

Visualization of Myelin Basic Protein (MBP) T Cell Epitopes in Multiple Sclerosis Lesions using a Monoclonal Antibody Specific for the Human Histocompatibility Leukocyte Antigen (HLA)-DR2-MBP 85–99 Complex

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Abstract

Susceptibility to multiple sclerosis (MS) is associated with the human histocompatibility leukocyte antigen (HLA)-DR2 haplotype, suggesting that major histocompatibility complex class II-restricted presentation of central nervous system-derived antigens is important in the disease process. Antibodies specific for defined HLA-DR2-peptide complexes may therefore be valuable tools for studying antigen presentation in MS. We have used phage display technology to select HLA-DR2-peptide-specific antibodies from HLA-DR2-transgenic mice immunized with HLA-DR2 molecules complexed with an immunodominant myelin basic protein (MBP) peptide (residues 85–99). Detailed characterization of one clone (MK16) demonstrated that both DR2 and the MBP peptide were required for recognition. Furthermore, MK16 labeled intra- and extracellular HLA-DR2-MBP peptide complexes when antigen-presenting cells (APCs) were pulsed with recombinant MBP. In addition, MK16 inhibited interleukin 2 secretion by two transfectants that expressed human MBP-specific T cell receptors. Analysis of the structural requirement for MK16 binding demonstrated that the two major HLA-DR2 anchor residues of MBP 85–99 and the COOH-terminal part of the peptide, in particular residues Val-96, Pro-98, and Arg-99, were important for binding. Based on these results, the antibody was used to determine if the HLA-DR2-MBP peptide complex is presented in MS lesions. The antibody stained APCs in MS lesions, in particular microglia/macrophages but also in some cases hypertrophic astrocytes. Staining of APCs was only observed in MS cases with the HLA-DR2 haplotype but not in cases that carried other haplotypes. These results demonstrate that HLA-DR2 molecules in MS lesions present a myelin-derived self-peptide and suggest that microglia/macrophages rather than astrocytes are the predominant APCs in these lesions.

Key words: multiple sclerosis • antigen presentation • myelin basic protein • autoimmunity • microglia

Introduction

Multiple sclerosis (MS)¹ is a chronic inflammatory demyelinating disease of the central nervous system (CNS) with

¹Abbreviations used in this paper: CNS, central nervous system; GFAP, glial fibrillary acidic protein; HA, hemagglutinin; LAMP, lysosome-associated membrane protein; MBP, myelin basic protein; MS, multiple sclerosis; PLP, proteolipid protein.

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unknown etiology. Inherited susceptibility to MS is associated with the HLA class II genes DRB1*1501, DRB5*0101, and DQB1*0602, which are all contained in the DR2 haplotype (1). Because these three genes are in strong linkage disequilibrium, it has not been possible to determine which of them are the principal MS risk genes. Any of these three genes or combinations of them could mediate the MHC class II-associated susceptibility to MS. The exact role for the disease-associated MHC class II molecules in the pathogenesis of MS is not clear. One prevailing hypothesis is that they present self-antigens to auto-aggressive T cells that enter the CNS and induce an inflammatory response that leads to clinical manifest disease (2, 3).

The target autoantigens are unknown in MS, but, largely based on their encephalitogenicity in animal models for this disease, myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein are the major candidates (for review see references 3 and 4). Investigations of the immune response against CNS-derived proteins in MS patients have largely focused on MBP as a candidate antigen. The MBP 84–103 peptide has been defined as the strongest binder of all MBP peptides to the disease-associated HLA-DR2 molecules and is recognized by T cell clones from patients and normal individuals with the HLA-DR2 haplotype (5–7). Several lines of evidence indicate, however, that T cells recognizing this epitope are only in vivo activated in MS patients (6, 8–10). The minimal epitope for HLA-DR2-restricted T cell recognition has been mapped to residues 85–99 of MBP (7). T cell lines against other regions of MBP have also been generated, although less frequently (5, 6, 11–15).

The identity of the cells in the CNS that present myelin-derived autoantigens to CD4⁺ T cells is not clear. Phenotypic and functional analyses have implicated both microglia/macrophages and astrocytes (for review see references 16–18). To directly examine MHC class II-restricted presentation of a myelin antigen in MS lesions, we have generated an mAb (MK16) that is specific for the HLA-DR2 (DRB1*1501)-bound MBP 85–99 peptide. Here we describe the generation and a detailed characterization of this antibody and demonstrate how it can be used to detect HLA-DR2–MBP 85–99 peptide complexes intra- and extracellularly when APCs have been incubated with native MBP. This antibody was also used to examine antigen presentation in MS lesions and normal brain tissue. The antibody specifically labeled microglia/macrophages and in some cases hypertrophic astrocytes from patients with the HLA-DR2 haplotype, whereas these cells were not stained in lesions from HLA-DR2–negative patients.

Materials and Methods

Cell Lines. *Drosophila melanogaster* Schneider 2 (S2) cell transfectants expressing either DRA1*0101/DRB1*1501 (DRB1*1501) or DRA1*0101/DRB1*0401 (DRB1*0401) molecules were grown at 25°C in Schneider's medium (Sigma Chemical Co.) supplemented with 10% heat-inactivated FCS, 2

mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The following transfectants and T cell hybridomas were all grown at 37°C in DMEM (Sigma Chemical Co.) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM β-ME, and 1% nonessential amino acids: L cell transfectants expressing either DRA1*0101/DRB5*0101 (DRB5*0101) or DRB1*1501 molecules, the mouse BW 58 TCR-α/β T cell hybridoma (19), and four T cell transfectants expressing DRB1*1501-restricted human/mouse chimeric α/β TCR. Two of these T cell transfectants recognize a human MBP-derived peptide corresponding to residues 85–99 (Hy.2E11 and Ob.1A12; reference 20 and our unpublished data), and the remaining two transfectants recognize PLP-derived sequences corresponding to residues 40–60 (5C6) and 95–116 (106J), respectively (our unpublished data).

The following EBV-transformed human B cell lines and mouse T cell hybridomas were all grown at 37°C in RPMI (Sigma Chemical Co.) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM β-ME: Vavy expressing DR3 molecules (DRA1*0101/DRB1*0301), Priess expressing DRB1*0401 molecules, and MGAR expressing DR2 molecules (DRB5*0101 and DRB1*1501); and two T cell hybridomas expressing DRB1*0401-restricted mouse α/β TCR recognizing either a human type II collagen-derived epitope corresponding to residues 261–273 (CII 261–273; hybridoma CII 3838) or an influenza A hemagglutinin (HA)-derived epitope corresponding to residues 307–319 (HA 307–319; hybridoma HA 3.3) (21).

Antigens. Recombinant human MBP was expressed in *Escherichia coli* using a cDNA construct with a COOH-terminal (His)₆-tag cloned into vector pET22b (Novagen). Expression was induced by addition of isopropyl-β-d-thiolactopyranoside (IPTG; 1 mM) to the growth media. The protein was purified from lysed bacteria by metal-chelate chromatography, followed by further purification using cation exchange HPLC (POROS CM column; Perseptive Biosystems). The purified protein was dialyzed against 0.01 N HCl and concentrated by vacuum dialysis.

Peptides were synthesized by Fmoc chemistry (Schafer-N, Denmark; Microchemistry Facility, Harvard University); purity (>95%) was verified by reverse-phase HPLC, and integrity was verified by mass spectrometry. The following peptides were synthesized: MBP 85–99 (ENPVVHFFKNIVTPR) and monosubstituted and -truncated analogues derived from this sequence; an HLA-DQw6 peptide corresponding to residues 43–58 (DQw6 43–58) (DVGYYRAVTPQGRPDA); CII 261–273 (AGFKGEQGPKGEP); HA 307–319 (PKYVKQNTLKLAT); an influenza A HA peptide corresponding to residues 161–175 (HA 161–175) (YRNLVWFIKKNTRYYP); a human Ig κ peptide corresponding to residues 145–159 (SK 145–159) (KVQWKVDNALQSGNS); and two human PLP peptides, PLP 40–60 (TGTEKLIETYFSKNYQDYEYL) and PLP 95–116 (AVRQIFGDYKTTICGKGLSATV).

Transfection, Purification, and Functional Analysis of DR Molecules from S2 Cells. S2/DRB1*1501 and S2/DRB1*0401 molecules were produced as previously described (22). In brief, cDNA encoding DRA1*0101, DRB1*1501, and DRB1*0401 was separately cloned into the expression vector, pmtal, and pairwise cotransfected with a third vector, pcohygro (encoding hygromycin B resistance), into S2 cells using lipofection according to the manufacturer's instructions (GIBCO BRL). S2 cells expressing the transfected constructs were selected and single-cell cloned. After CuSO₄ induction, detergent-solubilized HLA class II mole-

cules were purified using affinity chromatography. The purified protein was characterized by SDS-PAGE followed by silver staining. The total protein concentration was determined by bicinchoninic acid assay (Sigma Chemical Co.) using BSA as the reference protein. Peptide binding and inhibition assays with the purified S2/DRB1*1501 and S2/DRB1*0401 molecules were performed essentially as described in detail elsewhere (21, 22).

Generation and Purification of HLA–Peptide Complexes. A variety of different HLA class II–peptide complexes were generated by incubating detergent-solubilized and affinity-purified DRB1*1501 or DRB1*0401 molecules with 50–100× molar excess of different peptides at 25°C for 48–72 h in the presence of a protease inhibitor mixture (Complete; Boehringer Mannheim) and 0.1 M sodium citrate phosphate buffer, pH 6.0. After incubation, the HLA–peptide complexes were subjected to a buffer exchange and separated from free peptide by gel filtration on Sephadex G-25 spin columns containing 0.1% NP-40 in PBS as previously described (22).

Analysis of Antibody and Fab Specificity by ELISA. HLA–peptide specificity of serum from mice immunized with DRB1*1501–peptide complexes and the resulting antigen binding fragment (Fab)–phages or purified Fab molecules were analyzed by ELISA. Purified HLA–peptide complexes were diluted in PBS to a final concentration of 0.1 µg/ml and coated onto 96-well microtiter plates (Maxisorp; Nunc, Inc.) followed by blocking with 2% skim milk in PBS. All washes between incubations were performed with 0.05% Tween-20 in PBS. Serum, Fab–phages, or purified Fab molecules were diluted in blocking buffer, added to the microtiter wells, and incubated for 2 h at room temperature. Plates were washed, incubated with biotin-labeled secondary antibodies for 1 h at 4°C, washed again, and incubated for an additional 1 h at room temperature with Eu³⁺-labeled streptavidin (Wallac) in assay buffer (Wallac). Europium was released by adding 100 µl per well of enhancement solution (Wallac), and fluorescence was measured in a time-resolved fluorometer (Wallac).

To determine the fine specificity of MK16, varying concentrations of DRB1*1501 molecules incubated with monosubstituted or truncated MBP peptide analogues were added as competitors before the addition of MK16 Fab–phages to microtiter wells coated with DRB1*1501–MBP 85–99 peptide complexes. The concentration of HLA–peptide complex that prevented 50% of the Fab–phages from binding to DRB1*1501–MBP 85–99 peptide complexes was the IC₅₀ (50% inhibiting dose) value. In a preliminary experiment, the Fab–phages were titrated against a fixed amount of DRB1*1501–MBP 85–99 peptide complex to determine the concentration of Fab–phages corresponding to 10–20% of the maximal response. All competition assays were performed using these Fab–phage concentrations.

