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A Study Of Human T-Cell Lines Generated From Multiple Sclerosis Patients And Controls By Stimulation With Peptides Of Myelin Basic Protein

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Abstract

We generated T-cell lines from the peripheral blood of controls and of patients with multiple sclerosis (MS) by stimulation with overlapping synthetic peptides representing the entire sequences of all four isoforms of human myelin basic protein (MBP). The T-cell lines reacted to a wide range of epitopes in the major isoforms of MBP and to epitopes that were present only in the minor isoforms. Many MS patients and controls had T-cells responding to one or more cryptic MBP epitopes, as indicated by the generation of a peptide-specific T-cell line(s) by stimulation with synthetic peptides but not by stimulation with whole MBP. About one-third of the peptide-generated lines were cytotoxic. Although we have shown that this technique of peptide stimulation is effective in generating human antiviral cytotoxic CD8⁺ T-cell lines, all the cytotoxic MBP-specific lines generated by this method were predominantly CD4⁺. Our study did not reveal any significant differences, between MS patients and controls, in reactivity to epitopes within any of the isoforms of MBP.

Keywords: cryptic epitope; isoform; multiple sclerosis; myelin basic protein; peptide; T-cell

Index Terms: t lymphocyte; multiple sclerosis; cytotoxic t lymphocyte; myelin basic protein; synthetic peptide

1. Introduction

There is increasing evidence that multiple sclerosis (MS) is an autoimmune disease (Pender, 1995) but the target antigens in the central nervous system (CNS) have not been clearly determined. Myelin basic protein (MBP) has been implicated as a possible target antigen because immunization of experimental animals with MBP and adjuvants results in the disease experimental autoimmune encephalomyelitis (EAE), which resembles MS. This form of EAE is mediated by CD4⁺ T-cells specific for MBP (Pettinelli and McFarlin, 1981; Zamvil et al., 1985). A number of laboratories have isolated MBP-specific T-cell lines or clones from the peripheral blood of MS patients and controls by stimulation with whole human MBP (Weber and Buurman, 1988; Vandenbark et al., 1989; Martin et al., 1990; Ota et al., 1990; Pette et al., 1990; Zhang et al., 1990; Liblau et al., 1991; Burns et al., 1991). These lines and clones express CD4, are restricted by HLA-DR class II major histocompatibility complex (MHC) molecules, and have been analyzed in detail. Little attention has been given to the potential

role of CD8⁺ T-cells in the pathogenesis of MS, even though CD8⁺ cells, as well as CD4⁺ cells, are present in MS lesions (Sobel et al., 1988). Oligodendrocytes, which synthesize MBP and other myelin components in the CNS, can be induced to express class I MHC molecules (Wong et al., 1984; Turnley et al., 1991) and could thus present MBP to, and be destroyed by, cytotoxic CD8⁺ MBP-specific T-cells. Such oligodendrocyte destruction could contribute to the primary demyelination that occurs in MS.

We therefore performed the present study to determine whether CD8⁺ MBP-specific T-cells could be generated from the peripheral blood of MS patients and controls by stimulation with synthetic peptides, using a method we have previously shown to be able to generate human antiviral and anti-parasite cytotoxic CD8⁺ T-cells (Doolan et al., 1991a and Doolan et al., 1991b). We used overlapping synthetic peptides representing the entire sequence of the most abundant isoform (18.5 kDa) of MBP and also the sequences of the 21.5, 20.2 and 17.3 kDa isoforms, which are expressed in the developing human CNS (Roth et al., 1987). It has been suggested that the minor isoforms may also be expressed by remyelinating oligodendrocytes and thus be potential T-cell targets in MS (Voskuhl et al., 1993a and Voskuhl et al., 1993b). We also generated T-cell lines by stimulation with whole MBP in order to compare the specificities of these lines with those of the lines generated by stimulation with peptides.

2. Materials and methods

2.1. Subjects

Twenty-one patients (12 female; 9 male) aged 17–66 with clinically definite MS or laboratory-supported definite MS (Poser et al., 1983) were studied. Seven patients had relapsing-remitting MS, 7 had secondary progressive MS and 7 had primary progressive MS. The duration of MS ranged from 1–30 years (mean=11±7). The MS patients had not received any immunosuppressive or corticosteroid therapy within 6 months prior to study. Twelve healthy individuals (4 female; 8 male) aged 24–40 and 2 hospital in-patients (one female, aged 47, with Huntington's disease [Con 14], and one female, aged 44, with no evidence of neurological disease [Con 13]) served as controls.

2.2. Myelin proteins and peptides

MBP was purified from human brain tissue by the method of Deibler et al. (1972), and its purity assessed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. Overlapping synthetic peptides 20 amino acids in length were synthesized by the tea-bag method (Houghten, 1985), using the published sequences of the 18.5 kDa isoform of human MBP (Roth et al., 1987). Additional peptides were prepared to represent sequences from the deleted and inserted regions in the 21.5, 20.2 and 17.3 kDa isoforms of human MBP. Fig. 1 shows the 4 human MBP isoforms, and Table 1 lists the synthetic peptides used. Peptides 5B-5E were synthesized to represent the 26 amino acids in region A. Peptide 10B was made according to the sequence of the junctional zone resulting from the deletion of the 11 amino acids in region B. All peptides were tested for purity by analytical high-performance liquid chromatography (HPLC), and where necessary were further purified by reverse phase HPLC. The amino acids were numbered according to their position in the sequence of the 18.5 kDa isoform of human MBP.

