with an expanded disability status score (EDSS) of 6.5, and MS patient MS#2 (HLA-A1,2,B14,35,Cw4, DR1,6) was a 37-year-old male with chronic progressive MS of 12-years duration with an EDSS of 3.0. HLA typing was performed in the HLA Typing Laboratory, Department of Transfusion Medicine, National Institutes of Health.

Synthetic Peptides. Peptides were synthesized, purified, and analyzed as described (22). Peptide sequences were selected from human MBP (23), PLP (24), MAG (25), and MOG (26) protein sequences. All peptides were dissolved in stock solutions of phosphate-buffered saline with 50% dimethyl sulfoxide at 1 mg/ml.

Selection and Analysis of Peptide Binding to HLA-A2 Molecules. Peptide sequences were selected based on a computer algorithm developed to predict the relative binding strengths of nonapeptides to HLA-A2 (22). HLA-A2 complexes were reconstituted with isolated heavy chains, peptides, and ^{125}I-labeled β_2-microglobulin (^{125}I-β_2m) and analyzed by gel filtration (22). Stability of HLA-A2 complexes was assessed by measuring the rate of dissociation of ^{125}I-β_2m at 37°C as described (22).

Generation and Assay of Peptide-Specific CTLs. Induction of peptide-specific CTLs *in vitro* was performed with unseparated PBLs exactly as described for the induction of influenza virus matrix protein peptide 58–66 CTLs (27). Purified CD8$^+$ responder cells were isolated from PBLs with magnetic beads coupled with anti-CD8 antibody (Dynabeads M-450 CD8; Dynal, Lake Success, NY) and detached using a polyclonal anti-immunoglobulin antibody (DetachaBead; Dynal) as described by the manufacturer. T-cell clones were isolated from limiting dilution cultures with responder cells at 10 and 1 cell per well with 20 units of recombinant interleukin 2 per ml. Cytolytic activity was assayed by $Na_2{}^{51}CrO_4$ release as described (27) using Hmy2.C1R cells (28) (a gift of Peter Cresswell, Yale University) that were transfected with HLA-A2 (A2-Hmy), HLA-A1 (A1-Hmy), and HLA-B44 (B44-Hmy). A hybrid clone (MO3.13) from a fusion of the rhabdomysarcoma RD.TG6 and mixed human glial cells (29) was a gift of Neil Cashman (McGill Univ., Montreal). MO3.13 was transfected with HLA-A2 cDNA in the RSV.Neo vector as described (27). Cell surface expression of HLA-A2 was assessed by indirect immunofluorescence with the anti-HLA-A2 antibody BB7.2 as described (27). HLA-A2-transfected and untransfected MO3.13 cells were used as targets after stimulation with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma) and 200 units of IFN-γ per ml (Genzyme) for 3 days to induce expression of myelin and MHC genes (29). Results of cytotoxicity assays are expressed as mean % specific lysis of triplicate determinations (27).

Measurement of Cytokine Production by T-Cell Clones. T-cell clones (2×10^5) were incubated for 48 hr with 5×10^5 2000-rad irradiated (1 rad = 0.01 Gy) allogeneic PBLs and phytohemagglutinin (PHA 1:1000; Gibco Life Technologies Gaithesburg, MD) in 1-ml cultures. Supernatants were harvested and assayed by sandwich ELISA kits for human tumor necrosis factor α (TNF-α) (R & D Systems) and human IFN-γ (Genzyme) according to the manufacturer's instructions. Background levels of cytokines were obtained from cultures of irradiated PBLs containing PHA with no added T-cell clones and were subtracted from the values obtained for culture supernatants obtained from the culture with T-cell clones.

RESULTS

Selection and Binding of Myelin Peptides to HLA-A2. A method has been developed that predicts the ability of nonamer peptides to bind to HLA-A2 (22). We have used this algorithm to predict the best HLA-A2 binding peptides within the sequences of the human myelin proteins MBP, PLP, MAG, and MOG (Table 1). The criterion used to identify these peptides was that they are predicted to form a complex with HLA-A2 and β_2m with a dissociation rate $t_{1/2}$ of >100 min at 37°C (22). One such peptide sequence was predicted from MBP, two from PLP, six from MAG, and eight from MOG (Table 1). Gel filtration assays showed that 16 of the 17 predicted peptides bound detectably to HLA-A2 (measured as ≥10% β_2m incorporated, Table 1). The experimental $t_{1/2}$ of ^{125}I-β_2m dissociation varied widely, with 8 of the 17 predicted peptides having a measured $t_{1/2}$ of >100 min (Table 1). We have used 7 of these peptides with measured dissociation rates >100 min (PLP 80–88, PLP 253–261, MAG 509–517, MAG 556–564, MAG 287–295, MOG 113–121, and MOG 133–141) plus MBP 110–118 to induce CD8$^+$ CTL responses *in vitro* from PBLs of HLA-A2$^+$ individuals.