Immunization and Fab Phage Library Construction. We used a previously described method for displaying antibody (Fab fragments) libraries on phages (23, 24). In brief, by using PCR-based technology, Fab encoding genes derived from IgG cDNA from immunized mice are amplified and assembled into a bicistronic operon, which is inserted into a phagemid vector under transcriptional control and transformed into *E. coli*. By using this cloning strategy, libraries containing >10⁸ potentially different clones can be generated. After superinfection with a helper phage, the infected bacteria produces recombinant phage particles, where each phage represents a unique antibody encoded by genes present within the same phage particle. The displayed antibodies on phage particles allow for purification of antigen-specific phages by repeated rounds of selection (panning) on a selected target fol-

lowed by amplification in bacterial cells of the eluted bound phages.

Transgenic mice expressing DRB1*1501 molecules on a DBA1/J background (20) and BALB/c mice (State Serum Institute, Denmark) were immunized with DRB1*1501–MBP 85–99 peptide complexes as previously described (24). Sera were harvested from immunized mice and tested for the presence of DRB1*1501–MBP 85–99 peptide-specific antibodies by ELISA. Mice producing such specificities were used to generate phage display libraries of antibody Fab-fragments.

The Fab encoding genes were PCR amplified from cDNA and assembled using a comprehensive sets of primers corresponding to the Fab region of the heavy F_D and L κ chains. The resulting Fab fragments were digested with SfiI and NotI, purified from agarose gels, and ligated into a phagemid vector, pFab 5c.His, digested with the same enzymes to generate V_H and F_D phagemid libraries (23). The resulting phagemid libraries were transformed into electrocompetent *E. coli* TOP10F' cells (British Biotechnology), and transformed cells were propagated in LB (Luria-Bertani) medium containing 1% glucose, 100 µg/ml ampicillin, and 20 µg/ml tetracycline. Phage libraries displaying Fab fragments were generated by superinfection of exponentially growing phagemid libraries with helper phage (R408; Stratagene Inc.) followed by induction with 1 mM IPTG and amplified by an overnight incubation at room temperature. The resulting Fab phages were purified by polyethylene glycol precipitation and titrated with *E. coli* TOP10F' cells (23).

Selection by Biopanning. Panning selections were performed using 1.5 × 10⁷ sulfate latex beads (diameter 5 µm; Interfacial Dynamics) coated with a 0.5 µM solution of purified DRB1*1501–MBP 85–99 peptide complexes. After blocking with 5% BSA in PBS, 300 µl phage library containing 10¹¹–10¹² CFU in 5% BSA, 0.1% NP-40 in PBS was added to the coated beads and incubated for 4 h at 4°C with end-over-end rotation followed by three to five washes with 5% BSA in PBS. After the first round of panning, the subsequent rounds of panning were performed in the presence of DRB1*1501 molecules at a concentration of 1 µM. Bound phages were eluted and used to infect exponentially growing *E. coli* TOP10F' cells, followed by superinfection with helper phage (R408; Stratagene Inc.) and induction with IPTG and then overnight incubation and phage precipitation before initiating a new panning round (23, 24).

Expression and Purification of Soluble MK16 Fab Molecules. The F_D (H chain of the Fab fragment) and κ L chain encoding regions were recloned by PCR into the T7 polymerase-driven expression vector pYR. Inclusion bodies of F_D and L chain were purified, solubilized, and refolded in a redox-shuffle buffer. Correctly folded Fab molecules were purified by ion exchange (Q- and SP-Sepharose) and size exclusion chromatography as previously described (25).

FACS[®] Analysis. For flow cytometry analysis, ~10⁵ cells were incubated with primary antibody for 1 h at 4°C in FACS buffer (PBS containing 5% FCS and 0.1% NaN₃). After two washes with FACS buffer, secondary biotin-labeled antibodies were added for 1 h at 4°C, and the cells were washed again and incubated for 1 h at 4°C with R-PE-labeled streptavidin (Becton Dickinson). To quantitate DR expression, cells were incubated directly with a biotin-labeled anti-HLA-DR mAb (mAb L243; Becton Dickinson), followed by incubation with R-PE-labeled streptavidin as described above. Cells were analyzed on a FAC-Scan™ flow cytometer using CELLQuest™ software (Becton Dickinson). A total of 10,000 cells per sample were analyzed, and the viable cells were gated by light scatter.

Preparation and Isolation of Adherent Mononuclear Cells from Blood. 50 ml of peripheral blood was obtained from a DRB1*1501 homozygous donor. PBMCs were immediately separated by density gradient centrifugation on Ficoll (Lymphoprep; Nycomed), washed three times, and resuspended in tissue culture medium consisting of RPMI (Sigma Chemical Co.) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM β-ME, counted, and adjusted to 10⁶ cells/ml. Next, the mononuclear cells were incubated in culture flasks for 2 h at 37°C and, after removal of the supernatant, the remaining adherent cells were isolated, washed twice with culture medium, and incubated with peptides for 2 h at 37°C followed by FACS[®] analysis.

T Cell Stimulation Assay. Using round-bottomed microtiter plates, 2 × 10⁴ T cell hybridomas were cocultured in the presence of irradiated APCs (5 × 10⁴ per well; MGAR and L cell transfectants expressing DRB1*1501 and Pries expressing DRB1*0401 molecules) and dilutions of recombinant MBP or various peptides. After incubation for 24 h at 37°C, 100 µl of the supernatant was tested for IL-2 in an IL-2-specific sandwich ELISA using Eu³⁺-labeled streptavidin as detection system, as previously described (21).

In separate experiments, purified DRB1*1501-peptide complexes were coated directly to microtiter wells in serial dilutions and used to stimulate T cell hybridomas. Microtiter wells were blocked by 5% FCS in PBS and washed with 0.05% Tween-20 in PBS before incubation with 2 × 10⁴ T cell hybridomas at 37°C for 24 h. T cell stimulation was determined by using the IL-2 ELISA as described above.

Analysis by Confocal Laser Scanning Microscopy. L cells expressing DRB1*1501 were seeded onto Chamber Slides (Nalge Nunc International) and grown at 37°C overnight and then cultured with or without peptides or recombinant MBP at 37°C for 2 or 4 h. Cells were washed twice with culture medium and twice in PBS, followed by fixation with 0.5% paraformaldehyde in PBS for 20 min. After another wash with PBS, the cells were permeabilized with 0.2% saponin (Sigma Chemical Co.) for 30 min at room temperature. Cells were blocked with PBS containing 2% BSA and 0.2% saponin (blocking solution) for 30 min at room temperature, washed, and simultaneously incubated with MK16 Fab and rat anti-mouse CD107a antibody (lysosome-associated membrane protein [LAMP]-1; PharMingen) diluted in blocking solution overnight at 4°C. The cells were washed four times with blocking solution and incubated with FITC-labeled goat anti-mouse IgG (Fab specific) absorbed against rat serum proteins (Sigma Chemical Co.) and Texas Red-labeled F(ab')₂ fragment goat anti-rat IgG antibodies (Fc fragment specific; Jackson ImmunoResearch Labs.) diluted in blocking solution for 1 h at room temperature. After three washes in PBS, chamber slides bearing cells were mounted in Slowfade[™] (Molecular Probes, Inc.) antifade reagent in 50% glycerol, and staining of cells was evaluated using an MCR-1024 confocal laser scanning microscope (Bio-Rad Labs.) attached to a Zeiss Axiovert 100 TV. Green fluorescence was detected at wavelength 520–560 nm after excitation at wavelength 488 nm. Red fluorescence was detected at wavelength 585 nm after excitation at wavelength 568 nm.

Immunocytochemistry. Frozen sections were fixed in acetone for 10 min and stained using either the avidin-biotin-peroxidase technique (ABC; Vector Labs.) or double immunofluorescence. Overnight incubations at 4°C were performed with the following mAbs: MK16, pan-reactive anti-HLA class II antibody to HLA-DP-DQ-DR, anti-CD14 (microglia of the monocyte lineage,

monocytes and macrophages; DAKO Corp.), CD3 (pan T cells; Becton Dickinson), and anti-glial fibrillary acidic protein (GFAP) (astrocytes; Roche Molecular Biochemicals). The chromogen used was 3,3-diaminobenzidine for bright field microscopy and, for immunofluorescence, secondary mouse antibodies labeled with Alexa 568, which is an anti-mouse IgG antibody (Molecular Probes, Inc.), and Cy-2, which is an anti-mouse Fab fragment (Jackson ImmunoResearch Labs.). Slides were scored in a blinded fashion by two observers on a scale of 0–4 based on the degree of positivity.

Results

The goal of this study was to generate mAbs specific for the complex of HLA-DR2 (DRB1*1501) and the immunodominant human MBP 85–99 peptide. Such antibodies are difficult to generate, as many antibodies elicited by immunization are directed against the HLA molecule and do not require a specific peptide for recognition. We therefore immunized HLA-DR2 (DRA1*0101, DRB1*1501) transgenic mice, because tolerance to the HLA-DR2 molecule might favor the production of antibodies specific for the HLA-DR2-MBP peptide complex. To efficiently select for potentially rare clones, antibodies were isolated by phage display technology rather than conventional hybridoma technology.

Purification and Functional Characterization of Purified DRB1*1501 Molecules. It was important to efficiently load DRB1*1501 molecules with the MBP 85–99 peptide for immunization of DRB1*1501 transgenic mice. As HLA class II molecules that are occupied by low-affinity endogenous peptides or devoid of peptides can be produced in insect cells (for example, see references 22 and 26), DRB1*1501 molecules were expressed in S2 cells using full length DRA1*0101 and DRB1*1501 cDNAs. Detergent-solubilized DRB1*1501 molecules from these cells were purified by affinity chromatography and characterized by SDS-PAGE as well as by peptide binding and T cell stimulation assays (data not shown). SDS-PAGE analysis resolved the individual DRα and DRβ chains when the sample was heated and the heterodimer of ~52 kD when the sample was not heated before loading. Specific and dose-dependent inhibition of peptide binding was observed with the MBP 85–99, DQw6 43–58, PLP 40–60, and PLP 95–116 peptides, which are known to bind DRB1*1501 molecules (7, 15, 27, 28), whereas no inhibition was seen with SK 145–159, which binds to DRB1*0401 molecules (data not shown). MBP 85–99 peptide bound DRB1*1501 molecules with an IC₅₀ of 5 nM; the IC₅₀ values for the DQw6 43–58, PLP 40–60, and PLP 95–116 peptides were 70, 200, and 1,480 nM, respectively. DRB1*1501-MBP peptide complexes, but not other DRB1*1501-peptide complexes, stimulated IL-2 production by a DRB1*1501-restricted T cell hybridoma specific for the MBP peptide. Likewise, only DRB1*1501-PLP 40–60 and DRB1*1501-PLP 95–116 complexes, respectively, stimulated the two PLP-specific T cell hybridomas (data not shown). Taken together, these data demonstrated that the purified

DRB1*1501 molecules were functional with respect to binding of selected peptides and subsequent T cell stimulation.

