

Prevention of Experimental Allergic Encephalomyelitis in Rats by Targeting Autoantigen to B Cells: Evidence That the Protective Mechanism Depends on Changes in the Cytokine Response and Migratory Properties of the Autoantigen-specific T Cells

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Summary

Previous experiments from this laboratory have shown that Lewis rats were protected from experimental allergic encephalomyelitis (EAE) induced by the injection of myelin basic protein (MBP) in Freund's complete adjuvant if they were treated with the encephalitogenic peptide of MBP covalently linked to mouse anti-rat immunoglobulin (Ig) D. It was suggested that this protection developed because the antibody-peptide conjugate targeted the peptide to B cells and that this mode of presentation induced a Th2-like T cell response that controlled the concomitant encephalitogenic Th1 reaction to the autoantigen. The current experiments were carried out to test this hypothesis and to examine the alternative explanation for the protective effect of the conjugate pretreatment, namely that it induced a state of nonresponsiveness in the autoantigen-specific T cells. It was shown that EAE induction was suppressed in Lewis rats when the antibody-peptide conjugate was injected intravenously 14 and 7 d before immunization with MBP in adjuvant, but that anti-MBP antibody titers were at least as high in these animals as in controls that were not pretreated with the conjugate before immunization. Lymph node cells from these pretreated animals, while proliferating *in vitro* to MBP as vigorously as those from controls, produced less interferon γ and were very inferior in their ability to transfer disease after this *in vitro* activation. In contrast, these same lymph node cells from protected rats generated markedly increased levels of messenger RNA for interleukin (IL)-4 and IL-13. When these *in vitro* experiments were repeated using the encephalitogenic peptide rather than MBP as the stimulus, the proliferative response of lymph node cells from pretreated donors was less than that from controls but was still readily detectable in the majority of experiments. Furthermore, the cytokine expression induced by the peptide was similar to that elicited by whole MBP. While these results support the original hypothesis that the anti-IgD-peptide conjugate pretreatment protected rats from EAE by inducing a Th2-type cytokine response, a totally unexpected finding was that this pretreatment greatly reduced the level of leukocyte infiltration into the central nervous system. This result provides a direct explanation for the protective effect of the pretreatment, but it raises questions regarding migratory and homing patterns of leukocytes activated by different immunological stimuli.

Experimental allergic encephalomyelitis (EAE)¹ is a T cell-dependent, paralytic autoimmune disease that can be induced in experimental animals by the injection of myelin-derived autoantigens emulsified in CFA. In Lewis rats, which

are a particularly susceptible strain, active EAE can be induced either by immunization with guinea pig myelin basic protein (MBP) or with a peptide consisting of amino acid residues 70–86 of the mature protein sequence (1, 2). The disease can also be transferred (passive EAE) from animals with active EAE to naive syngeneic recipients by the intravenous injection of donor spleen or lymph node cells after their *in vitro* culture with MBP.

¹ Abbreviations used in this paper: CNS, central nervous system; EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; RT-PCR, reverse transcription PCR.

The ascending paralysis characteristic of EAE is caused by the action of CD4⁺ T lymphocytes that produce focal edema in the central nervous system (CNS) (3, 4), but in both active and passive EAE, the disease is transient and affected animals recover completely within 4–5 d of its onset (5). Attempts to induce further episodes of the disease are unsuccessful despite the fact that spleen cells from recovered animals have the potential to cause the disease. It appears that the pathological response to guinea pig MBP is somehow held in check in animals that have recovered from their single episode of paralysis.

Previous experiments carried out in this laboratory reported that rats treated with the encephalitogenic peptide of MBP covalently coupled to monoclonal anti-IgD antibody were resistant to the induction of EAE on concomitant challenge with MBP in CFA (6). This resistance was provisionally ascribed to the fact that the antibody-peptide conjugate targeted the autoantigen to B cells and that the presentation of the peptide by these cells would be expected to induce a Th2-like cytokine response (7–9). Given that Th2 cytokines antagonize Th1 responses (10–13) and that EAE is a Th1 cell-mediated disease (14–16), targeting the peptide in this way is expected to have the observed effect. It has also been shown, however, that when B cells are used to present monomeric antigen to T cells, the latter fail to respond to a subsequent challenge with the same antigen in adjuvant (17, 18). The ability of B cells to induce T cell tolerance in this system depends on the B cells remaining in a nonactivated state (17, 18), but the level of activation of the B cells in our earlier experiments was not determined. Consequently, an alternative explanation for the capacity of the antibody-peptide conjugate to prevent EAE was that it induced a state of non-responsiveness in peptide-specific T cells.

The antibody-peptide treatment in these experiments was started at the same time as the challenge with MBP in CFA so that, in principle, some T cells might have been activated without first encountering the encephalitogenic peptide presented on B cells. It was anticipated that this possibility could cause difficulties in interpreting experiments designed to identify the mechanism of disease resistance, so for the present study, the protocol was modified. In the experiments now reported, the anti-IgD-peptide conjugate injections were given well before the challenge with MBP in CFA, thereby favoring previous antigen presentation by B cells. It was found that this mode of preimmunization also prevented the subsequent induction of EAE. The mechanism of this refractoriness was then studied; particularly, experiments were carried out to determine whether it was a result of T cell nonresponsiveness or of an induced change in the cytokine response to the encephalitogen.

Materials and Methods

Animals and Induction of EAE. 8–12-wk-old Lewis strain rats of either sex were used and bred in the specific pathogen-free (SPF) unit of the Medical Research Council Cellular Immunology Unit

(Oxford, UK). To induce active EAE, animals were injected in the hind footpads with 50 µg MBP in CFA (19). In individual experiments all animals were of the same sex.