Induction of Myelin Peptide-Specific CTLs. Unseparated PBLs from 1 (Q164) of 10 HLA-A2$^+$ normal individuals and 1 (MS#1) of 9 HLA-A2$^+$ MS patients were able to generate MBP 110–118 peptide-specific CTLs after five *in vitro* stimulations (Fig. 1*A*). These anti-MBP 110–118 CTLs were peptide-specific and HLA-A2-restricted because (*i*) they lysed MBP 110–118 pulsed A2-Hmy targets but not the same targets pulsed with a known HLA-A2-restricted epitope (30), M1 58–66 from influenza A virus matrix protein (Fig. 1*A*), and (*ii*) they did not lyse HLA-A1 targets pulsed with MBP 110–118 (Fig. 1*A*). Similarly, unseparated PBLs from 1 (MS#2) of 10 HLA-A2$^+$ MS patients and none of 8 HLA-A2$^+$ normal individuals were able to generate PLP 80–88 peptide-specific CTLs after three *in vitro* stimulations (Fig. 1*B*). None of these same HLA-A2$^+$ individuals' PBLs could generate CTLs to PLP 253–261 after six *in vitro* stimulations (data not shown).

For induction of MAG peptide-specific CTLs, CD8$^+$ T cells were isolated from the PBLs of four HLA-A2$^+$ normal individuals and were stimulated *in vitro* with peptide-pulsed irradiated autologous unseparated PBLs. Specific cytolytic activity to MAG 287–295 and MAG 509–517 could be detected in CD8$^+$ T cells from two of the four donors (Q127 and Q130) after four *in vitro* stimulations (Fig. 1 *C* and *D*), and MAG 556–564-specific CTLs were detectable in CD8$^+$ T cells from one of the four individuals (Q145) after five *in vitro*

Table 1. Binding of peptides to HLA-A2

Peptide	Sequence	$t_{1/2}$* (pre.)	% β_2m†	$t_{1/2}$‡ (mea.)	SD_{50},§ nM
MBP 110–118	SLSRFSWGA	560	40	40	1–10
PLP 253–261	SLLTFMIAA	350	60	350	
PLP 80–88	FLYGALLLA	120	80	1300	1–10
MAG 556–564	VLFSSDFRI	610	80	390	10–100
MAG 406–414	NLSVEFAPV	250	80	40	
MAG 234–242	SMDVKYPPV	230	80	40	
MAG 8–16	PLFWIMISA	180	0		
MAG 509–517	LMWAKIGPV	120	90	850	0.01–0.1
MAG 287–295	SLLLELEEV	110	80	1100	0.1–1
MOG 157–165	KLWTVPTNM	1100	60	30	
MOG 113–121	KLLDKSDTA	420	80	170	
MOG 7–15	KMSLCLFIL	300	60	60	
MOG 240–248	LKWMMETKA	130	10	60	
MOG 164–172	NMPSKLHIV	110	80	270	
MOG 221–229	TLYNNRWSC	110	70	<5	
MOG 133–141	NMLEKVVLI	104	80	290	
MOG 422–430	WKVNASFLL	101	10	70	

*Predicted $t_{1/2}$ (min) of ^{125}I-β_2m dissociation, using table 5 in ref. 24.
†% of ^{125}I-β_2m incorporated into HLA-A2 determined by gel filtration assay, rounded to the nearest 10%.
‡Measured $t_{1/2}$ (min) of ^{125}I-β_2m dissociation at 37°C.
§Concentration of peptide that sensitizes target cells half-maximally.