Immunization Strategy. Transgenic mice expressing HLA-DRA*0101/DRB1*1501-encoded molecules on a DBA1/J background (H-2^a) and nontransgenic BALB/c (H-2^d) mice were Bacillus Calmette-Guerin (BCG) primed and immunized with purified DRB1*1501-MBP 85-99 peptide complexes coupled to tuberculin purified protein derivative. This immunization scheme has been successfully used to generate MHC-peptide-specific antibodies (24, 29). The syngenic immunization strategy was chosen to facilitate the generation of antibodies that specifically recognize the DRB1*1501-MBP 85-99 peptide complex, rather than pan-reactive anti-DR antibodies. Nontransgenic BALB/c mice were also immunized to directly address this issue. Four mice of each type were immunized, and two of four syngenic immunizations, but none of the allogenic immunizations, elicited an antibody response to the DRB1*1501-MBP 85-99 peptide complex that could be detected in the serum and distinguished from the pan-reactive anti-DR response. In one of these mice (DRB1*1501-1), the serum exhibited a 50-fold higher reactivity with DRB1*1501-MBP 85-99 peptide complexes than with "empty" DRB1*1501 molecules or DRB1*1501 with a control peptide (Fig. 1), whereas this difference was five-fold in the other mouse (DRB1*1501-2; data not shown). Splens from such mice were used to generate phage libraries displaying Fab molecules on phage particles.

Construction of Phage Display Library and Panning Approach. The resulting Fab-phage library consisted of 1.7×10^8 independent clones and was panned on immobilized DRB1*1501-MBP 85-99 peptide complexes. Soluble, empty DRB1*1501 molecules were added as competitors to neutralize pan-specific anti-DR antibodies that might also have been elicited. Fab-phages isolated after each round of panning were analyzed for binding to DRB1*1501-MBP 85-99 peptide complexes, DRB1*1501-DQw6 43-58 peptide complexes, or empty DRB1*1501 molecules by ELISA. As illustrated in Fig. 2, Fab-phages recognizing DRB1*1501-MBP 85-99 peptide complexes were successively enriched during panning rounds 1 to 5, whereas pan-reactive DRB1*1501 Fab-phages were effectively removed during rounds 4 and 5. Fab-phages produced from randomly picked clones isolated after the fourth and fifth rounds of panning were analyzed by ELISA, and 79 of 175 (45%) Fab-producing clones reacted strongly with DRB1*1501-MBP 85-99 peptide complexes but not with DRB1*1501-DQw6 43-58 peptide complexes or empty DRB1*1501 molecules. 61 clones (35%) had the same specificity but reacted more weakly. 9 clones (5%) were not specific for the DRB1*1501-MBP peptide complex but also reacted with DRB1*1501-DQw6 43-58 complexes and with empty DRB1*1501 molecules. The remaining 26 clones (15%) did not bind to DRB1*1501 molecules in complex with MBP 85-99, DQw6 43-58 peptides, or empty DRB1*1501 molecules.

Restriction fragment length polymorphism analysis showed that the clones that were specific for the

DRB1*1501-MBP 85-99 peptide complex could be divided into 10 different groups. One clone from each group was reanalyzed by ELISA for reactivity to DRB1*1501-MBP 85-99 peptide complexes, DRB1*1501-DQw6 43-58 peptide complexes, and empty DRB1*1501 molecules, as well as DRB1*0401 molecules in complex with HA 307-319 or CII 261-273 peptides. All 10 clones reacted

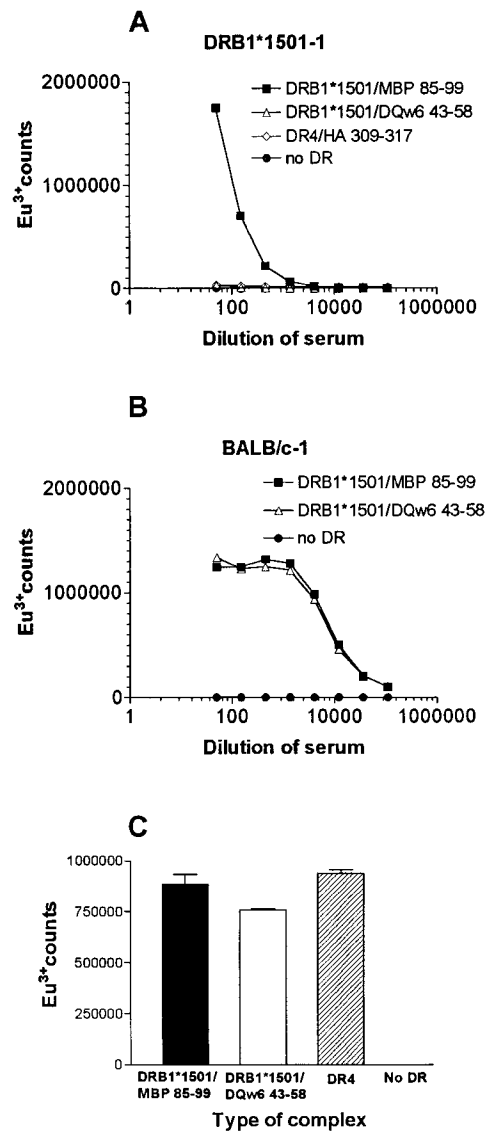


Figure 1. Immunizations of transgenic mice with purified DRB1*1501-MBP 85-99 peptide complexes elicit antibodies specific for the HLA-DRB1*1501-MBP peptide complex. After three immunizations, sera from (A) transgenic mice or (B) BALB/c mice were analyzed by ELISA for the presence of antibodies against the DRB1*1501-MBP peptide complex. Microtiter wells were coated with purified DRB1*1501-MBP 85-99, DRB1*1501-DQw6 43-58, or DRB1*0401-HA 307-319 complexes or without complexes. Bound antibodies were detected with biotin-labeled rabbit anti-mouse Igs. Each symbol represents a single determination. (C) The amount of DR-peptide complex immobilized was quantitated with biotin-labeled mAb L243. The mean and SEM ($n = 2$) are shown.

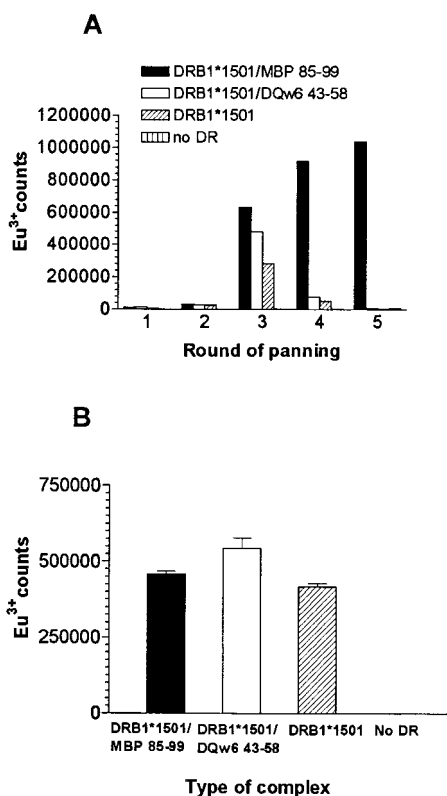


Figure 2. Competition biopanning selects for Fab-phages that bind specifically to the DRB1*1501-MBP 85-99 complex. (A) A pool of phages from panning rounds 1-5 was isolated, and DRB1*1501-peptide specificity was assayed by ELISA. Microtiter wells were coated with purified DRB1*1501 molecules in complex with MBP 85-99 or with DQw6 43-58 peptides, without peptide, or without DR molecules. Bound phages were detected with biotin-labeled rabbit anti-fd bacteriophage. Bars illustrate a representative set of data. (B) The amount of immobilized DRB1*1501-peptide complex was quantitated by biotin-labeled mAb L243. The mean and SEM ($n = 2$) are shown.

only with the DRB1*1501-MBP 85-99 peptide complexes (data not shown). These 10 clones were next analyzed in a FACS[®] assay, and five of the clones, which represented five different groups, reacted significantly better with MGAR cells (expressing DRB5*0101 and DRB1*1501 molecules) incubated with MBP 85-99 peptide than with MGAR cells incubated with a control peptide or no peptide. The other five clones did not make this distinction (data not shown).

The DNA sequences of the H and L chain CDRs of the five clones that were specific for the DRB1*1501-MBP 85-99 peptide complex were determined. The CDR-H3 regions were identical, but minor amino acid differences were present in the other CDR regions (data not shown). The IC₅₀ values of the individual clones were determined by ELISA, and no significant differences in affinity were observed (data not shown). These data demonstrated that these phages represented individual clones and that a strong selection for a particular H chain rearrangement had occurred.

Specificity of Purified MK16. Soluble Fab molecules were produced from one DRB1*1501-MBP 85-99-spe-

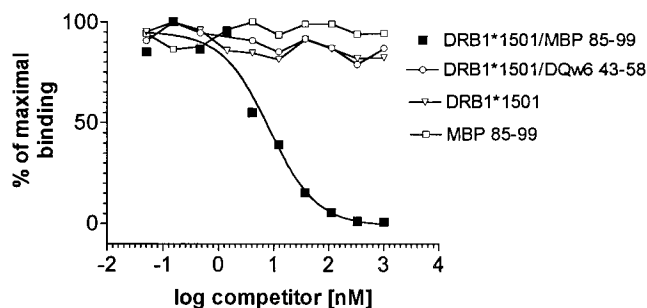


Figure 3. Binding characteristics of MK16 by competition ELISA. Microtiter wells coated with DRB1*1501-MBP 85-99 peptide complexes were incubated with a fixed amount of MK16 Fab-phages and various concentrations of soluble DRB1*1501-MBP 85-99 peptide complexes, DRB1*1501-DQw6 43-58 peptide complexes, DRB1*1501 molecules, or MBP 85-99 peptide. Bound MK16 Fab-phages were detected with biotin-labeled rabbit anti-Fd bacteriophage by ELISA. Each symbol represents a single determination.

cific clone (MK16), because it showed the highest reactivity by FACS[®] analysis (data not shown). In a competition assay, binding of MK16 to immobilized DRB1*1501-MBP 85-99 peptide complexes was inhibited in a specific and dose-dependent manner by soluble DRB1*1501-MBP 85-99 peptide complexes (Fig. 3), but neither component could inhibit alone, nor could irrelevant DRB1*1501-DQw6 43-58 complexes.

It was important to demonstrate that the antibody also recognized DRB1*1501-MBP 85-99 peptide complexes expressed by mammalian cells. Significant staining with MK16 was seen when L cells expressing DRB1*1501 (Fig. 4 A) and adherent mononuclear cells from a DRB1*1501-positive donor had been cultured with MBP 85-99 peptide (Fig. 5), but not when these cells had been incubated with DQw6 43-58 peptide or media only. No significant staining was seen with L cells that expressed DRB5*0101 molecules and had been incubated with MBP 85-99 peptide, DQw6 43-58 peptide, or no peptide (Fig. 4 B).