Passive EAE was adoptively transferred from immunized donors to naive recipients as described (20). Briefly, splenocytes or lymph node cells (popliteal, mesenteric, and paraortic) from rats immunized with MBP in CFA were stimulated *in vitro* for 3 d with MBP and then injected intravenously into syngeneic recipients. The cell doses used for transfer were between 3–5 × 10⁷ viable leukocytes. Animals were scored daily for clinical signs of disease on a scale from 0 to 5 depending on severity: 0, normal; 1, limp tail; 2, hind limb weakness; 3, unilateral hind limb paralysis; 4, bilateral hind limb paralysis; and 5, bilateral hind limb paralysis and incontinence.

mAbs and the Preparation of Antibody-Peptide Conjugates. The mAbs used in these studies were as follows: W3/25 (anti-rat CD4) (21), OX-6 (anti-rat MHC class II) (22), OX-8 (anti-rat CD8) (23), OX-21 (anti-human C3b inactivator) (24), ED1 (anti-rat macrophage) (25), OX60 (anti-rat IgD) (6), and OX81 (a neutralizing anti-rat IL-4 mAb [Ramirez F., D. Fowell, M. Puklavec, S. Simmonds, and D. Mason, manuscript submitted for publication]).

The mAbs OX60 and OX81 are mouse IgG₁ Igs, and they were prepared from ascitic fluid by sodium sulphate precipitation and ion exchange chromatography. OX60 mAb and OVA were individually covalently linked to the encephalitogenic peptide of MBP by sulpho-SMCC reagent (Pierce Chemical Co., Rockford, IL). The encephalitogenic peptide (1) was synthesized by the Fmoc HOBt/NMP method (26), but with a Cys residue added to its NH₂ terminus to facilitate coupling by the sulpho-SMCC reagent (full sequence: CGSLPQKSQRSQDENPVV). Rats were injected *i.v.* with purified mouse anti-rat IL-4 mAb (OX81) according to the schedule set out in results.

Proliferative Responses. Lymph node cells or splenocytes were stimulated with 20 µg/ml MBP, 2 µg/ml of the encephalitogenic peptide, or 5 µg/ml Con A in RPMI/10% FCS containing 2 × 10⁻⁵ M 2-ME, 1 mM sodium pyruvate plus antibiotics. Proliferation was measured by ³[H]thymidine uptake during the last 18 h of a 72-h culture period and results expressed as mean cpm of quadruplicate wells. At various times throughout the culture, supernatants were removed for cytokine analysis and cells were harvested after 24 or 48 h stimulation and RNA purified for analysis of cytokine gene expression by reverse transcription PCR (RT-PCR; see below).

Cytokine Assays. Culture supernatants were assayed for the presence of IL-2 using the murine IL-2-dependent cell line (CTLL) as previously described (27). Briefly, different volumes of samples were added to 2 × 10⁴ CTLL cells (final concentration was between 5 and 50%) and incubated for 18 h at 37°C. The cells were then pulsed with 0.5 µCi of ³[H]thymidine for 6 h and the incorporation of radiolabel was measured. Values are expressed as units per milliliter of IL-2, as derived from a standard curve constructed using a commercial preparation of recombinant human IL-2 (Boehringer Mannheim GmbH, Mannheim, Germany).

IFN-γ protein in the supernatant was measured by specific ELISA. 96-well plates were coated overnight at 4°C with 10 µg/ml of an anti-rat IFN-γ mAb (DB1) and blocked for 30 min with 1% BSA in PBS. Undiluted tissue culture supernatant (50 µl per well), followed by rabbit anti-mouse IFN-γ antiserum that cross-reacts with rat IFN-γ (diluted 1/200) and swine anti-rabbit IgG-alkaline phosphatase-conjugated antiserum diluted 1/1,000 (Dako, Glostrup, Denmark), were sequentially incubated for 2 h at room temperature, separated by three washes with PBS containing 0.05% Tween

20. The assay was developed by adding the enzyme substrate 4-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) at 5 mg/ml for 30 min at room temperature before measuring the absorbance at 405 nm. Values are expressed as U/ml IFN- γ derived from a standard curve constructed using rat recombinant IFN- γ . The DB1 mAb and recombinant rat IFN- γ were kindly supplied by Dr. P. Van der Meide (TNO, Rijswijk, The Netherlands). The rabbit anti-mouse IFN- γ antiserum was kindly provided by Dr. J. Tite (Wellcome Research Laboratories, Beckenham, UK).

RT-PCR. Total cellular RNA was isolated from 5×10^6 stimulated or unstimulated lymphocytes using RNeasy B (Biogenesis, Poole, UK). RNA was reverse transcribed to cDNA using oligo-dT as primer and MMLV reverse transcriptase (GIBCO BRL, Paisley, Scotland) in a final volume of 40 μ l as described (28). For semiquantitative PCR analysis of cytokine mRNA levels, serial dilutions of the cDNA were amplified in 50- μ l reaction volumes as described (28). Reactions were performed in an MJ Research, Inc. programmable thermal controller (Genetic Research Instrumentation Ltd., Dunmow, UK) for the indicated number of cycles. Each cycle consisted of 93°C for 1 min, 60°C for 2 min, and 72°C for 3 min using 37 cycles for IL-4 and IL-13 and 23 cycles for β -actin. After amplification, 10 μ l of amplified product was then separated by electrophoresis on 1.5% agarose minigels and visualized by ethidium bromide staining.