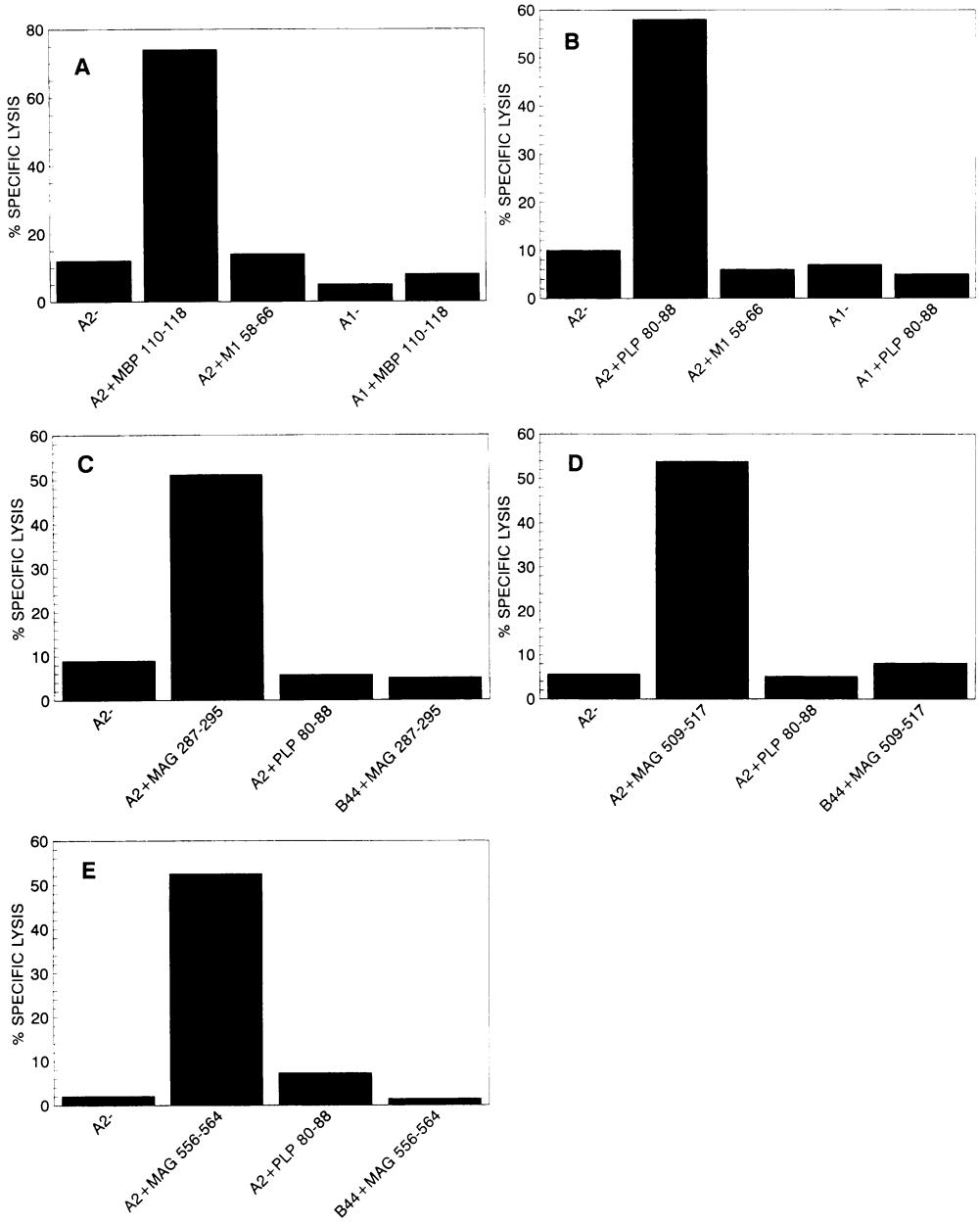

FIG. 1. Myelin peptide-specific CTL recognition of peptide-pulsed targets. Representative results are shown in each panel for cytolytic activity of the various CTL lines assayed on Hmy targets that were transfected with the indicated class I genes and were either unpulsed (−) or pulsed for 1 hr at 37°C with 1 μg of the indicated peptide per ml. (*A*) MBP 110–118-reactive CTL line from Q164 (HLA-A2,29,B8,45,Cw6,7,DR3,DQw2) stimulated six times *in vitro* and assayed at an effector-to-target ratio (E:T) of 5:1. (*B*) PLP 80–88-reactive CTL line from MS#2 stimulated *in vitro* six times and assayed at an E:T of 5:1. (*C*) MAG 287–295-reactive CTLs from Q127 (A2,26,B39,62,Cw3,7,DR2,4,DQ1,3) stimulated *in vitro* four times and assayed at an E:T of 5:1. (*D*) MAG 509–517-reactive CTL line from Q127 stimulated four times *in vitro* and assayed at an E:T of 5:1. (*E*) MAG 556–564-reactive CTLs from Q145 (A2,3,B35,Cw4,DR3,9) stimulated six times *in vitro* and assayed at an E:T of 2.5:1.

stimulations (Fig. 1*E*). No induction of MOG 113–121 or MOG 133–141 peptide-specific CTLs could be detected in $CD8^+$ PBLs from six normal individuals tested after six stimulations (data not shown).