It was also important to determine if MK16 could recognize DRB1*1501-MBP peptide complexes produced by intracellular processing of native MBP. MK16 stained MGAR cells incubated with recombinant MBP almost at the same level of intensity as MGAR cells incubated with MBP 85-99 peptide (Fig. 6). No specific staining was seen with MGAR cells that presented endogenous peptides or DQw6 43-58. We also examined EBV-transformed human B cell lines that express DRB1*0301 molecules (B cell line Vavy) or DRB1*0401 molecules (B cell line Priess) and that had been cultured in the presence of recombinant MBP, MBP 85-99, or DQw6 43-58 peptides. No specific staining was observed, indicating that the antibody was specific for the DRB1*1501-MBP peptide complex (data not shown). These results demonstrated that recognition by MK16 was dependent on the simultaneous presence of DRB1*1501 and MBP 85-99 peptide and that MK16 could detect DRB1*1501-MBP 85-99 peptide complexes independent of whether they were formed from synthetic MBP 85-99 peptide or processed recombinant MBP.

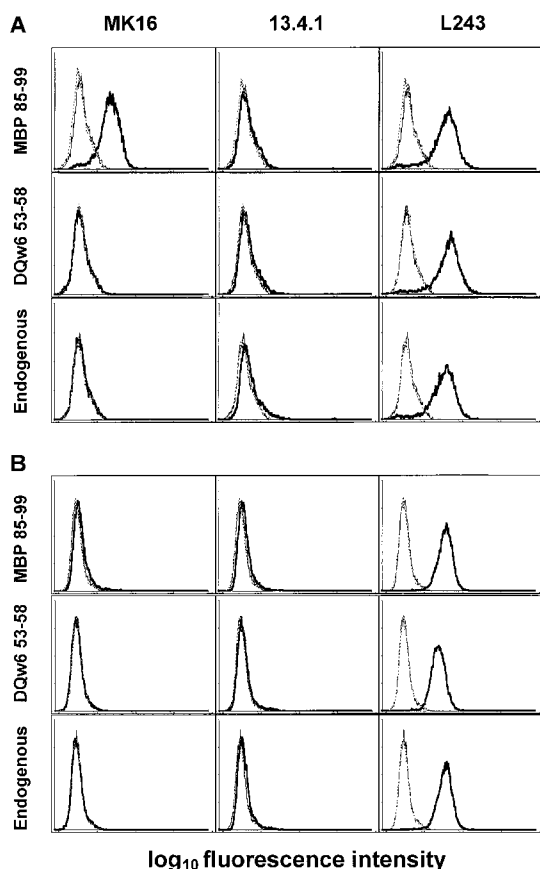


Figure 4. In FACS[®] analysis, MK16 only recognizes MBP 85–99 peptide on cell surfaces when presented by DRB1*1501 molecules. L cell transfectants expressing either (A) DRB1*1501 molecules or (B) DRB5*0101 molecules were incubated in the presence of MBP 85–99 peptide, DQw6 43–58 peptide, or no peptide for 2 h and incubated with purified MK16 or an isotype control, 13.4.1, as indicated (thick lines). Bound antibody was detected with biotin-labeled goat anti-mouse Ig (Fab specific), followed by R-PE-conjugated streptavidin. HLA-DR surface expression was quantified by using biotin-labeled mAb L243 (thick lines). The thin line and dotted lines represent data obtained by incubating cells without primary antibody or without a secondary reagent, respectively.

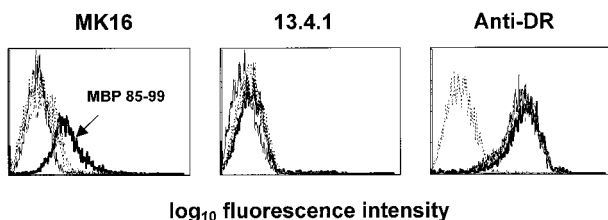


Figure 5. In FACS[®] analysis, MK16 stains DRB1*1501–MBP peptide complexes when presented by human mononuclear cells. Adherent mononuclear cells from a DR2-positive donor (DRB1*1501 homozygous) were isolated and incubated with MBP 85–99 peptide (thick lines), DQw6 43–58 peptide (uneven broken line), or without peptide for 2 h (thin line), washed twice, and stained with purified MK16 or an isotype control (Fab 13.4.1). HLA-DR expression was quantified by using biotin-labeled mAb L243. Bound antibodies were detected as described in Fig. 4. Broken lines represent data obtained by incubating cells without primary antibody.

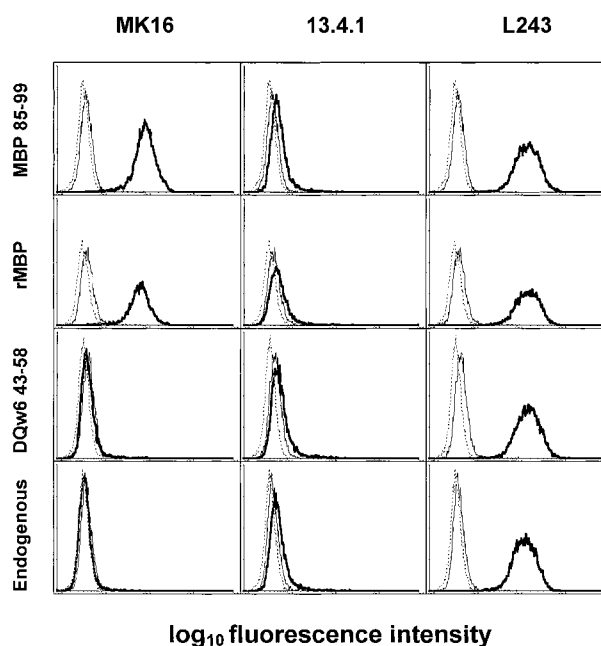


Figure 6. MK16 recognizes DRB1*1501–MBP peptide complexes produced by intracellular processing. A HLA-DR2 homozygous B cell line (MGAR) that expresses DRB1*1501 and DRB5*0101 was incubated with recombinant MBP for 24 h or with MBP 85–99 peptide, DQw6 43–58 peptide, or without peptide for 2 h. After this incubation, cells were washed and stained with purified MK16 or an isotype control (Fab 13.4.1; thick lines). Bound antibodies were detected as described above. HLA-DR surface expression was quantified by using biotin-labeled mAb L243 (thick lines). Thin and dotted lines represent data obtained by incubating cells without primary antibody or without the secondary reagent, respectively.

Fine Specificity of MK16. MBP peptide residues required for recognition by MK16 were examined using a panel of single amino acid analogues of MBP 85–99 peptide as well as peptides with NH₂- or COOH-terminal truncations. Soluble DRB1*1501 molecules were incubated with monosubstituted Ala analogues and truncated variants of MBP 85–99 peptide and tested for their ability to inhibit the binding of MK16 to immobilized DRB1*1501–MBP 85–99 peptide complexes. Ala substitution of Phe-92 and Val-96 resulted in a 20–40-fold reduction in relative binding affinity of MK16, whereas binding was disrupted by Ala substitution of Pro-98 and Arg-99. All other Ala substitutions only had a very modest effect on the binding of MK16 (Table I, top section).

We also analyzed the MBP 85–99 peptide binding specificity of purified DRB1*1501 molecules. Val-89 and Phe-92 were shown to occupy the hydrophobic P1 and P4 pockets of the DRB1*1501 peptide binding site (Table I, top section), which is in accordance with previously published results (7, 15, 30). Consistent with the peptide binding requirements for these two pockets, substitution of Val-89 with the negatively charged Asp greatly diminished the binding affinity of MK16, and substitution of Phe-92 to Asp completely abrogated binding (Table I, bottom sec-

Table I. Recognition by MK16 Is Affected by Single Amino Substitutions and Truncations of the MBP 85–99 Peptide

Peptide	Residue no. and position in the binding pocket															IC ₅₀ MK16	IC ₅₀ DRB1*1501
	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99		
	P-3	P-2	P-1	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11			
	Alanine analogs															nM	nM
(85–89)	E	N	P	V	V	H	F	F	K	N	I	V	T	P	R	6	5
85 (E→A)	A	N	P	V	V	H	F	F	K	N	I	V	T	P	R	4	7
86 (N→A)	E	A	P	V	V	H	F	F	K	N	I	V	T	P	R	2	7
87 (P→A)	E	N	A	V	V	H	F	F	K	N	I	V	T	P	R	4	10
88 (V→A)	E	N	P	A	V	H	F	F	K	N	I	V	T	P	R	4	10
89 (V→A)	E	N	P	V	A	H	F	F	K	N	I	V	T	P	R	4	50
90 (H→A)	E	N	P	V	V	A	F	F	K	N	I	V	T	P	R	13	10
91 (F→A)	E	N	P	V	V	H	A	F	K	N	I	V	T	P	R	9	10
92 (F→A)	E	N	P	V	V	H	F	A	K	N	I	V	T	P	R	217	199
93 (K→A)	E	N	P	V	V	H	F	F	A	N	I	V	T	P	R	18	4
94 (N→A)	E	N	P	V	V	H	F	F	K	A	I	V	T	P	R	19	4
95 (I→A)	E	N	P	V	V	H	F	F	K	N	A	V	T	P	R	10	4
96 (V→A)	E	N	P	V	V	H	F	F	K	N	I	A	T	P	R	131	4
97 (T→A)	E	N	P	V	V	H	F	F	K	N	I	V	A	P	R	5	4
98 (P→A)	E	N	P	V	V	H	F	F	K	N	I	V	T	A	R	> 1,000	5
99 (R→A)	E	N	P	V	V	H	F	F	K	N	I	V	T	P	A	> 1,000	5
	NH ₂ - or COOH-terminal truncations																
(85–99)	-	N	P	V	V	H	F	F	K	N	I	V	T	P	R	2	3
(87–99)	-	-	P	V	V	H	F	F	K	N	I	V	T	P	R	2	5
(88–99)	-	-	-	V	V	H	F	F	K	N	I	V	T	P	R	4	30
(89–99)	-	-	-	-	V	H	F	F	K	N	I	V	T	P	R	9	253
(90–99)	-	-	-	-	-	H	F	F	K	N	I	V	T	P	R	>1,000	>1,000
(91–99)	-	-	-	-	-	-	F	F	K	N	I	V	T	P	R	>1,000	>1,000
(85–98)	E	N	P	V	V	H	F	F	K	N	I	V	T	P	-	>1,000	4
(85–97)	E	N	P	V	V	H	F	F	K	N	I	V	T	-	-	>1,000	15
(85–96)	E	N	P	V	V	H	F	F	K	N	I	V	-	-	-	>1,000	20
	Single amino acid substitutions																
89 (V→D)	E	N	P	V	D	H	F	F	K	N	I	V	T	P	R	214	281
92 (F→Y)	E	N	P	V	V	H	F	Y	K	N	I	V	T	P	R	6	12
92 (F→D)	E	N	P	V	V	H	F	D	K	N	I	V	T	P	R	>1,000	>1,000

MK16 Fab-phages were mixed with various concentrations of purified DRB1*1501 molecules loaded with peptides containing the indicated single amino acid substitutions or truncations, followed by incubation in microtiter wells coated with DRB1*1501-MBP 85–99 peptide complexes. Bound MK16 Fab-phages were detected with biotin-labeled rabbit anti-Fd bacteriophage by ELISA. For peptide binding and specificity of purified DRB1*1501 molecules isolated from S2/DRB1*1501 cells, a constant amount of DRB1*1501 molecules was incubated in the presence of a fixed concentration of biotin-labeled MBP 85–99 peptide and increasing amounts of competitor peptide. The concentration of competitor complex needed to result in IC₅₀ was determined. Binding values for residues critical for binding are in bold type. Each peptide was analyzed in three independent experiments, and results are represented as mean values.

tion). Substitution of Phe-92 by Tyr (Table I, bottom section) did not reduce binding by MK16, consistent with the preference of the P4 pocket for aromatic side chains. Peptides with NH₂- and COOH-terminal truncations delin-

eated the minimal peptide sequence required for MK16 binding. Deletion of Arg-99 abolished MK16 binding, whereas deletion of the NH₂-terminal segment (residues 85–88) had no effect (Table I, center section). Truncation

of Val-89 disrupted binding of MK16, as this residue represents a primary anchor for the binding of the MBP 85–99 peptide to DRB1*1501 (Table I, center section; reference 7, 15, 30). These results demonstrated that the two major HLA-DR2 anchor residues of MBP 85–99 peptide were required for MK16 binding and that the COOH-terminal part of the peptide, in particular residues Val-96, Pro-98, and Arg-99, contributed to binding. Taken together, these data strongly suggest that MK16 binds directly over the HLA-peptide complex.