Primers used were as follows: β -actin sense primer 5'-ATG CCA TCC TGC GTC TGG ACC TGG C-3', β -actin antisense primer 5'-AGC ATT TGC GGT GCA CGA TGG C-3', IL-4 sense primer 5'-TGA TGG GTC TCA GCC CCC ACC TTG C-3', IL-4 antisense primer 5'-CTT TCA GTG TTG TGA GCG TGG ACT C-3' (28); IL-13 sense primer 5'-CAG GGA GCT TAT CGA GGA GC-3', and IL-13 antisense primer 5'-CGA GTT AGT AGG ACT TTT GAA G-3' (29). These primers were designed to amplify cDNA fragments, representing mature 607-bp mRNA transcripts for β -actin, 378 bp for IL-4 cDNA, and 279 bp for IL-13 cDNA.

MBP Antibody Detection. Sera from immunized rats were obtained by centrifugation of blood samples that were collected by bleeding rats from the tail artery under halothane anesthesia.

For detection of MBP-specific antibodies, a standard ELISA technique was applied. Briefly, ELISA plates were coated overnight with 10 μ g/ml of purified MBP at 4°C. After saturation with 5% milk powder for 90 min, the plates were washed and serum samples were added in different dilutions (between $1:5 \times 10^2$ and $1:5 \times 10^4$). After a 2-h incubation period, plates were extensively washed and MBP-specific antibodies were detected by addition of horseradish peroxidase-conjugated rabbit anti-rat IgG diluted in 5% normal mouse serum (Serotec, Kidlington, UK). Substrate was added and color development was assayed in an ELISA plate reader. Each serum was tested in duplicate. Results were expressed as OD at 492 nm.

Antibodies to mouse IgG were assayed in essentially the same way as those to MBP, except that the ELISA plates were coated with 10 μ g/ml of OX60 IgG and the sera were diluted to 1:500.

Immunohistology. Cryostat sections of rat spinal cord (5 μ m) were prepared from rats 15 d after immunization with MBP. They were air-dried and stained using the immunoperoxidase method (30). Briefly, sections were fixed in ethanol, washed, and incubated with the chosen mAb for 1 h at 4°C. After further washes, the slides were incubated with peroxidase-labeled rabbit anti-mouse Ig (Dakopatts Ltd., Copenhagen, Denmark). After further washes, bound antibody was revealed using 3, 3' diaminobenzidine HCl. Infiltrating CD4⁺ T cells were detected using the mAb W3/25, macrophages by their reactivity with the mAb ED-1, and MHC class II antigen with mAb OX6.

Results

Pretreatment with Anti-IgD-Peptide Conjugate Protects Rats from Active EAE. Lewis rats were injected i.v. with 100 μ g of mouse anti-rat IgD mAb conjugated to the encephalitogenic peptide of MBP (OX60-pep) on days -14 and -7 and were challenged subcutaneously with MBP in CFA on day 0 (OX60-pep group). The control rats received either 100 μ g of OX60 mAb alone (OX60 group), a mixture of 100 μ g of OX60 mAb and 2 μ g of encephalitogenic peptide of MBP (OX60+pep group), the encephalitogenic peptide coupled to OVA (OVA-pep group), or PBS only (PBS group). As Table 1 shows, pretreatment with the OX60+pep conjugate strongly suppressed disease: 16 out of the 20 Lewis rats pretreated with OX60-pep and challenged with MBP in CFA were completely protected from EAE (Table 1, row 1), and the remaining four rats developed a late and mild form of the disease. In contrast, 10–14 d after challenge, all the control rats in all the treatment control groups (PBS, OX60, OX60+pep, and OVA-pep) exhibited severe EAE and recovered.

These data are consistent with those already published (6) in that they show that targeting of the encephalitogenic peptide of MBP to B cells by chemically linking the peptide to an anti-IgD mAb prevents the induction of EAE after challenging the animals with MBP.

Lymph Node Cells but not Spleen Cells from Protected Rats Proliferate to MBP and ConA. Two rats were injected with 100 μ g of OX60-pep on days -14 and -7, and were challenged with MBP on day 0. Two control rats were injected with PBS instead of OX60-pep. 11 or 15 d after MBP challenge, splenocytes and cells from pooled draining lymph nodes were placed in culture and stimulated with MBP, with the encephalitogenic peptide of MBP or with ConA. The experiment was repeated six more times.

Fig. 1 shows the results of one representative experiment. Proliferation to MBP (Fig. 1 C) and ConA (Fig. 1 D) of lymph node cells from protected rats was equal to that of lymph node cells from control rats. In contrast, spleen cells from protected rats mounted a reduced proliferative response to MBP (Fig. 1 A) and ConA (Fig. 1 B). This reduction of the proliferative response was never observed in PBS-pretreated rats in the seven independent experiments, but it was found in five out of the seven experiments in OX60-pep-pretreated animals. The proliferative response was not restored by increasing the concentration of MBP to 100 μ g/ml or by adding exogenous IL-2 (data not shown). This same nonresponsiveness of splenocytes was found in animals that received, as pretreatment, a mixture of peptide and anti-IgD mAb (data not shown). As noted in the preceding section, the recipients of this antibody-peptide mixture developed EAE with the same severity as controls (Table 1), indicating that this nonresponsiveness was not necessarily associated with protection from EAE.

When lymphocytes from the draining lymph nodes of rats challenged with MBP in CFA after anti-IgD-peptide pretreatment were stimulated in vitro with the encephalitogenic pep-

Table 1. Incidence of EAE in OX60-pep-pretreated Rats and Controls

Group	Incidence (%)	Day of onset*	Clinical score [‡] mean (range)	Mean duration (d)
OX60-pep [§]	4/20 (20)	15.2 ± 0.95	0.3 (0–2)	3.0 ± 1.15
PBS [§]	21/21 (100)	12.1 ± 0.90	4.3 (3–5)	4.6 ± 0.97
OX60	5/5 (100)	11.8 ± 1.09	4.2 (3–4)	4.6 ± 0.54
OX60 + pep	5/5 (100)	12.6 ± 0.89	3.6 (1–4)	4.2 ± 0.44
OVA-pep	4/4 (100)	12.5 ± 0.57	3.5 (1–5)	4.3 ± 0.44

* Mean number ± SE of days between MBP immunization and onset of clinical EAE.