Analysis of the cell surface phenotype of each of the myelin peptide-specific CTL lines in Fig. 1 demonstrated that all are $CD8^+$, $CD4^-$ (data not shown). Titration of the amounts of peptide required to sensitize targets for lysis by these CTLs (SD_{50}) showed that they were all within the nanomolar range (Table 1). These results demonstrate that at least five HLA-A2-restricted $CD8^+$ T-cell epitopes are contained within the MBP, PLP, and MAG proteins.

Recognition of Endogenously Processed Myelin Proteins by Myelin Peptide-Specific CTLs. To determine whether the CTL lines generated against myelin peptides could recognize an endogenously processed form of the protein, the HLA-A2 transfected and untransfected cell line MO3.13 stimulated with PMA and IFN-γ was utilized. Fluorescence-activated cell sorting analysis of stimulated A2-MO3.13 cells expressed roughly half of the level of HLA-A2 observed on A2-Hmy cells (Fig. 2*A*, mean fluorescence intensity values of 127 and 225, respectively). MBP 110–118 peptide-specific CTLs lysed stimulated A2-MO3.13 cells but did not lyse similarly treated untransfected MO3.13 cells (Fig. 2*B*), and the levels of lysis relative to MBP 110–118 peptide-pulsed A2-Hmy cells varied between 4- and 20-fold less. MAG 556–564 peptide-specific CTLs could also specifically recognize stimulated A2-MO3.13 cells (Fig. 2*C*), with levels of lysis ≈20-fold less than those

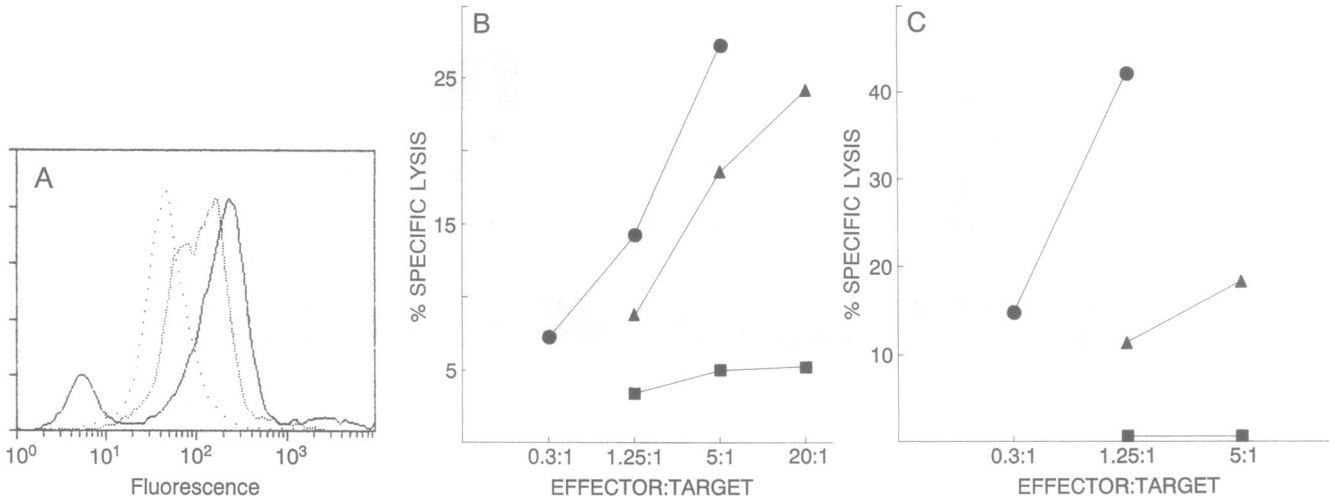

FIG. 2. MO3.13 cell surface expression of HLA-A2 and recognition by MBP 110–118 and MAG 556–564 peptide-specific CTL lines. (*A*) Cell surface expression of HLA-A2 detected by monoclonal antibody BB7.2 on untreated A2-MO3.13 cells (· · ·) and PMA- and IFN-γ-treated A2-MO3.13 cells (···) compared to HLA-A2 transfected Hmy cells (– – –). (*B* and *C*) Recognition by MBP 110–118 peptide-specific CTL line 164.13 (*B*) and MAG 556–564 peptide-specific CTL line 145 (*C*) of PMA- and IFN-γ-treated MO3.13 (■) and A2-MO3.13 (▲) target cells compared to untreated A2-Hmy targets (●) pulsed with either MBP 110–118 (1 μg/ml; *B*) or MAG 556–564 (1 μg/ml; *C*).