MK16 Binds with a Lower Affinity than Two Pan-reactive Anti-DR Antibodies. The specificity of MK16 was further examined in a competition binding assay with two pan-reactive anti-HLA-DR antibodies, mAb L243 and mAb L227 (31, 32), that are both capable of blocking DR-restricted T cell responses. mAb L243 recognizes a monomorphic determinant on the outer loops of the DR α chain (33), and mAb L227 recognizes a determinant of the DR β chain (31, 32). In this experiment, purified mAb L243, mAb L227, and soluble MK16 was used to inhibit the binding of MK16 Fab-phages to immobilized DRB1*1501-MBP 85–99 peptide complexes. The anti-DR antibodies L243, L227, and soluble MK16 blocked binding of MK16 Fab-phages in a dose-dependent manner (Fig. 7), whereas an isotype control Fab (13.4.1) did not inhibit binding. The experimentally determined IC₅₀ values were 21 nM for MK16 and 0.8 nM for both mAb L227 and mAb L243. These results demonstrated that MK16 had an ~30-fold lower affinity than the two anti-DR antibodies and that the binding site of MK16 appeared to overlap with the determinants recognized by mAb L243 and mAb L227.

MK16 Can Block the MBP-specific Stimulation of HLA-DR2-restricted T Cells. We next compared the ability of mAb L243 and MK16 to inhibit the MBP 85–99 peptide-specific stimulation of two DRB1*1501-restricted TCR transfectants (Hy.2E11 and Ob.1A12) that were generated with TCR sequences from human MBP-specific T cell

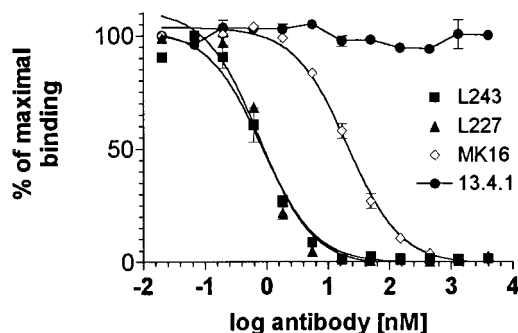


Figure 7. MK16 binds with a lower affinity than two monomorphic anti-HLA-DR mAbs, L243 and L227. Microtiter wells coated with DRB1*1501-MBP 85–99 peptide complexes were incubated with a fixed concentration of MK16 Fab-phages and various concentrations of the following competing antibodies: mAb L243, mAb L227, soluble MK16 Fab, or isotype control 13.4.1. Bound MK16 Fab-phages were detected with biotin-labeled rabbit anti-fd bacteriophage by ELISA. The mean and SEM ($n = 2$) are shown.

clones (7, 20). MK16 was able to block both T cell hybridomas, although to a different extent. The maximal IL-2 response of Hy.2E11 could be inhibited by >90%, whereas Ob.1A12 could be inhibited only by ~50% (Fig. 8). mAb L243 could inhibit the maximal IL-2 response of both T cell hybridomas by >90%. The inhibition by MK16 and L243 antibodies was specific, because neither of the T cells was affected by the addition of an isotype control (Fab 13.4.1; data not shown).

We next compared the IC₅₀ values for inhibiting the Hy.2E11 and Ob.1A12 T cells with MK16 and L243 antibodies (Fig. 8). Dose-response experiments demonstrated that a 50-fold higher concentration of MK16 was needed to inhibit Ob.1A12 to the same degree as Hy.2E11. Furthermore, a sevenfold higher concentration of MK16 was needed to reach a degree of inhibition of Hy.E211 similar to that obtained with mAb L243. This was even more pronounced when Ob.1A12 was investigated, as a 100-fold higher concentration of MK16 as compared with mAb L243 was needed to block the antigen-specific IL-2 response of this T cell hybridoma. Inhibition of DRB1*1501-restricted T cells was specific, as MK16 blocked neither the antigen-specific stimulation of two DRB1*1501-restricted T cell hybridomas recognizing two different PLP peptides (PLP 40–60 and PLP 95–116) nor two DRB1*0401-restricted T cell hybridomas recognizing two different peptides (CII 261–273 and HA 307–319), which were readily inhibited by mAb L243 (Fig. 8).

MK16 Has a Sensitivity Similar to That of T Cells Specific for the HLA-DR2-MBP Peptide Complex. We next investigated whether MK16 could detect DRB1*1501-MBP 85–99 peptide complexes with the same sensitivity as the two TCR transfectants, Hy.2E11 and Ob.1A12. In this experiment, MGAR cells were first cultured in the presence of various concentrations of recombinant MBP, MBP 85–99, or DQw6 43–58 peptides. These APCs were then stained with MK16 (Fig. 9, A and C) or cultured with Hy.2E11 (Fig. 9 B) or Ob.1A12 TCR transfectants (data not shown). The minimal antigen-peptide concentration required for significant staining with MK16 was 1–2 nM, whereas a concentration of 10–100 nM peptide was required to elicit a specific IL-2/T cell response. The original human T cell clones from which the TCR chains were isolated were more sensitive than the TCR transfectants and were activated by MBP 85–99 peptide at 5 nM (data not shown). These results demonstrated that MK16 detected complexes of DRB1*1501 and MBP 85–99 peptide with a sensitivity similar to that of human T cell clones.

Intracellular Localization of HLA-DR2-MBP Peptide Complexes. We also examined whether MK16 could be used to label intracellular DRB1*1501-MBP peptide complexes at a single cell level by using laser scanning confocal microscopy. Immunostaining of DRB1*1501-positive L cells preincubated with either recombinant MBP or the MBP 85–99 peptide showed intracellular staining that was confined to vesicular structures in the cytoplasm. The staining pattern was similar when the transfectants were

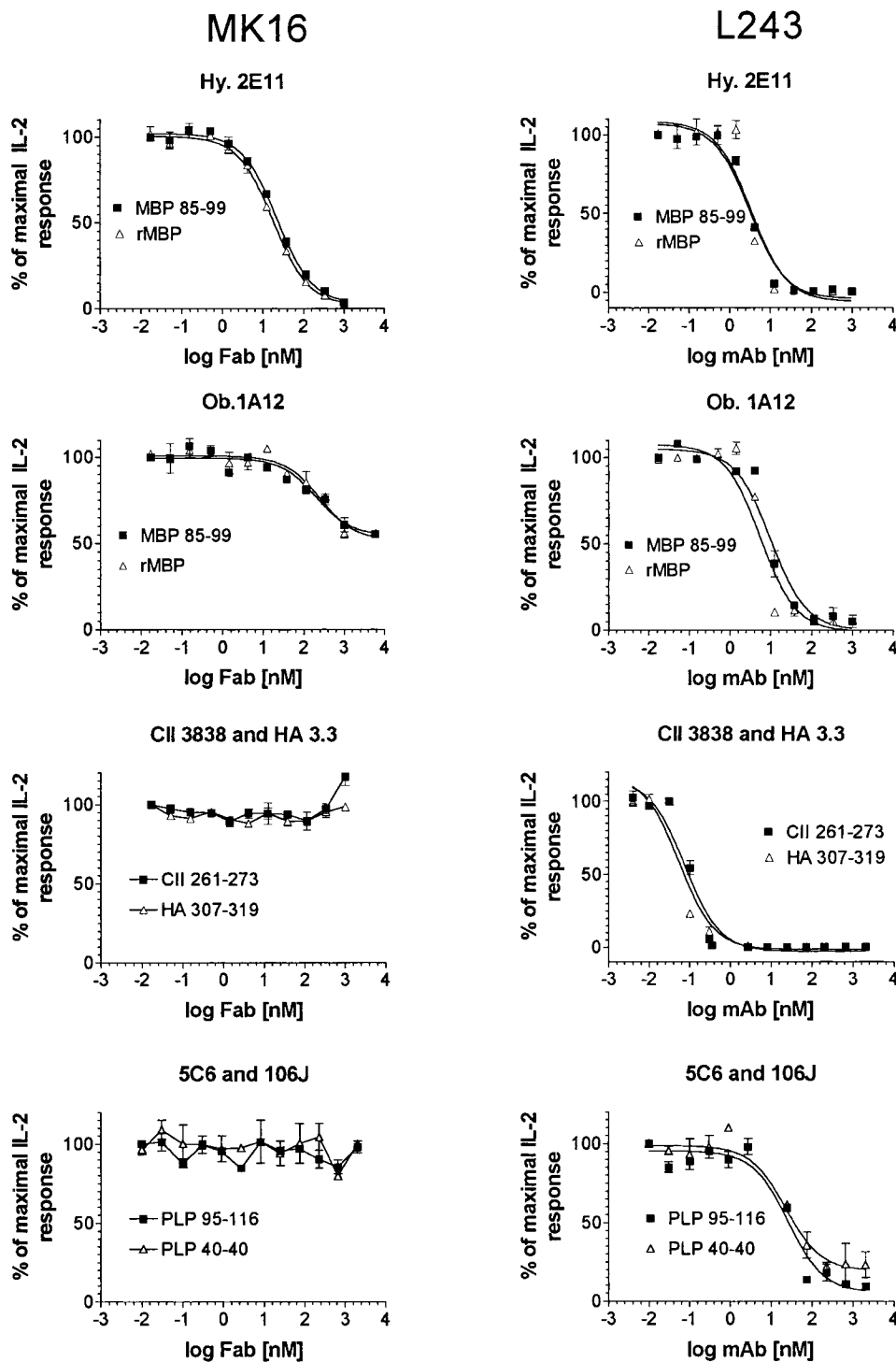


Figure 8. MK16 can inhibit DRB1*1501-restricted MBP-specific T cell hybridoma response to MBP-peptide. MGAR cells (expressing DRB1*1501 and DRB5*0101 molecules) were cultured with MBP 85-99, PLP 95-116, or PLP 40-60 peptides for 2 h or recombinant MBP for 24 h. Priess cells (expressing DRB1*0401 molecules) were cultured in the presence of CII 261-273 or HA 307-319 peptides for 2 h. Cells were washed, irradiated with 3,000 rads, and incubated in the presence of increasing concentrations of blocking antibodies for 30 min at 37°C before adding the T cell hybridomas. After incubation for 24 h, aliquots of 100 μ l were assayed in an IL-2 release assay. The mean and SEM ($n = 2$) are shown. Antibody concentrations needed to effect 50% inhibition (IC_{50}) of secreted IL-2 by Hy.2E11 or Ob.1A12 hybridomas when using MK16 as inhibitor were 22 and 1,000 nM, respectively, and when using mAb L243 as inhibitor were 3 and 10 nM, respectively. MK16 did not inhibit presentation of PLP 40-60 and PLP 95-116 peptides to DRB1*1501-restricted 5C6 and 106J T cell hybridomas nor of CII 261-273 or HA 307-319 peptides to DRB1*0401-restricted CII 3838 and HA 3.3 T cell hybridomas, whereas IC_{50} values, when using mAb L243 as inhibitor of IL-2 secretion by these hybridomas, were 26, 22, 0.08, and 0.06 nM, respectively.