‡ Mean grade of clinical EAE at the time of maximum intensity of inflammation for all rats in the group.

§ Rats from six independent experiments.

|| Data refer to the four rats in this group that developed detectable signs of EAE.

rather than with whole MBP, the results were rather different to those illustrated in Fig. 1. Proliferative responses from the lymph nodes of pretreated rats were usually but not always less than those from control donors (stimulation index = 1.37 ± 0.32 in protected rats vs 2.1 ± 0.87 in diseased rats). The level of reduced reactivity was highly variable, but this variation did not correlate with the level of protection from EAE, which was almost complete in all experiments. The origin of this variability is not apparent. In no case did splenocytes from anti-IgD-peptide pretreated rats proliferate in vitro to the encephalitogenic peptide.

Lymph Node Cells and Splenocytes from Protected Rats Have a Diminished Capacity to Transfer EAE. As shown in Fig. 2, severe EAE developed in all 10 naive Lewis rats given 3–5 × 10⁷ MBP-stimulated lymph node cells (Fig. 2, A and B, four recipients) or spleen cells (Fig. 2, C and D, six recipients)

from rats pretreated with PBS and challenged with MBP (PBS group). Paralysis appeared, as expected, 5 or 6 d after adoptive transfer. In contrast, naive Lewis rats receiving 3–5 × 10⁷ MBP-stimulated lymph node cells (Fig. 2, A and B, four recipients) or spleen cells (Fig. 2, C and D, six recipients) from protected rats (OX60-pep group) developed a mild form of the disease that appeared later than in the controls. The data are from two independent experiments (Fig. 2, A and C, and B and D). This reduced ability of cells from protected rats to transfer EAE could not be explained by a general hyporesponsiveness to the autoantigen because lymph node cells from these animals proliferated as well to MBP as did those from controls (Fig. 1 C).

Lymph Node Cells and Splenocytes from Protected Rats Produce Less IFN-γ. On day 11 after immunization with MBP, lymph node cells and splenocytes from protected and diseased

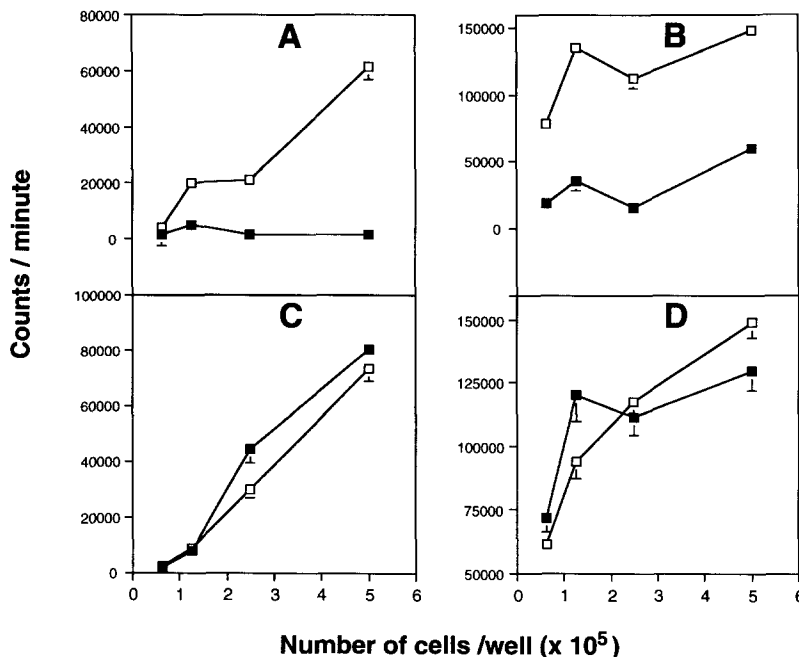


Figure 1. Effect of OX60-pep pretreatment on the proliferative response to MBP and to ConA. Splenocytes and lymph nodes from four Lewis rats challenged with MBP after being pretreated with OX60-pep (filled squares, two rats) or PBS (open squares, two rats) on days -14 and -7 were removed 11 d after MBP challenge. Splenocytes (A and B) or lymph node cells (C and D) were cultured, at several concentrations, in the presence of 20 µg/ml MBP (A and C) or 5 µg/ml ConA (B and D). Proliferation was assessed with a 18-h ³H-thymidine (0.5 µCi) pulse added after 48 h of stimulation. Results of one representative experiment out of five are shown and expressed as cpm ± SE of quadruplicate measurements.