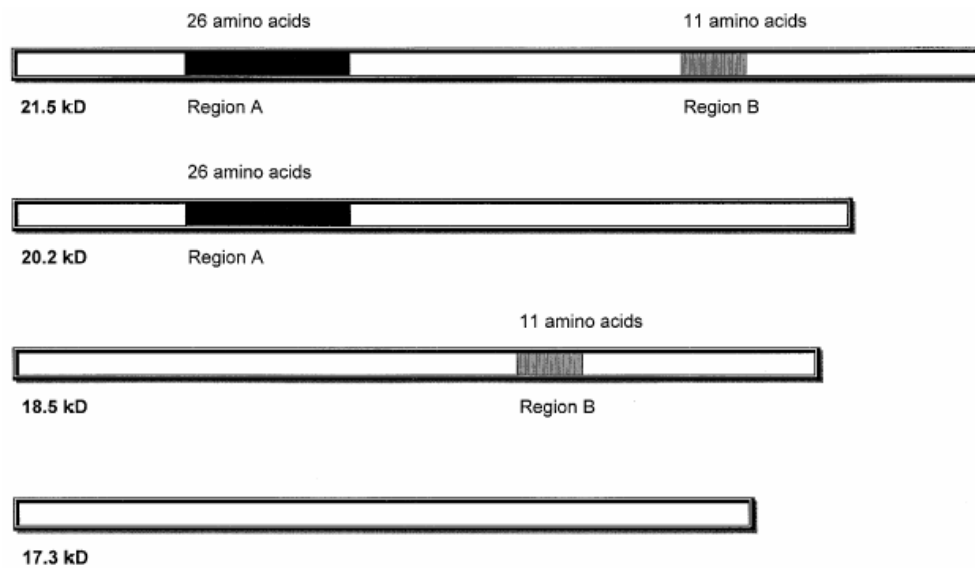


Fig. 1. There are four isoforms of human MBP, arising from alternate exon splicing. The major 18.5 kDa isoform contains the 11 amino acids (region B) encoded by exon 5, but not the 26 amino acids of region A encoded by exon 2.

Table 1. Synthetic peptides

Group of pooled peptides	Peptide number	Amino acid sequence	Numerical sequence in 18.5 kDa isoform
Group 1	1	ASQKRPSQRHGSKYLATAST	(1–20)
	2	GSKYLATASTMDHARHGFLP	(11–30)
	3	MDHARHGFLPRHRDTGILDS	(21–40)
	4	RHRDTGILDSIGRFFGGDRG	(31–50)
	5	IGRFFGGDRGAPKRGSGKDS	(41–60)
Group 2	5B	RGAPKRGSGKVPWLKGRSP	(Region A) (VPW..)
	5C	VPWLKGRSPLPSHARSQPG	(Region A)
	5D	LPSHARSQPGLCNMYKKDSH	(Region A)
	5E	LCNMYKKDSHHPARTAHYGS	(Region A) (.LCNMYK)
	6	APKRGSGKDSHHPARTAHYG	(51–70)
	Group 3	7	HHPARTAHYGSLPQKSHGRT
8		SLPQKSHGRTQDENPVVHFF	(71–90)
9		DENPVVHFFKNIVTPRTPP	(82–100)
10		KNIVTPRTPPPSQGKGRGLS	(91–110)
10B		PRTPPPSQGKGAEQRPFGF	(B Deletion)
Group 4	11	PSQGKGRGLSLSRFSWGAEG	(101–120) (Region B 106–116)
	12	LSRFSWGAEGQRPFGFYGGR	(111–130)
	13	RPGFGYGGRASDYKSAHKG	(122–140)
	14	ASDYKSAHKGFKGVDAQGTL	(131–150)
	15	FKGVDAQGTLSKIFKLGGRD	(141–160)
	16	SKIFKLGGRDSRSGSPMARR	(151–170)

Overlapping 20-mer peptides were synthesized to represent the entire sequences of all 4 isoforms of human MBP. Peptides 5B–5E cover the 26 amino acids in region A (exon 2), and peptide 10B covers the junctional zone formed by the deletion of 11 amino acids in region B exon 5 see Fig. 1. For the generation of T cell lines, the peptides were pooled into 4 groups, in order to reduce the total number of peripheral blood lymphocytes required.

2.3. Generation of T-cell lines

Peripheral blood (100 ml) was obtained by venepuncture from each subject. Whole blood was centrifuged over a Ficoll-paque gradient (Pharmacia), and peripheral blood mononuclear cells (PBMC) were collected from the interface and washed twice with RPMI containing 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% pooled, heat-inactivated human serum (RPMI-HS). 21 peptides covering all isoforms of MBP were pooled into 4 groups (Table 1). Five sets of cultures were established from each subject, using 6×10^6 PBMC for stimulation with each of the 4 groups

of peptides and 1.2×10^7 PBMC for stimulation with whole MBP. For the groups of peptides, the cells were cultured in duplicate 2 ml wells (3×10^6 cells per well) in 24-well flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark), whereas for whole MBP, the cells were cultured in quadruplicate 2 ml wells (3×10^6 cells per well). Every 10–12 days each line was restimulated with the appropriate group of peptides (each peptide at a concentration of $30 \mu\text{g/ml}$) and irradiated autologous phytohaemagglutinin-activated lymphoblasts (for peptide-generated lines, as described by Doolan et al. (1991b)), or MBP ($30 \mu\text{g/ml}$) and irradiated autologous PBMC (for MBP-generated lines), followed by feeding with RPMI-HS containing 10 U/ml recombinant human IL-2. After 4–5 weeks, the lines were tested for their proliferative and cytotoxic specificity.