pulsed with either recombinant MBP or the MBP 85-99 peptide. DRB1*1501-positive L cells that had not been incubated with MBP or had been incubated with the DQw6 43-58 peptide were not stained by MK16 (Fig. 10). Staining was also negative with an isotype control antibody (13.4.1) or in the absence of the primary antibody (data not shown). DRB1*1501-positive L cells incubated with an anti-CD107a mAb that recognizes LAMP-1

showed a similar vesicular staining pattern. Double staining with anti-CD107a and MK16 showed a certain degree of colocalization of these molecules. However, a substantial fraction of MK16-positive vesicles was not stained by the LAMP-1 antibody, suggesting that MK16 also labels DRB1*1501-MBP peptide complexes in other vesicles of the endosomal/lysosomal pathway. These results demonstrated that MK16 could be used to specifically label

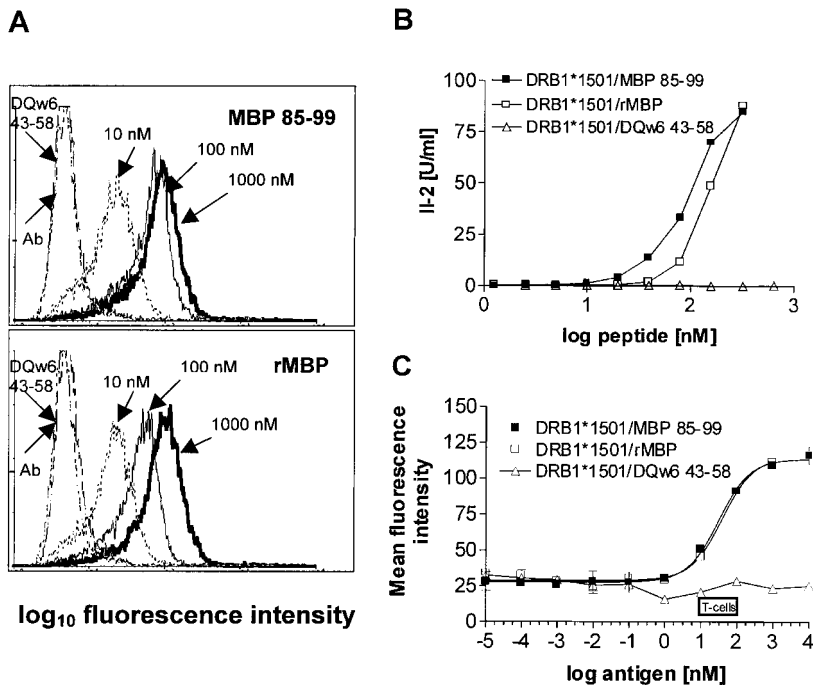


Figure 9. MK16 detects DRB1*1501-MBP 85-99 peptide complexes with a sensitivity similar to that of DRB1*1501-restricted MBP-specific T cell hybridoma. MGAR cells (expressing DRB1*1501 and DRB5*0101) were cultured with various concentrations of MBP 85-99 peptide, DQw6 43-58 peptide, or recombinant MBP for 2 or 24 h, respectively. (A) After this incubation, cells were stained with purified MK16 followed by incubation with biotin-labeled goat anti-mouse Ig (Fab specific) and detected with R-PE-labeled streptavidin. (B) T cell stimulation. In parallel, pulsed and irradiated cells were cocultured with T cell hybridomas Hy.2E11 or Ob.1A12 for 24 h (only data for Hy.2E11 are shown; similar results were obtained for Ob.1A12). Aliquots of 100 μ l were assayed in an IL-2 release assay. Each symbol represents the mean value of two determinations. (C) Summary of experiments performed in A and B. Curves illustrate the amount of binding presented as mean values measured by flow cytometry analysis. The mean and SEM ($n = 2$) are shown. The box labeled T cells represents the range of minimal peptide concentration needed for eliciting detectable IL-2 responses determined by three individual experiments.

DRB1*1501-MBP peptide complexes at a single cell level.

Immunoreactivity of Sections of MS and Normal Brain Tissue. To directly examine the identity of the cells that presented the DRB1*1501-bound MBP 85-99 peptide in MS lesions, we used MK16 for immunohistochemical staining of MS brain tissue. Normal brain tissue was also analyzed. Frozen sections of CNS tissue from 11 MS cases with chronic active lesions (Table II) and from 7 normal cases (data not shown) were HLA typed. Eight patients were DRB1*1501 positive and one was DRB1*1502 positive, while two were DRB1*1501 and DRB1*1502 negative. One of seven normal cases was DRB1*1501 positive. Sections from all patients and the DRB1*1501-positive case were stained with a pan-reactive anti-HLA class II mAb (anti-HLA-DP-DQ-DR) and MK16, as well as with antibodies to microglia/macrophages, monocytes, and astrocytes. In sections from all MS patients, the pan-reactive anti-HLA class II antibody reacted strongly with microglia/macrophages (as exemplified in Fig. 11 B and Fig. 12, A and C). In the majority of MS cases examined, regardless of HLA type, the same pan-reactive antibody also showed a low level of immunoreactivity of astrocytes within lesion areas and of occasional monocytes but no reactivity with blood vessel endothelium. In the DRB1*1501-positive normal case, the pan-reactive anti-HLA class II antibody stained only microglia/macrophages in the white matter but with greatly reduced intensity compared with MS lesions (data not shown).

When MK16 was applied to the same tissues, a clear difference in immunoreactivity between sections from DR2-positive patients and HLA-DR2-negative patients was seen (Table II and Fig. 11, A and D). On sections from HLA-

DR2-positive patients, MK16 stained predominantly cells that on the basis of their morphology were characterized as microglia/macrophages (Fig. 11 A). The intensity of this staining and the number of stained cells were lower as compared with the pan-reactive anti-HLA class II antibody (Fig. 11, A and B). In contrast, microglia, macrophages, astrocytes, and monocytes from the HLA-DR2-negative patients were all MK16 negative, while the occasional blood vessel exhibited very low level MK16 reactivity (Table II, patient no. M46 and F56; exemplified in Fig. 11 D). In the normal DRB1*1501-positive case, MK16 also labeled microglia/macrophages but not astrocytes. As compared with the staining pattern in MS lesions, fewer cells were MK16 positive, and their reactivity was weaker (Fig. 11 C). A higher level of background staining was observed in the normal DRB1*1501-positive case than in the HLA-DR2-positive patients, because the specimen of normal CNS had to be developed for a longer period to visualize staining.

Double immunofluorescence staining of the DR2-positive MS lesions directly demonstrated that although the MK16-positive cells also were HLA class II positive (Fig. 12, A-C), some HLA class II-positive cells were MK16 negative (Fig. 12 C, lower right corner). Double staining also showed that MK16-positive cells were CD14 positive (as exemplified in Fig. 12, D-F), confirming that these cells were microglia/macrophages. White matter from three of the DR2-positive patients also showed immunoreactivity for MK16 on hypertrophic astrocytes (Table II, patient nos. F31, F38, and F55; exemplified in Fig. 12, G-I), whereas one patient showed reactivity only on astrocytes (Table II, patient no. F36). In addition, lesions from two patients showed monocytes to be positive for the MK16 antibody (Table II, patient nos. F37 and M69). It was important to

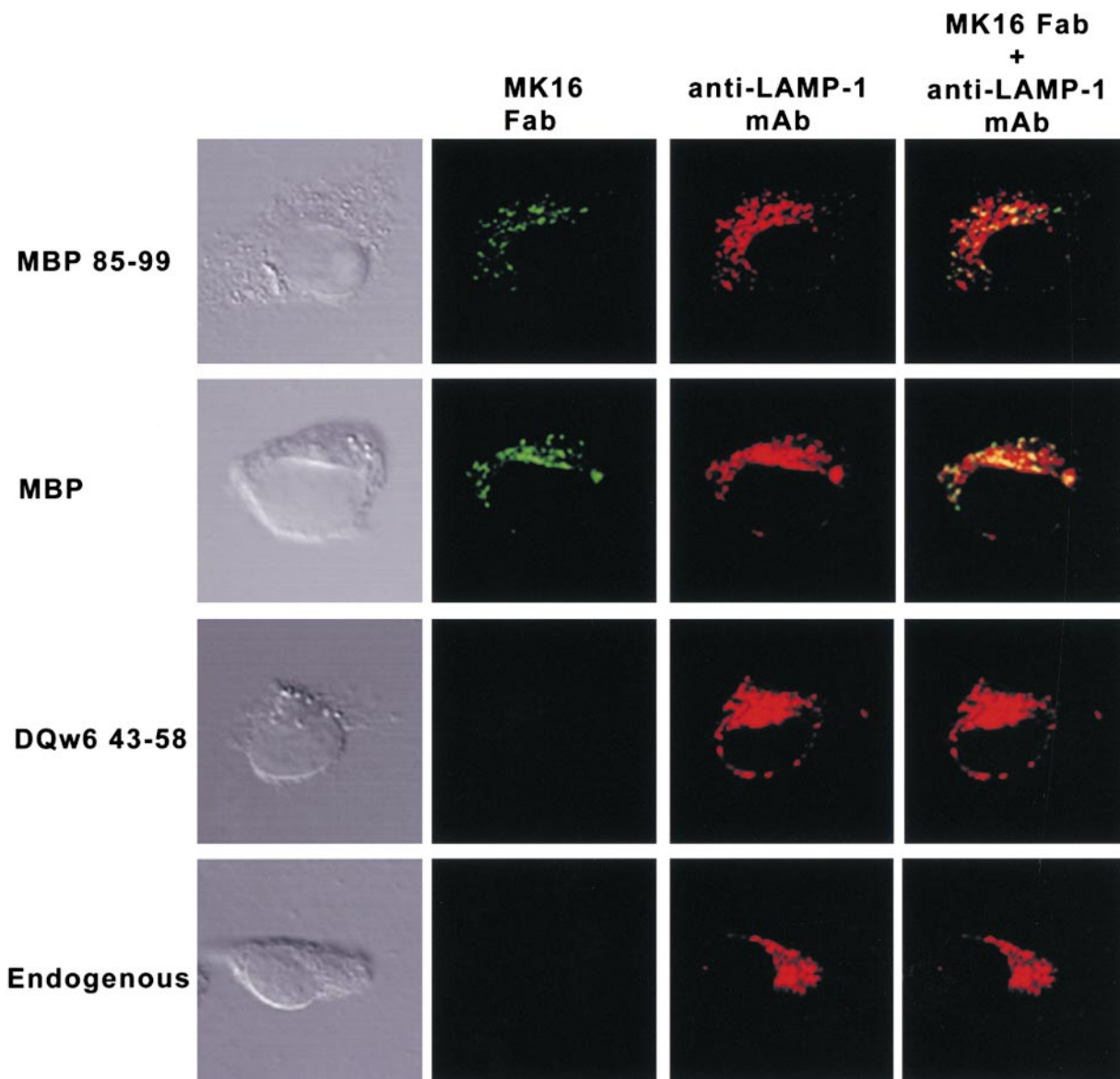


Figure 10. Colocalization of DRB1*1501-MBP peptide complexes and LAMP-1. L cells expressing DRB1*1501 molecules were cultured with or without the presence of MBP 85-99 peptide or DQw6 43-58 peptide for 2 h or with recombinant MBP for 24 h. Cells were washed, fixed, permeabilized, and incubated with purified MK16 and a rat anti-LAMP-1 antibody. MK16 and LAMP-1 were visualized by FITC-labeled goat anti-mouse IgG (Fab specific) antibody (green) and Texas Red-labeled goat anti-rat IgG (Fc fragment specific) antibody (red), respectively. Colocalization is visualized in yellow. The images are one optical section thick. Original magnification, $\times 40$.