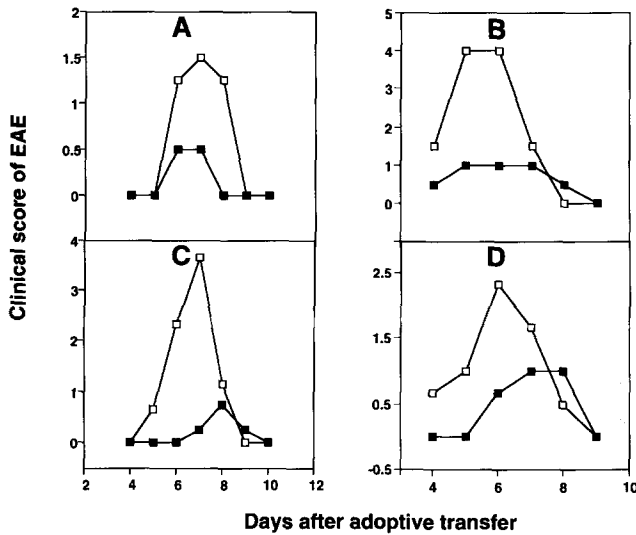


Figure 2. Effect of OX60-pep pretreatment on adoptive transfer of EAE. The figure shows the adoptive transfer of EAE with lymph node cells (A and B) or splenocytes (C and D) from animals where EAE was completely suppressed by anti-IgD-peptide pretreatment (filled squares) or from control diseased rats (open squares), where PBS was used instead of anti-IgD-peptide. Animals pretreated with PBS or anti-IgD-peptide 14 and 7 d before MBP challenge were killed on day 11 or 13 after this challenge and lymph node and spleen cells were prepared. The cells were cultured in vitro with MBP (2 $\mu\text{g}/\text{ml}$) and $3\text{--}5 \times 10^7$ viable leukocytes were injected intraperitoneally into each naive syngeneic recipient. The results shown are of two independent experiments (two rats in each experiment for lymph node cells; three rats in each experiment for splenocytes) and represent the mean clinical scores for paralysis.

rats were stimulated with the autoantigen MBP. Culture supernatants were removed at 24 and 48 h, and were assayed for IL-2 and IFN- γ .

As shown in Fig. 3, the amount of IL-2 detected in the supernatants from MBP-stimulated pooled lymph node cells (Fig. 3 A) or splenocytes (Fig. 3 B) obtained from two OX60-pep-pretreated animals did not differ from what was detected in the supernatants from the corresponding lymphocytes of two control PBS pretreated rats. In contrast, the amount of IFN- γ produced by MBP-stimulated lymph node cells (Fig. 4 A) or splenocytes (Fig. 4 B) from protected rats was very reduced compared to that produced by cells from diseased rats. In no instance did unstimulated cells produce either IL-2 or IFN- γ (not shown). Two additional experiments gave similar results.

Lymph Node and Spleen Cells from Protected Rats Express Elevated Levels of mRNA for IL4 and IL13. Experiments were carried out to determine the effects of pretreatment with OX60-pep on mRNA levels for IL-4 and IL-13 using a semi-quantitative RT-PCR technique (28). On day 11 after immunization with MBP, lymph node cells from protected rats (OX60-pep-pretreated rats), after 24 h stimulation with MBP, expressed higher levels of IL-4 ($\sim 5\times$) and IL-13 ($\sim 10\times$) in comparison to those expressed by MBP-stimulated lymph node cells from diseased rats (Fig. 5). The β -actin mRNA levels

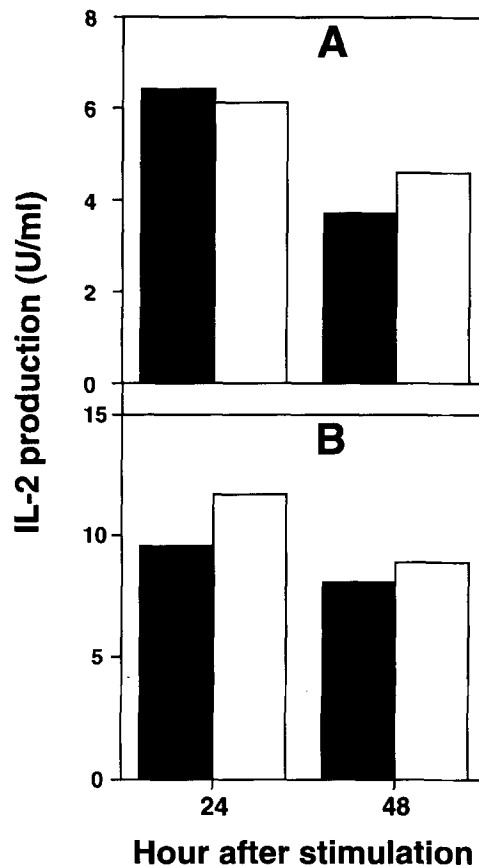


Figure 3. IL-2 production by MBP-stimulated lymph node and spleen cells from OX60-pep-pretreated rats and from controls. Spleens and lymph nodes from four Lewis rats immunized with MBP after being pretreated with OX60-pep (solid bars, two rats) or PBS (open bars, two rats) on days -14 and -7 were removed 11 d after MBP challenge. Splenocytes (A) or lymph node cells (B) were stimulated at 5×10^5 cells per well with 20 $\mu\text{g}/\text{ml}$ of MBP. Supernatants were collected after 24 and 48 h stimulation and were analyzed for the presence of IL-2 protein by using the CTLL bioassay. Results of one representative experiment out of three are shown and expressed as units per milliliter. All values represent the mean of duplicate determination; the range was within 10-15% of the mean.

were similar in both groups. Similar results were obtained when the encephalitogenic peptide was used as stimulus rather than MBP (data not shown).

A Neutralizing Anti-Rat IL-4 mAb Impairs the Protection Induced by Pretreatment with Anti-IgD-Peptide. Experiments were carried out to determine the role of IL-4 in preventing EAE induction by anti-IgD-peptide pretreatment. Rats pretreated with PBS or OX60-pep as described elsewhere (Table 1) were injected with 5 mg of neutralizing anti-rat IL-4 mAb IgG (OX81) on day -14 and thereafter with 3 mg of OX81 mAb on alternate days until day 0, the day of immunization with MBP. As Table 2 shows, the control injections (rows 1 and 2) had no effect on the severity of disease while, as expected, the pretreatment with OX60-pep prevented the development of EAE. In contrast, the anti-IL-4 mAb injections diminished the protection induced by OX60-pep pretreatment. Six out of the seven rats in this group developed EAE.