2.4. Assessment of T-cell line proliferative specificity

To test the specificity of the T-cell lines, 10^4 T-cells were incubated with 10^5 irradiated autologous PBMC and MBP peptide or whole MBP ($30 \mu\text{g/ml}$) in a total volume of 200 μl . The experiments were performed in triplicate in 96-well round-bottomed microtitre tissue culture plates (Nunc) for 72 h. During the final 12 h of culture, 1 μCi of ^3H -thymidine (Amersham Australia, Sydney) was added to each well. Cells were harvested onto glass fibre filter mats, and the amount of incorporated radioactive label was determined by scintillation counting using an LKB 1205 Betaplate liquid scintillation counter. The stimulation index (SI) was then determined for each mixture of cells and peptide or MBP. The SI was defined as the ratio of the mean cpm of test cultures (with added peptide or whole MBP) and the mean cpm of control cultures (no added peptide or MBP). A positive antigen-specific proliferative response was taken as one in which the SI was greater than or equal to 2 and in which the mean cpm of test cultures exceeded the mean cpm of control cultures by 3 SD of the control mean.

2.5. Assessment of T-cell line cytotoxic capacity

T-cell lines showing positive proliferative responses were assessed for cytotoxic capacity by standard 6 h chromium release assays, using, as targets, autologous Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) that had been incubated overnight with grouped peptides (each peptide at a concentration of $10 \mu\text{g/ml}$), individual peptides ($10 \mu\text{g/ml}$) or MBP ($10 \mu\text{g/ml}$). The effector/target ratio was 10:1. Prior to the assay the LCL cells were washed, labelled for 90 min with 200 μCi of $^{51}\text{Cr}(\text{Na}_2\text{Cr}_2\text{O}_7)$ at 37°C , and then washed 4 more times with medium. Labelled target cells were plated at 5×10^3 cells/well in 96-well round-bottomed plates, and effector T-cells were mixed and incubated with these as triplicate cultures in 200 μl volumes for 6 h at 37°C in the presence and in the absence of grouped peptides (each peptide at a concentration of $5 \mu\text{g/ml}$), individual peptides ($5 \mu\text{g/ml}$) or MBP ($5 \mu\text{g/ml}$). Maximum release and spontaneous release of chromium label were measured in wells containing target cells in the presence of 10% SDS or medium alone respectively. Fifty μl of supernatant was removed from each well after 6 h and counted in a gamma counter. The assays were performed with test T-cells and target LCL cells in the presence of free peptide (peptide lysis) and in the absence of peptide (control lysis). Percentage peptide-specific lysis was calculated by the formula:

$$\text{peptide-specific lysis} = \text{peptide lysis} - \text{control lysis}$$

Results for both peptide and control lysis assays were calculated using the following formula:

$$\text{Percentage lysis} = \frac{\text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Assays were only considered valid if spontaneous release was less than 20% of maximum release. A peptide-specific lysis >10% was considered to indicate antigen-specific cytotoxic capacity (Doolan et al., 1991b).

2.6. Flow cytometry analysis

All cytotoxic and some non-cytotoxic T-cell lines were analyzed for CD4 and CD8 expression. Cells (5×10^5) were washed in ice-cold phosphate-buffered saline supplemented with 1% foetal calf serum and 0.01% NaN_3 , split into 3 tubes, and resuspended in 100 μl volumes. Directly conjugated (fluorescein isothiocyanate) anti-human-CD4 or -CD8 antibodies (Becton Dickinson, Belgium) were added to the cell suspension and incubated for 30 min at 4°C. Cells were subsequently washed twice and analyzed on a FACScan (Becton Dickinson) with gates set to include the total population.

2.7. DR tissue typing

Genomic DNA was prepared from donor LCLs by chloroform/phenol extraction and amplified by the polymerase chain reaction (PCR) using 5 generic primer pairs. One pair amplifies the second exon of all the DRB genes, and one pair is used to amplify each of the DR1, DR2, DR4, and DRw52 allelic groups. The PCR product was then quantified on an agarose gel before denaturation with NaOH/EDTA, and dot blotted onto a positively charged membrane. Membrane-bound DNA was hybridized sequentially using a panel of 45 sequence-specific oligonucleotides. Hybridization signal was detected using non-radioactive techniques and the hybridization patterns analyzed, enabling the individual DRB1 alleles to be assigned.

2.8. Statistical analysis

The percentage of subjects from whom T-cell lines reactive to a given peptide could be generated was compared in MS patients and controls using the Chi-square test with Yates' correction applied as required.

3. Results

3.1. Generation of T-cell lines by stimulation with whole MBP

By stimulation with whole MBP, 8 T-cell lines were derived from the peripheral blood of 14 controls (Fig. 2), and 10 lines were derived from the blood of 20 patients with MS (Fig. 3). The most frequent immunodominant peptides were peptides 9 (82–100), 14 (131–150) and 15 (141–160), but other peptides, particularly 1 (1–20), 7 (61–80) and 13 (122–140), were immunodominant in some subjects (Fig. 4). There were no significant differences, between MS patients and controls, in the reactivities of the lines. The reactivities of the T-cell lines from the MS patients to peptides 82–100, 131–150 and 141–160 did not correlate with expression of the MS-associated DRB1*1501 allele (Hillert, 1994). Two of the lines from the controls (Fig. 2) and 3 of the lines from MS patients were cytotoxic (Fig. 3). The levels of cytotoxicity of a line from a control (Con 11) and of a line from an MS patient (MS 21) are shown in Fig. 5A and 5B respectively. Interestingly, the line from MS 21 exhibited cytotoxicity towards at least 3 different MBP epitopes (peptides 7, 9 and 14/15). All of the cytotoxic lines from controls and MS patients, and all 4 of the analyzed non-cytotoxic lines derived from MS patients were predominantly CD4^+ (mean=85±7%; range 71–98%); none was predominantly CD8^+ (mean=10±5%; range 3–20%).