show that potentially autoreactive T cells are found adjacent to MK16-positive APCs in the MS lesions. Double staining with anti-CD3 and MK16 demonstrated that this was indeed the case (as exemplified in Fig. 12, J-L). Taken together, these results demonstrated that MK16 could be used to localize APCs in MS lesions and normal brain tissue that present the DRB1*1501-MBP peptide complex and that these cells were predominantly microglia/macrophages. However, the frequency and staining level of MK16-positive cells was markedly higher in the MS lesions than in normal brain tissue, and T cells adjacent to these APCs were only observed in the MS lesions.

Discussion

Antibodies against specific MHC-peptide complexes are relatively new tools that have already proven to be valuable in the study of antigen processing and presentation. Using such antibodies, it has been possible to localize, quantitate, and detect specific MHC-peptide complexes in situ (25, 29, 34-47). Previously reported antibodies directed against MHC class II-bound peptides were derived from either endogenous proteins such as invariant chain and a mouse MHC class II molecule (I-E α) or exogenous model antigens (e.g., hen egg lysozyme). Conventional hybridoma technology has been used in combination with optimized

Table II. *MK16 Reactivity with CNS Tissue from 11 Different MS Cases*

Patient no.	DRB1 alleles	DR2 positive		
		Microglia/ macrophage	Astrocytes	Other
F31	01, 1501	+	+	blood vessels
F37	03, 1501	+/-		monocytes
F32	1501, 1601	+/-		
F38	03, 1501	+++	+/-	
F55	1501	+	+/-	
F36	08, 1501		++	
F27	1501	++		
F56	1501	+		
M69	11, 1502	+/-		monocytes
DR2 negative				
M46	03, 13			blood vessels
F56	11, 13			blood vessels

Frozen sections of CNS tissue from 11 MS cases were HLA typed and, after fixation, stained with the following antibodies: anti-HLA-DR-DP-DQ or MK16 and anti-CD14 (microglia of the monocyte lineage, monocytes and macrophages) or CD68 (microglia and macrophages) or anti-GFAP (astrocytes) antibodies. Slides were scored in a blinded fashion by two observers on a scale from 0 to 4 based on the degree of positivity.

immunization and screening strategies to generate such antibodies. In these studies, it was reported that comprehensive and rigorous screening of a large number of hybridoma clones (500–1,000) resulted in the recovery of only 1–2 clones with the desired specificity (for example, see references 41 and 42). These numbers indicate that antibodies with such specificities tend to be relatively rare and difficult to isolate.

We chose to generate an antibody that specifically recognized a human MHC class II molecule, DRB1*1501, in complex with an autoantigenic peptide (MBP 85–99). We used the phage display technique because we have previously shown that this technique represents an attractive alternative to hybridoma technology to generate an antibody against a defined MHC class I–peptide complex (24). The HLA-DRB1*1501–MBP complex was selected because it is a target for autoreactive T cells in MS (6, 7, 11, 12, 48; for review see reference 3) and because the structural requirements for binding of the MBP 85–99 peptide to DRB1*1501 molecules and for its recognition by cognate TCR have been analyzed in great detail. Peptide binding studies and structure determination by x-ray crystallography demonstrated that Val-89 and Phe-92 are major anchor residues that are accommodated in the hydrophobic P1 and P4 pockets of the DRB1*1501 molecule (7, 15, 30). In accordance with these results, we have shown that MK16 is sensitive to amino acid changes at these positions. The epitope recognized by the antibody is located at the COOH-terminal end of the MBP 85–99 peptide and involves residues Val-96, Pro-98, and Arg-99. In contrast, residues His-90, Phe-91, and Lys-93 are particularly im-

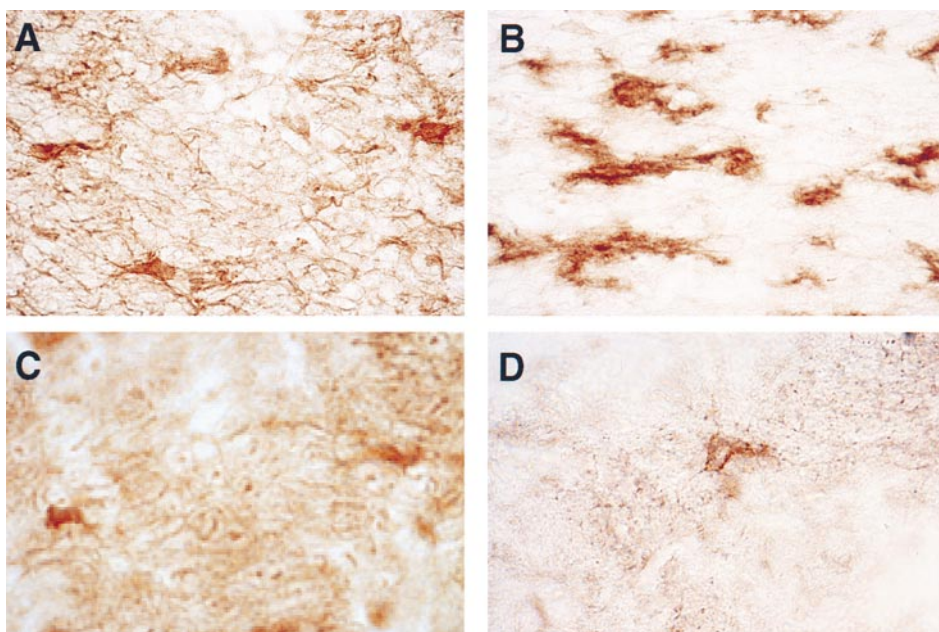


Figure 11. Immunohistochemical anti-HLA class II antibody and MK16 stainings of frozen sections from chronic active MS lesions and normal CNS tissue. (A) MK16 immunoreactivity at the edge of an active lesion from a DRB1*1501-positive MS patient shows staining of four microglial cells and some astroglial cell processes. $\times 600$. (B) Anti-HLA-DP-DQ-DR antibody staining of the same MS lesion (adjacent section) showing more intense staining of microglia/macrophages. $\times 600$. (C) DRB1*1501-matched normal CNS tissue immunoreacted with MK16 shows scattered MK16-positive microglia/macrophages with lower level immunoreactivity compared with that seen in MS. A higher level of background staining was observed in normal CNS than in MS lesions, because the specimen of normal CNS had to be developed for a longer period to visualize staining. $\times 600$. (D) Non-HLA-matched chronic active MS lesion immunoreacted with MK16. A rare blood vessel shows positive immunoreactivity. Microglia/macrophages, astrocytes, and monocytes were MK16 negative. $\times 600$.