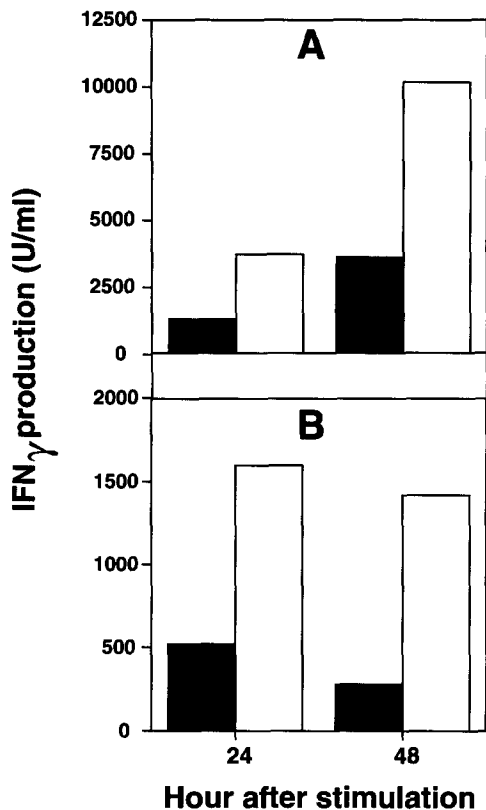


Figure 4. Comparison of IFN- γ production by MBP-stimulated lymph node and spleen cells from OX60-pep- or PBS-pretreated rats. The same supernatants as those tested for IL-2 in Fig. 3 were also assayed for IFN- γ . Data for the lymph node cells are shown in (A) and for splenocyte cultures in (B). Solid bars refer to supernatants from anti-IgD-peptide-pretreated rats and open bars to the controls. Supernatants were collected after 24 and 48 h of stimulation and analyzed for the presence of IFN- γ protein by specific ELISA. Results of one representative experiment out of three are shown and expressed as units per milliliter. All values represent the mean of duplicate determination; the range was within 5–15% of the mean.

Among these six rats, two developed severe disease (bilateral hind limb paralysis, grade 4) comparable to that observed in the control group, and the remaining rats developed mild disease. The effect of the anti-IL-4 mAb was statistically significant ($P < 0.029$ by Fisher's exact test). In contrast, when an irrelevant antibody (OX21) was used in place of OX81, no abrogation of protection was observed (Table 2).

Rats Protected from EAE by Anti-IgD-Peptide Pretreatment Have Few Infiltrating Leukocytes in Their Spinal Cords. Immunohistological examination of the spinal cords of anti-IgD-peptide-pretreated and control rats 15 d after challenge with MBP in CFA showed a very marked reduction in the magnitude of the leukocyte infiltrate in the protected animals. This reduction was observed for CD4⁺ and CD8⁺ T cells and for macrophages, and there was a parallel reduction of MHC class II antigen expression on the microglia. Fig. 6 shows an example in which leukocyte infiltration and MHC antigen class II expression were observed in diseased rats but not in protected rats. Three other randomly selected samples from

other protected rats showed the same result. As recorded in Table 1, a small proportion of protected rats developed a very mild form of EAE that was presumably associated with a degree of leukocyte infiltration. Nevertheless, it is evident that the protection from EAE afforded by the anti-IgD-peptide pretreatment was associated with a much diminished level of encephalomyelitis as evaluated histologically.

Rats Protected from EAE by Anti-IgD-Peptide Pretreatment Make Undiminished Antibody Responses to MBP. The antibody response to MBP in the OX60-pep-pretreated group was assayed on day 11 (Fig. 7 A, five rats) and on day 21 (Fig. 7 B, five rats) after challenge with MBP in CFA and compared with that from the corresponding controls. As shown, animals from the OX60-pep group had antibody levels at least as high as those from controls with EAE.

The same sera were assayed for antibodies to OX60 mAb. Of the 10 anti-IgD-peptide-pretreated rats examined, one made a detectable response (day 21) but the results from the remaining nine were indistinguishable from those obtained from control rats pretreated with PBS (data not shown).

Discussion

The data presented are consistent with those already published (6) in that they show that targeting of the encephalitogenic peptide of MBP to B cells by chemically linking it to an anti-IgD mAb prevents the induction of EAE after challenge of the experimental animals with MBP in CFA (Table 1). As indicated in the Introduction, the aim of this study was to distinguish between two possible alternative explana-