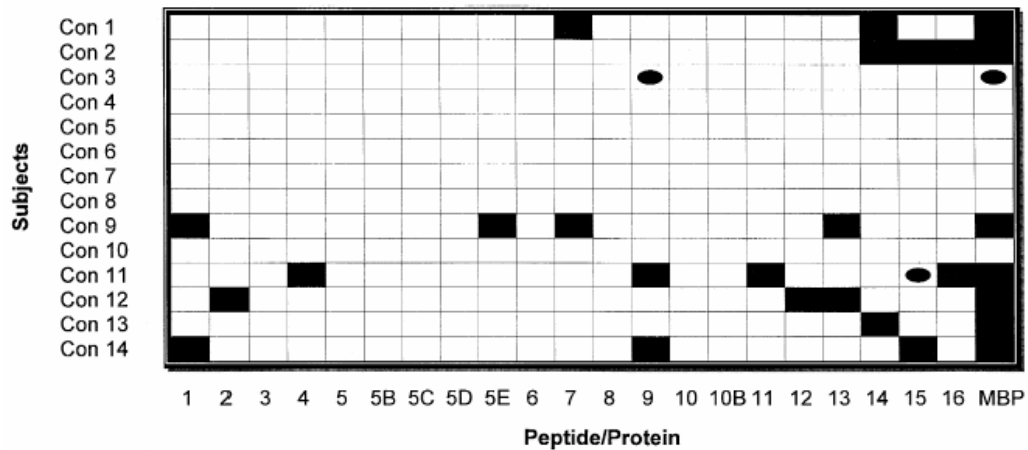


Fig. 2. Whole-MBP-generated T-cell lines from each control were tested for proliferative and cytotoxic responses to whole MBP and to individual peptides. Positive proliferative responses (stimulation index ≥ 2) are shown as black rectangles. T-cell lines showing both positive proliferative responses and positive cytotoxic responses ($>10\%$ peptide-specific lysis) to MBP or a given peptide are designated by black ovals.

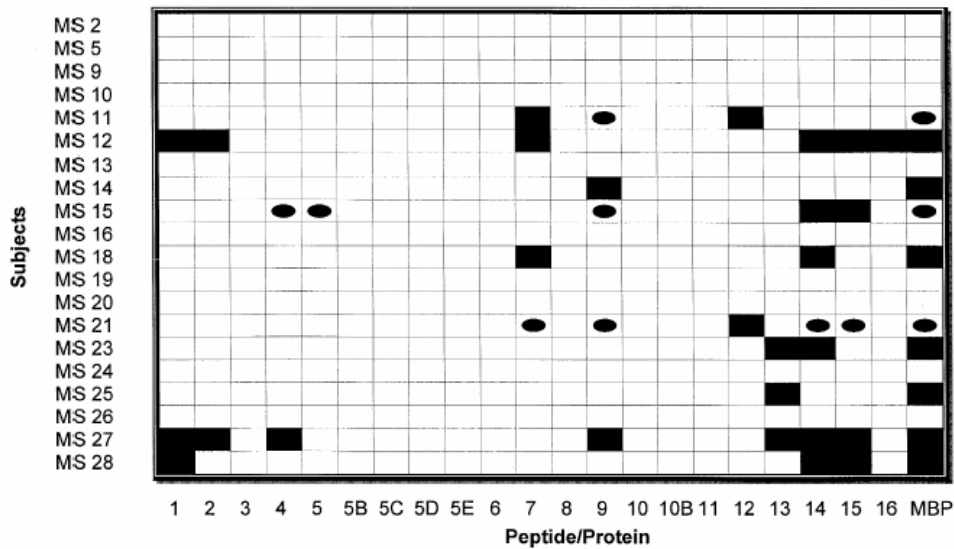


Fig. 3. Whole-MBP-generated T-cell lines from each patient with MS were tested for proliferative and cytotoxic responses to MBP and to individual peptides, as in Fig. 2.

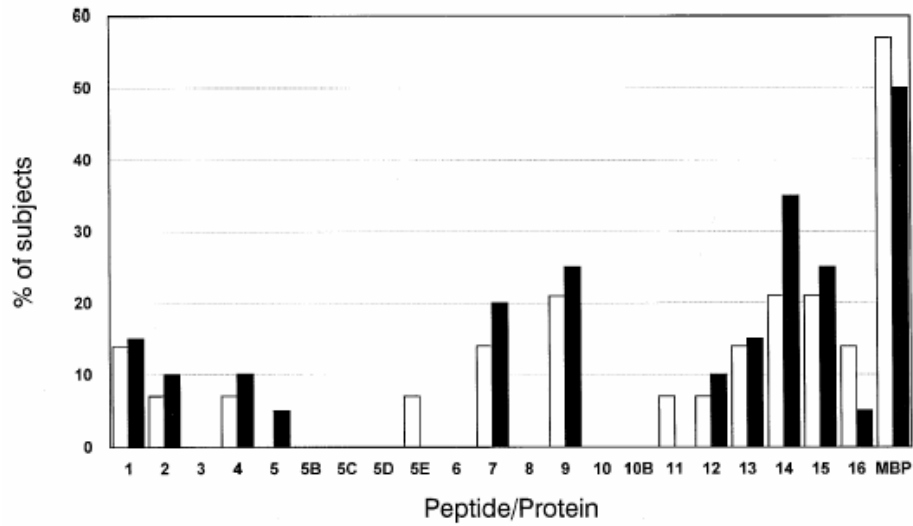


Fig. 4. Percentages of subjects (controls, open bars; MS patients, closed bars) from whom T-cell lines of different specificities (stimulation index ≥ 2) could be successfully generated by stimulation with whole MBP.