observed on MAG 556–564 peptide-pulsed A2-Hmy cells. No specific cytolytic activity was observed on stimulated A2-MO3.13 targets by PLP 80–88, MAG 287–295, or MAG 509–517 peptide-specific CTLs (data not shown). These results indicate that at least the MBP 110–118 and MAG 556–564 peptide-specific CTLs can recognize epitopes that are generated by endogenous processing of these myelin proteins.

Cytokine Production by Myelin Peptide-Reactive CD8⁺ T-Cell Clones. T-cell clones specific for MBP 110–118 and MAG 556–564 were isolated by limiting dilution from bulk cultures. Each of these clones was tested for peptide specificity and HLA-A2 restriction in CTL assays as described above, and all displayed the $CD8^+$, $CD4^-$ cell surface phenotype (data not shown). Fourteen clones specific for MBP 110–118 and 4 clones specific for MAG 556–564 were assayed for their capacity to produce TNF-α and IFN-γ when stimulated with PHA. These cytokines were chosen because of their potential roles in the pathogenesis of demyelinating disorders (see *Discussion*). All of these clones produced detectable amounts of TNF-α (Fig. 3 *A* and *B*), and all but three clones secreted >200 pg/ml. Five of the 14 MBP 110–118-specific CTL clones also produced IFN-γ (Fig. 3*C*), whereas none of the 4 MAG 556–564-specific clones produced detectable levels of IFN-γ (Fig. 3*D*). At present we do not know how many different clonotypes are present within this panel of 18 CTL clones, but the fact that only a subset produces both TNF-α and IFN-γ indicates that at least two different clonotypes are represented.