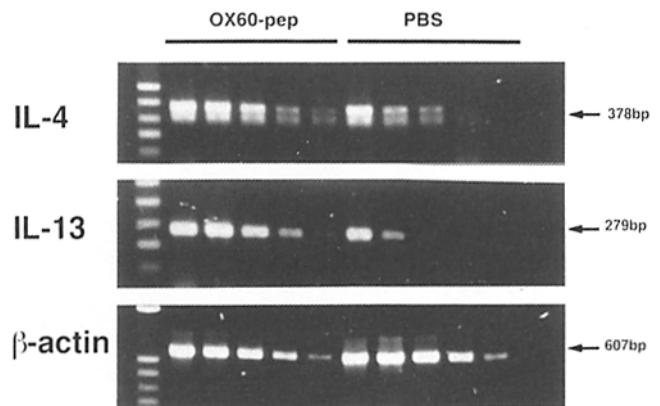


Figure 5. IL-4 and IL-13 mRNA expression by MBP-stimulated lymph node cells from OX60-pep-pretreated rats and from controls. The figure shows mRNA expression by lymph node cells removed 11 d after MBP challenge and stimulated with MBP for 24 h. (Lanes 1–5) Amplified products from increasing threefold dilutions of cDNA from MBP-stimulated lymph node cells from protected rats (OX60-pep-pretreated rats). (Lanes 6–10) Dilutions of cDNA from MBP-stimulated diseased rats (PBS-pretreated control rats). (Lane 11) A negative control (all buffers without RNA). The cDNA templates ranged from 3 μ l of undiluted cDNA (lanes 1 and 6) to 3 μ l of 1/81 dilutions (lanes 5 and 10). Hinfl-digested pATx is the molecular weight marker. The cDNA for IL-4 and IL-13 was examined after 37 amplification cycles and cDNA for β -actin after 23 amplification cycles. Results of one representative experiment out of two are shown.

Table 2. Incidence of EAE in OX60-pep-pretreated Rats and Controls Treated with OX81 (Anti-Rat IL-4)

Group	Incidence (%)	Day of onset*	Clinical score [†] mean (range)	Mean duration (d)
PBS [§]	14/14 (100)	12.2 ± 0.9	3.3 (0.5–5)	4.0 ± 0.9
PBS + OX81	3/3 (100)	11.7 ± 0.6	4.0 (4)	7.0 ± 3.5
OX60-pep [§]	4/13 (31)	17.0 ± 3.5	0.5 (0–4)	3.2 ± 1.7
OX60-pep + OX21	2/5 (40)	15.0 ± 1.4	0.4 (0–1)	4.0 ± 1.4
OX60-pep + OX81	6/7 (86)	15.0 ± 2.6	1.6 (0–4)	5.0 ± 2.6

* Mean number ± SE of days between MBP immunization and onset of clinical EAE. Data refer only to the rats that developed detectable signs of EAE.

† Mean grade of clinical EAE at the time of maximum paralysis for all rats in the group.

§ Rats from three independent experiments.

|| Rats from two independent experiments.

tions for the observed protective effect of the anti-IgD-peptide conjugate, namely the induction of T cell nonresponsiveness or the modification of the cytokine repertoire to the encephalitogen.

It was found that the anti-IgD-peptide pretreatment es-

entially abrogated the leukocyte infiltration into the CNS. Since EAE is a cell-mediated disease, this observation provided a simple explanation for the protective effect of the conjugate but raises the question as to its cause. This lack of infiltration could, in principle, have arisen because the potentially en-