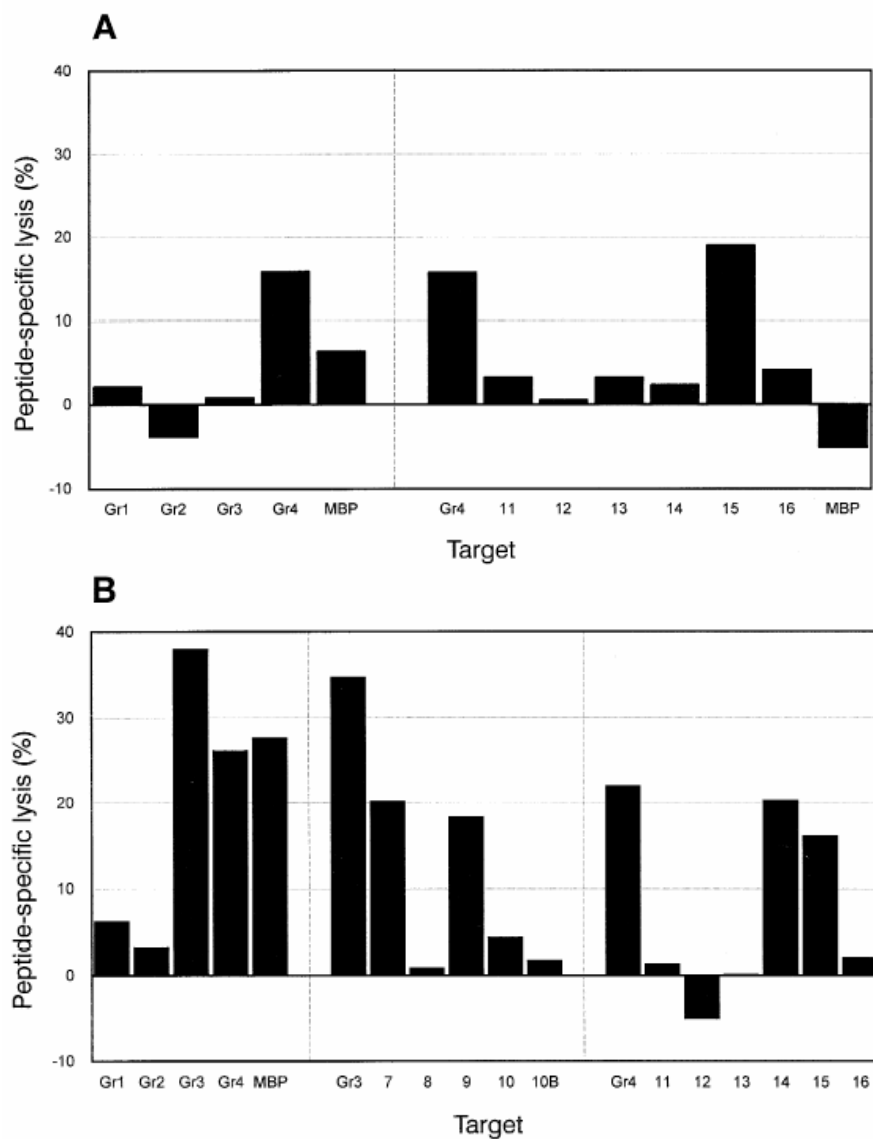


Fig. 5. Peptide-specific lysis (%) by T-cell lines generated by stimulation with whole MBP. Panel A shows the results for a line generated from a control subject (Con 11), and panel B for a line generated

from a patient with MS (MS 21). The lines were first tested against the groups (Gr1–4) of peptides and against MBP. If a positive cytotoxic response (>10% peptide-specific lysis) against a particular group was found, the line was tested against the individual peptides within that group and again tested against the whole group.

3.2. Generation of T-cell lines by stimulation with synthetic MBP peptides

By stimulation with the four groups of synthetic MBP peptides, 30 T-cell lines were derived from the peripheral blood of 14 controls (Fig. 6), and 48 lines were derived from the blood of 19 patients with MS (Fig. 7). The specificities of the lines were diverse, and each of the 21 peptides was recognized by at least one T-cell line. The lines most commonly reacted to peptides 1 (1–20), 5E (C-terminal region of exon-2-encoded segment), 9 (82–100), 11 (101–120), 14 (131–150) and 15 (141–160) (Fig. 8). Many MS patients and controls had T-cells responding to one or more cryptic MBP epitopes, as indicated by the generation of a peptide-specific T-cell line(s) by stimulation with synthetic peptides but not by stimulation with whole MBP (compare Fig. 6 with Fig. 2, and Fig. 7 with Fig. 3). Some of these peptide-generated lines responded to whole MBP as well as to peptide, for example: Con 2, peptide 5; Con 3, peptide 15; Con 5, peptide 2; Con 7, peptide 2; Con 7, peptide 14; MS 11, peptide 11; MS 15, peptide 1; MS 20, peptide 10B. There were no differences, between MS patients and controls, in the reactivities of the lines, except for a higher frequency of lines reactive to peptide 9 (82–100) in the patients with MS, which was not statistically significant. Six of 9 tissue-typed MS patients with T-cell lines reactive to the 82–100 peptide expressed DRB1*1501; however, 5 of 7 tissue-typed MS patients without such lines also expressed this allele. One patient with T-cells reactive to this peptide expressed DRB1*1502. Thirty % of the lines derived from controls (Fig. 6) and 37.5% of those derived from MS patients were cytotoxic (Fig. 7). The levels of cytotoxicity of lines from a control (Con 11) and from an MS patient (MS 13) are shown in Fig. 9A and 9B, respectively. In some cases a T-cell line was found to show cytotoxicity towards the whole group of peptides with which it was generated, but could not be found to demonstrate a positive cytotoxic response against any individual peptide within that group (for example, Fig. 9A, T2). This might be explained by low levels of cytotoxicity against several individual peptides within the group. All the cytotoxic T-cell lines generated from controls and MS patients were predominantly CD4⁺ (mean=84±8%; range 64–97%); none was predominantly CD8⁺ (mean=11±5%; range 1–22%).