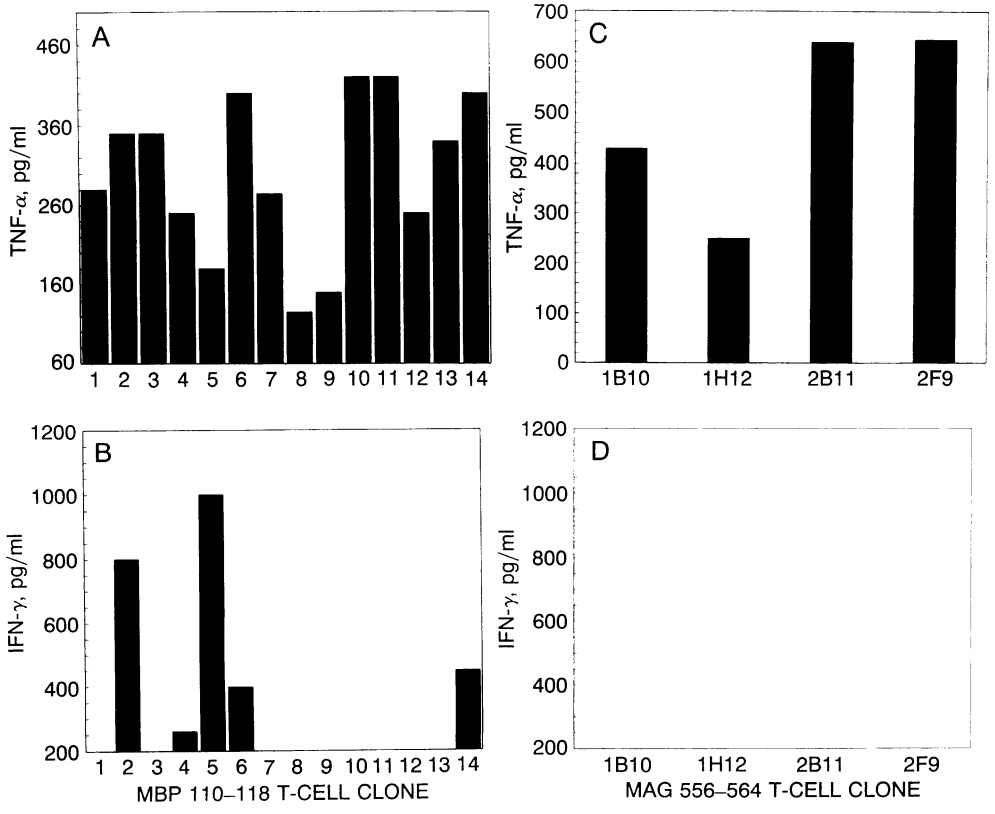

FIG. 3. Production of TNF-α and IFN-γ by MBP 110–118- and MAG 556–564-reactive $CD8^+$ CTL clones. Fourteen MBP 110–118-reactive CTL clones from donor Q164 (*A* and *B*) and four MAG 556–564-reactive CTL clones from donor Q145 (*C* and *D*) were stimulated with PHA and assayed for their capacity to produce TNF-α (*A* and *C*) and IFN-γ (*B* and *D*). Background levels of cytokines obtained by irradiated PBLs plus PHA alone were subtracted from the values for each T-cell clone.

DISCUSSION

It is clear from the results in this study that some normal individuals, as well as some MS patients, have circulating $CD8^+$ T cells that are capable of recognizing self myelin protein-derived peptides. These T cells have not been deleted or anergized under our experimental conditions. These findings are similar to those that demonstrated that $CD4^+$ T-cell responses to MBP could be generated *in vitro* from PBLs obtained from MS patients as well as normal individuals (6, 7, 9). It appears that the generation of autoimmune T-cell responses to these myelin proteins is not limited by the availability of T cells with the appropriate antigen specificity but is controlled at the level of antigen availability for presentation and/or the quantity of antigen/MHC complexes required to stimulate primary T-cell responses.

The finding that all of the $CD8^+$ CTL clones that are specific for MBP 110–118 or MAG 556–564 can also produce TNF-α may have significance for the pathogenesis of demyelination, despite the small number of $CD8^+$ CTL clones that have been analyzed. This cytokine has been shown to contribute to inflammatory demyelinative processes by up-regulation of MHC class I molecule expression (31), by cytotoxic damage to endothelial cells leading to a breakdown of the blood–brain barrier (32), and by direct injury to oligodendrocytes (33). Elevated levels of TNF-α have been found in the cerebrospinal fluid of MS patients with active disease (34), and TNF-α has been detected histologically in MS lesions (35). Studies that demonstrated that antibodies to TNF-α could prevent development of EAE (36) have indicated a major role for TNF-α in this animal model. The fact that a subset of these CTL clones also produces IFN-γ indicates that they also have the capacity to up-regulate the expression of MHC molecules in the CNS (37) and thus promote the development of T-cell responses in these tissues. It has been shown that treatment of MS patients with IFN-γ exacerbated the clinical course of the disease (38), which indicates that this cytokine may be important in the normal pathogenesis of MS.

We have attempted to determine whether $CD8^+$ CTLs generated against synthetic myelin-derived peptide sequences could recognize endogenously processed forms of the proteins. The MO3.13 target cells expressed readily detectable quantities of MBP but only low levels of PLP and MAG (29). When transfected with HLA-A2 and stimulated with PMA and IFN-γ, these targets could be specifically recognized by MBP 110–118 and MAG 556–564 peptide-specific CTL lines but not the other peptide-specific CTLs. The lack of cytolytic activity by these CTL lines on PMA- and IFN-γ-treated A2-MO3.13 targets could be due to a number of factors: (*i*) these CTLs have T-cell receptors that recognize synthetic peptides but these peptides are not produced by endogenous processing and presentation of these proteins in A2-MO3.13 cells, (*ii*) the level of expression of HLA-A2/peptide complexes is too low on these cells relative to the A2-Hmy target cells for lysis to be observed in short-term ^{51}Cr-release assays, or (*iii*) A2-MO3.13 cells possess low levels of cell surface ligands for the adhesion molecules utilized for CTL/target conjugation. We have observed that PMA- and IFN-γ-treated A2-MO3.13 cells express the CD2 ligand LFA-3 (CD58) at 1/5th to 1/10th the level of A2-Hmy cells and are completely negative for expression of the LFA-1 (CD11a/CD18) ligands ICAM-1 (CD54), -2, and -3 (unpublished observations). Perhaps only those CTL lines with the highest-affinity T-cell receptor for peptide/HLA-A2 complexes can detectably lyse A2-MO3.13 targets.

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