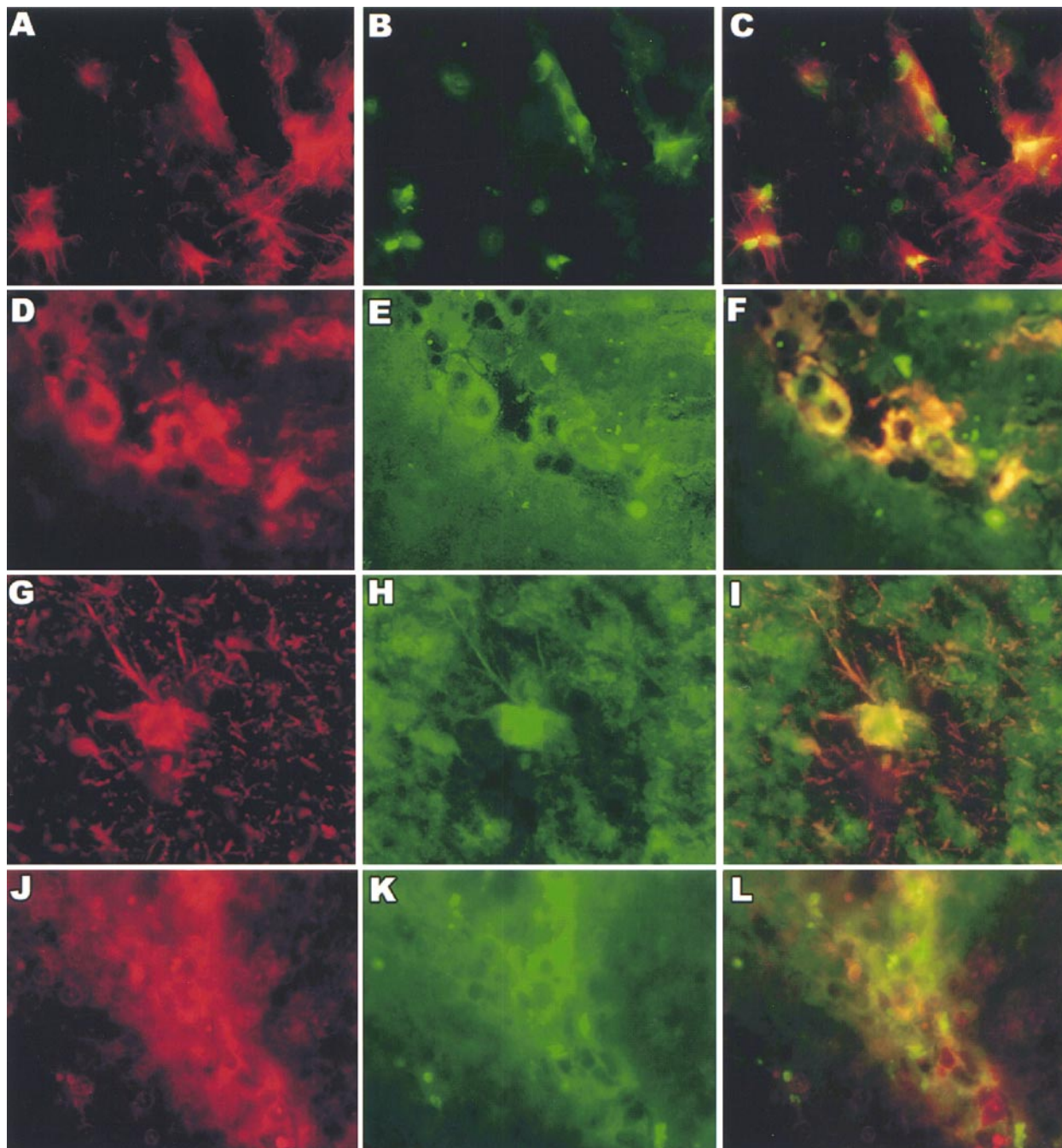


Figure 12. Double immunofluorescence of frozen sections of white matter from a DRB1*1501-positive patient with chronic progressive MS. Panels A–C are stained by using an anti–HLA class II antibody and MK16. (A) The section was immunoreacted with the anti–HLA-DP-DQ-DR antibody and visualized with an Alexa 568–conjugated secondary antibody (red). An area from an acute lesion displays immunoreactivity on numerous macrophages and microglia around a blood vessel (upper right). A few hypertrophic astrocytes are also immunoreactive (bottom center and left). $\times 500$. (B) The same field reacted with MK16 and visualized with a Cy-2–conjugated secondary antibody (green). Note the immunoreactive microglial cells around the blood vessel. Not all HLA class II–positive cells are MK16 positive. $\times 500$. (C) Double immunofluorescence of the same field shows overlapping HLA class II/MK16 reactivity (yellow/orange) on the majority of cells, while some cells are HLA class II positive only (red). Two perivascular microglial cells are predominantly MK16 positive. $\times 500$. Panels D–F are stained by using an anti-CD14 antibody and MK16. (D) A row of perivascular macrophages in the same acute lesion (adjacent section) immunoreacted with anti-CD14 mAb and visualized with Alexa 568 (red). $\times 500$. (E) The same macrophages are MK16 positive, which can be visualized with the Cy-2–labeled secondary antibody (green). The surrounding tissue have a high green autofluorescence, which is partly the result of our enhancement of the image to maximize the staining of MK16–positive cells (this is also the case in H, K, I, and L) and partly due to the quality of postmortem inflamed tissue. $\times 500$. (F) Double immunofluorescence shows clear overlap between CD14 and MK16 on the same macrophages. $\times 500$. Panels G–I are stained by using an anti-GFAP antibody and MK16. (G) A hypertrophic astrocyte (red; Alexa 568) and numerous astroglial cell processes are immunoreactive for GFAP. $\times 500$. (H) With MK16, the same hypertrophic astrocyte and its processes are Cy-2 positive (green). $\times 500$. (I) Double immunofluorescence shows clear overlap (yellow/orange) between anti-GFAP and MK16. $\times 500$. Panels J–L are stained by using an anti-CD3 antibody and MK16. Because this image is of a perivascular cuff, the plane of focus is different both between cells and from the surrounding parenchyma and therefore appears fuzzy. (J) A perivascular cuff of infiltrating cells from the same acute lesion as above shows diffuse immunoreactivity for anti-CD3 (red; Alexa 568). $\times 500$. (K) The same perivascular infiltrate shows a different pattern of immunoreactivity with MK16 (green; Cy-2). $\times 500$. (L) Double immunofluorescence shows distinct staining of CD3- and MK16–positive cells within the same perivascular cuff. $\times 500$.

portant for recognition by human MBP-specific T cell clones (7, 49). MK16 could also inhibit recognition of MBP 85–99 peptide by the two T cell hybridomas (Ob.1A12 and Hy.2E11), although to a different extent. Although Hy.2E11 could be blocked >90%, Ob.1A12 could only be blocked to ~50% of the maximal response. Even though mAb L243 efficiently inhibited both T cell hybridomas, we showed that MK16 had a >30-fold lower affinity than mAb L243. Subtle differences in the positioning of the two TCRs on the HLA-DR2–MBP peptide complex may contribute to the different degree of blocking with MK16. His-90 and Phe-91 represent primary TCR contact residues for clone Ob.1A12, whereas Phe-91 and Lys-93 are primary TCR contact residues for clone Hy.2E11 (7, 49).

An mAb recognizing an MS-relevant T cell epitope may have therapeutic potential. An mAb specific for an I-A^s–MBP complex was found to block the proliferative T cell response *in vitro* to the I-A^s–restricted encephalitogenic determinant of MBP and to inhibit experimental allergic encephalomyelitis (EAE) in H-2^s mice (50). By analogy, we are currently testing whether MK16 can inhibit EAE in a newly developed humanized animal model for MS (20). If this is the case, MK16 might have clinical relevance in the treatment of MS. Alternatively, MK16 antibody could be expressed as an intact antibody with an Fc segment that fixes complement. Such a strategy would target APCs that phagocytose myelin and present myelin-derived peptides to T cells but would spare oligodendrocytes that do not express MHC class II molecules (51). Antibodies to human MHC class II molecules (DR7 and DRw11) in complex with MBP fragments have also been reported (52) but may not be suitable for clinical purposes. DR7 and DRw11 are not associated with susceptibility to MS; the antibodies showed significant background reactivity with endogenous MHC–peptide complexes, and the precise MBP epitopes recognized by these antibodies were not defined.

MK16 represents a powerful tool to investigate MHC class II presentation of MBP and may contribute to our understanding of molecular mechanisms involved in the development of MS. Recent studies have provided evidence that HLA-DR–restricted presentation of MBP by transformed B cells and fibroblast transfectants can occur via two different alternative pathways, which are both independent of intracellular processing involving the lysosomes (53, 54). We have shown in this study that the MK16 antibody could be used to detect HLA-DR2–MBP peptide complexes at the single cell level. Importantly, the antibody-stained APCs pulsed with intact MBP and could detect intracellular complexes, which colocalized with lysosomes, indicating that MBP is also processed in the endosomal/lysosomal pathway like the vast majority of other exogenous antigens.

MK16 was also used to visualize cells that present MBP T cell epitopes in MS lesions, a topic of interest because it is unclear which cells in the CNS might serve as APCs and stimulate myelin-reactive infiltrating CD4⁺ T cells. In particular, there is some controversy as to the relative role of microglia/macrophages and astrocytes as APCs. Phenotypic

analysis of human brain tissue has shown that MHC class II molecules are readily detectable on microglia/macrophages, whereas astrocytes have much lower, if any, MHC class II expression (55). In MS, both microglia/macrophages and astrocytes become activated and express higher levels of MHC class II molecules (55–57). In addition, microglia/macrophages but apparently not astrocytes express costimulatory molecules such as CD80 and CD40 that are critical for T cell activation (58, 59). These findings suggest that microglia/macrophages rather than astrocytes are the predominant APCs in MS lesions. However, functional studies *in vitro* have questioned this relatively simple paradigm and given evidence that both activated microglia/macrophages and activated astrocytes may act as efficient APCs and stimulate naive as well as activated T cells (for recent publications see references 60–63). It should be noted that the majority of these studies have been carried out in rodents and might therefore not be directly relevant, as microglia/macrophages and astrocytes show important interspecies phenotypic differences, such as inducibility of CD80 expression (for review see references 16–18). Our results demonstrated unequivocally that microglia/macrophages were the cell type most consistently staining positively with MK16 in HLA-DR2 (DRB1*1501 and 1502)-positive MS patients. Importantly, a few lesions also showed reactivity on hypertrophic astrocytes and monocytes within lesion areas. The level of MK16 reactivity was less intense than that observed with a pan-reactive anti-HLA-DP-DQ-DR antibody. This difference might have been expected, because MK16 only recognizes a subset of HLA-DR molecules, i.e., those binding the MBP 85–99 peptide, which is in contrast to the anti-HLA-DP-DQ-DR antibody, which recognizes all HLA class II molecules.

It is interesting that microglia/macrophages reacting with the pan-reactive anti-HLA class II antibody did not always also stain with MK16. This dichotomy may suggest that not all microglia/macrophages have the capacity to process MBP and might reflect the presence of microglia/macrophage cells at different developmental stages or exhibiting differential activation. Alternatively, this result may indicate that microglia/macrophages are a heterogeneous population of cells, some of which are long-term residents of the CNS and some of which may be of recent hematogenous derivation in which only a fraction has the ability to process MBP and other antigens. The differential staining of some microglia/macrophages with the pan-reactive anti-HLA class II antibody and MK16 also shows that MK16 staining is not, in general, merely a function of cross-reactivity with DRB1*1501 molecules bound to other self-peptides. If this had been the case, we would have expected MK16 to react with all DRB1*1501-positive cells. This conclusion is further substantiated by the findings that MK16 only stained adherent mononuclear cells from a DRB1*1501-positive donor and DRB1*1501-positive L cell transfectants and a DRB1*1501-positive B cell line (MGAR) when these different cell types had been incubated with the MBP 85–99 peptide.

Our studies do not resolve whether microglia/macrophages or astrocytes presenting the HLA-DRB1*1501–MBP

complex have the capacity to stimulate T cells but merely show that they both have the potential. Importantly, the observation also strongly suggests that both cell types can process native MBP, though it cannot be excluded that the HLA-DRB1*1501-bound MBP 85–99 peptide is derived from extracellular degradation of MBP (64, 65). Moreover, our studies do not emphasize the role of the DRB1*1501 molecule at the expense of the DRB5*0101 and DQB1*0602 molecules as the principal MS-associated MHC class II gene in the DR2 haplotype. Population studies in different ethnic groups might be the best way to examine which of these three MS-associated class II genes confer the strongest susceptibility to MS. It is also important to note that other MHC class II molecules than those encoded in the DR2 haplotype and CNS-derived antigens other than myelin are also likely to play an important role in the pathogenesis of MS.

Our data also show that the HLA-DRB1*1501-MBP complex is presented by microglia/macrophages in normal brain, albeit at a lower level and by fewer cells than in the MS lesions. So far, this analysis has only been done on a single postmortem case, as only one of the seven control subjects carried the HLA-DR2 haplotype. These observations indicate that low level DR-restricted MBP presentation in the brain is normal rather than pathologic. These results also support the notion that T cell entry into CNS might be a critical checkpoint in the initiation of disease, because physiologic expression of MHC class II-MBP complexes in the CNS may be sufficient to activate infiltrating autoreactive T cells (66).

We thank Drs. E. Mellins, M.M. Davis, R. Sobel, H. Laursen, and C. Schaefer-Nielsen for helpful discussions.

This work was supported by the Danish Medical and Natural Science Research Councils (to J. Engberg and L. Fugger), The Novo Nordisk Foundation (to J. Engberg and L. Fugger), The National Multiple Sclerosis Society USA (to K. Wucherpfennig and C. Raine), and The Danish Multiple Sclerosis Foundation, The Karen Elise Jensen Foundation, the European Union, and the Alfred Benzon Foundation (to L. Fugger). Technical questions regarding the phage display technique used in this manuscript should be forwarded to J. Engberg (E-mail: je@mail.dfh.dk).

Submitted: 13 March 1999

Revised: 21 January 2000

Accepted: 4 February 2000

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