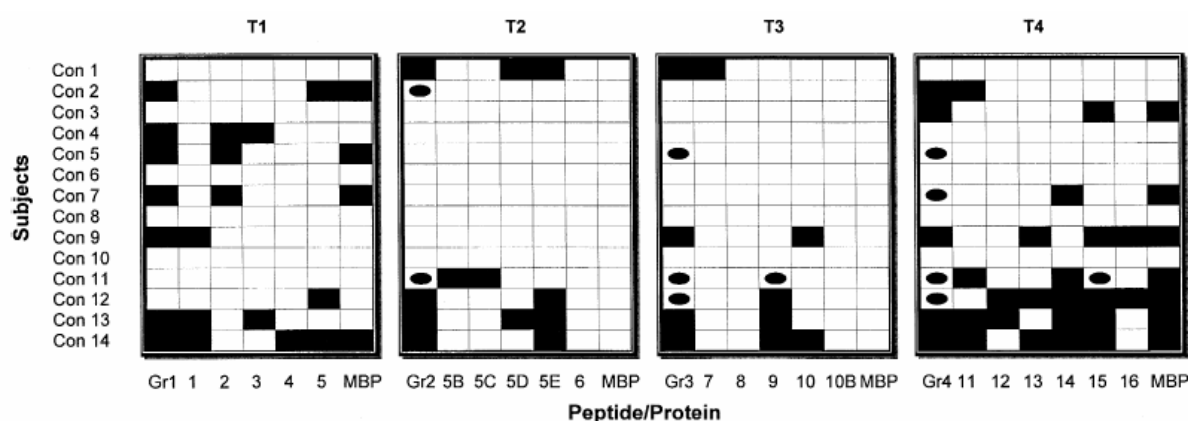


Fig. 6. T-cell lines were generated from each control by stimulation with each of the four groups (Gr1–4) of synthetic peptides. These lines (T1–T4) were tested for proliferative and cytotoxic responses against the whole groups of peptides, peptides within each group and MBP. Positive proliferative responses (stimulation index ≥ 2) are shown as black rectangles. T-cell lines showing both positive proliferative responses and positive cytotoxic responses (>10% peptide-specific lysis) to a given group of peptides, to an individual peptide or to MBP are designated by black ovals.

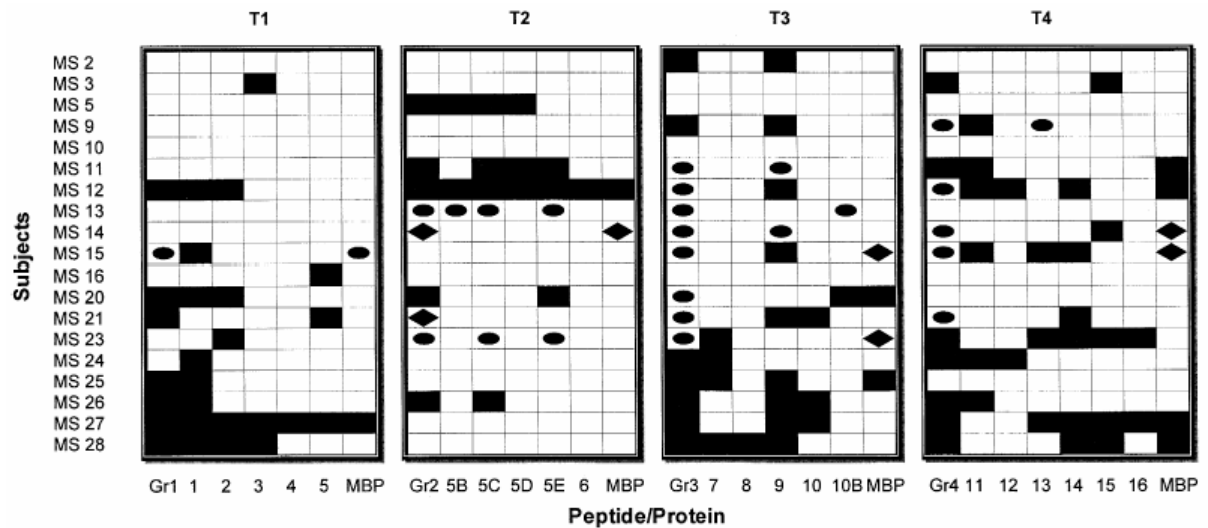


Fig. 7. T-cell lines were generated from each patient with MS by stimulation with each of the four groups (Gr1–4) of synthetic peptides. These lines (T1–T4) were tested for proliferative and cytotoxic responses as in Fig. 6. A black diamond indicates a positive cytotoxic response in the absence of a positive proliferative response.

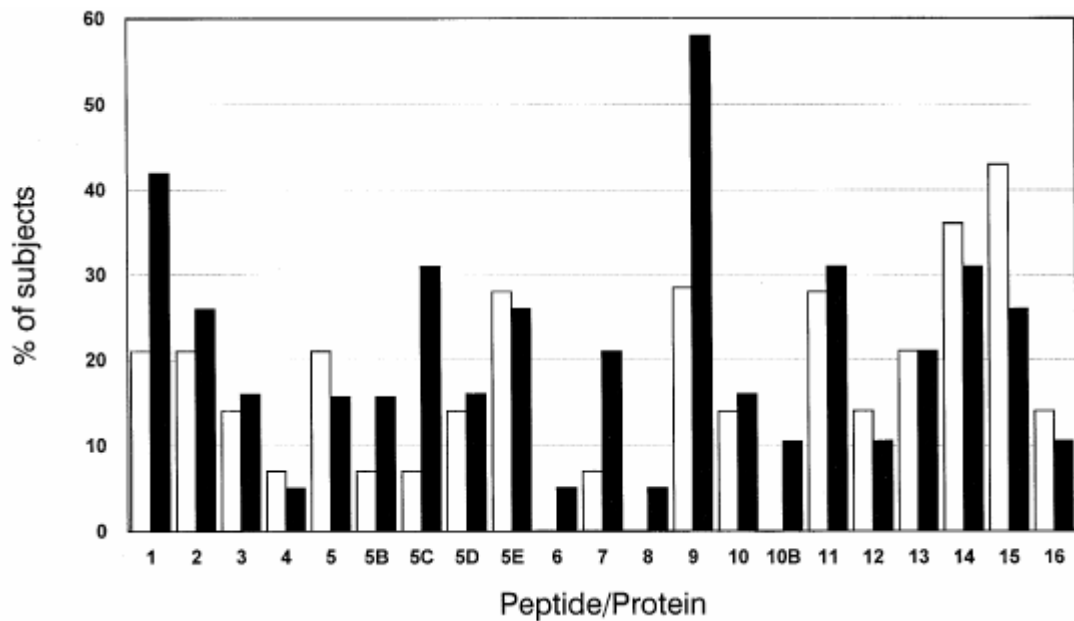


Fig. 8. Percentages of subjects (controls, open bars; MS patients, closed bars) from whom T-cell lines of different specificities (stimulation index ≥ 2) could be successfully generated by stimulation with groups of synthetic MBP peptides.

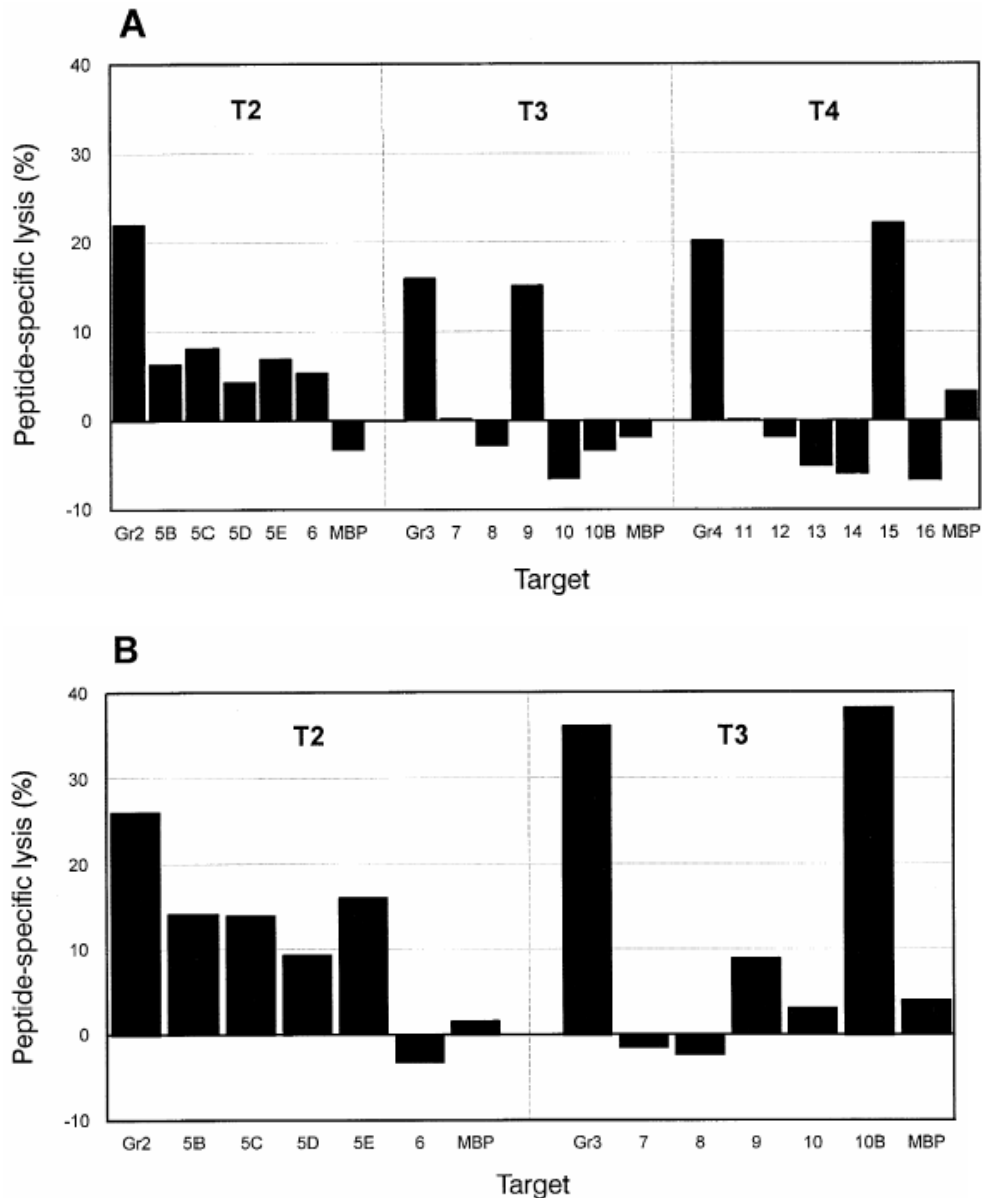


Fig. 9. Peptide-specific lysis (%) by T-cell lines generated by stimulation with groups (Gr1–4) of synthetic MBP peptides. Panel A shows the results for the 3 lines (T2, T3 and T4) generated from a control subject (Con 11), and panel B shows the results for the 2 lines (T2 and T3) generated from a patient with MS (MS 13). Each line was tested against the group of peptides with which it was generated and against the individual peptides within that group, as well as against whole MBP.

To confirm the ability of the technique to induce and maintain the growth of CD8⁺ cytotoxic T-cell lines, a peptide from EBV nuclear antigen 3 (residues 329–353) (Burrows et al., 1990) was used to stimulate repetitively PBMC from an HLA-B8⁺ healthy subject, as previously described (Doolan et al., 1991b). This resulted in the generation of a CD8⁺ T-cell line that induced 31% peptide-specific lysis of autologous target LCLs at an effector/target ratio of 10:1 (not shown).

4. Discussion

In the present study we investigated whether CD8⁺ MBP-specific T-cells could be generated from MS patients and controls, using a method we have previously shown to be able to generate human antiviral and anti-parasite CD8⁺ cytotoxic T-cells (Doolan et al., 1991a and

Doolan et al., 1991b). With this technique we developed 78 T-cell lines reactive to one or more of 21 overlapping synthetic peptides representing the total lengths of the four isoforms of human MBP. 27 of these lines were cytotoxic. All of the cytotoxic lines were predominantly CD4⁺ and none was predominantly CD8⁺. Using techniques similar to ours, other workers have shown that CD8⁺ MBP-specific T-cell lines can occasionally be derived from MS patients and healthy individuals (Inobe et al., 1993; Tsuchida et al., 1994). Tsuchida et al. (1994) were able to generate CD8⁺ T-cell lines reactive to the 110–118 peptide of MBP from 1 of 10 HLA-A2⁺ normal individuals and 1 of 9 HLA-A2⁺ MS patients, after using a computer-based algorithm to predict that this peptide would bind to HLA-A2. One possible explanation for our findings is that the 20-residue peptides bound to MHC class II molecules better than to MHC class I molecules and hence favoured antigen presentation to CD4⁺ T-cells rather than to CD8⁺ T-cells. However, our results suggest that the MBP-reactive T-cell repertoire in MS patients and controls is strongly biased towards CD4⁺ T-cells.

Generally the number of epitopes recognized by peptide-generated T-cell lines from an individual subject was greater than the number recognized by T-cell lines generated by stimulation with whole MBP, although there were occasional subjects from whom no specific lines could be generated by either method of stimulation. Many MS patients and controls had T-cells responding to one or more cryptic MBP epitopes, as indicated by the generation of a peptide-specific T-cell line(s) by stimulation with synthetic peptides but not by stimulation with whole MBP. The more effective generation of lines by stimulation with peptides is explained by the circumvention of the need for antigen processing, as the synthetic peptides can bind directly to MHC molecules on the antigen-presenting cells. The spectrum of MBP epitopes recognized by the peptide-generated lines was diverse, which is consistent with the finding in the Lewis rat of a wide range of cryptic encephalitogenic MBP epitopes (Mor and Cohen, 1995).

Using the method of peptide stimulation we were also able to generate T-cell lines reactive to epitopes present in the minor 21.5 kDa and 20.2 kDa isoforms of human MBP (in the exon-2-encoded region) but not present in the common 18.5 kDa isoform. Such lines could be generated from both MS patients and controls, as previously reported by Voskuhl et al. (1993a) and Voskuhl et al. (1993b). We were also able to generate, from 2 MS patients, T-cell lines reactive to a novel MBP epitope (peptide 10B) present in the 20.2 and 17.3 kDa isoforms but not in the 21.5 or 18.5 kDa isoforms. As the 21.5, 20.2 and 17.3 kDa isoforms are expressed in the developing human CNS (Roth et al., 1987), it has been suggested that they may also be expressed by remyelinating oligodendrocytes in MS lesions (Voskuhl et al., 1993a).

We found that the most common immunodominant epitopes revealed by generation of T-cell lines by stimulation with whole MBP were within the 82–100 and 131–160 regions, as previously reported by others (Martin et al., 1990; Ota et al., 1990; Zhang et al., 1990 and Zhang et al., 1992; Liblau et al., 1991). However, we also found that other regions, particularly 1–20, 61–80 and 122–140, contained immunodominant epitopes in some MS patients and healthy individuals.

Our study did not reveal any significant differences between MS patients and controls in reactivity to MBP epitopes within any of the isoforms of MBP. However, this does not rule out a role for MBP-specific T-cells in the pathogenesis of MS, as differences in activation status or cytokine production would not have been detected by our methods.

Acknowledgements

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