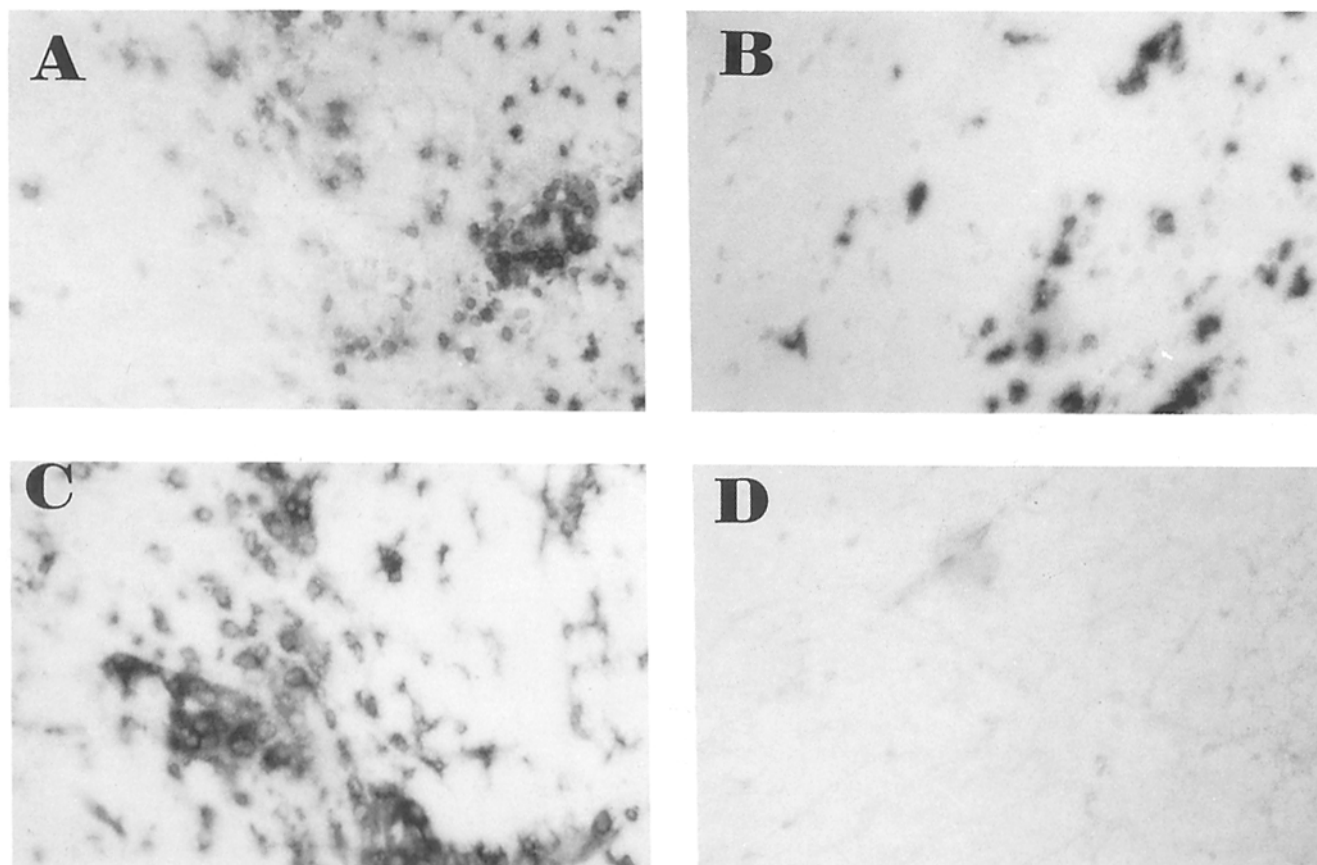


Figure 6. Immunohistological staining of spinal cords from rats with EAE and from those protected by pretreatment with anti-IgD-peptide. Shown are cryostat sections of spinal cord from Lewis rats challenged with MBP after being pretreated with PBS (A–C) or with OX60-pep (D). The sections are stained for CD4⁺ T cells (A), for macrophages (B), and for MHC class II antigen (C and D). ×400.

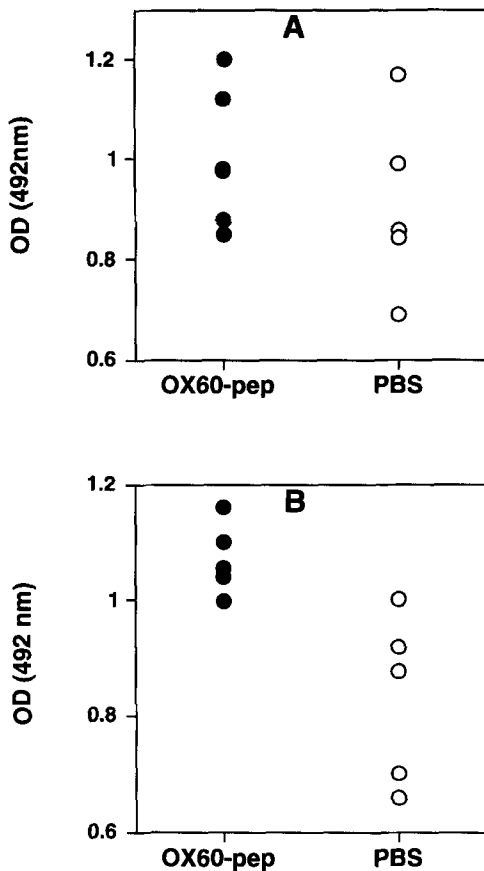


Figure 7. MBP-specific antibody production by OX60-pep-pretreated rats and by controls. Sera from animals of both experimental groups (*filled circles*, OX60-pep-pretreated rats; *open circles*, PBS-pretreated rats) were tested for anti-MBP autoantibody production. The rats were bled on day 11 (*A*, $n = 5$ for each group) and on day 21 (*B*, $n = 5$ for each group) after challenge with MBP. Data are represented as absorbance values for individual rats. All values represent the mean of duplicate determination; the range was within 5–10% of the mean.

cephalitogenic T cells had been rendered nonresponsive by their exposure to the anti-IgD-peptide reagent or because the migratory patterns and inflammatory potential of the reactive cells were changed by the conjugate.

With regard to the possible induction of T cell nonresponsiveness in our experiments, it has been shown that polyclonal monomeric antigen, injected *in vivo* in the form of Fab anti-IgD, induces nonresponsiveness in those T cells that are specific for the xenogeneic immunoglobulin determinants expressed on the Fab fragments of the anti-IgD antibody (17, 18). Use of Fab fragments in these experiments ensured that the surface Ig on the B cells, to which the anti-IgD bound, was not cross-linked, so that these cells remained in an inactivated state. In contrast, the use of polyclonal or monoclonal $F(ab')_2$ anti-IgD fragments in similar experiments did not lead to nonresponsiveness, a result ascribed to the fact that the bivalent $F(ab')_2$ molecules were able to cross-link surface Ig on the B cells and thereby activate them (17, 18). More

recent results have modified this conclusion by demonstrating that the outcome of such experiments depends on the dose of antibody used. Mice injected with a high dose (100 μg) of rat anti-mouse IgD made both a primary response to rat IgG and also a secondary one after challenge with the same dose of the same antibody. However, when one tenth of the dose was used to prime the mice, they made neither a primary or a secondary response, although challenge was again made with the high dose (31). The anti-IgD-peptide conjugates in our own experiments were used at a dose of 100 μg . As noted above, such a dose of anti-IgD in mice was stimulatory while lower doses induced tolerance. Rats have $\sim 10\times$ the body weight of mice and this difference may help to explain the failure of all but one of our anti-IgD-peptide-treated rats to make an antibody response to mouse IgG. However, the single animal that did respond was completely protected from EAE after challenge with MBP in CFA, indicating that a humoral response to mouse IgG was not necessarily associated with a failure of the protective mechanism. Furthermore, the observations on T cell responses from rats pretreated with anti-IgD-peptide conjugates and then challenged with MBP in CFA are not compatible with the view that T cell nonresponsiveness to MBP or to the encephalitogenic peptide thereof was induced by this protocol. *In vitro* stimulation of lymphocytes from nodes draining the site of challenge with MBP in CFA showed that such cells from anti-IgD-peptide-pretreated donors proliferated as well to MBP as those from control rats pretreated with PBS (Fig. 1). When the encephalitogenic peptide of MBP was used to stimulate the cultures, the results were more variable, but the responses equal in magnitude to those obtained from controls were compatible with complete protection from EAE. Furthermore, the anti-MBP antibody titers obtained from pretreated rats (Fig. 7) indicate that the anti-IgD-peptide conjugate treatment led to no deficiency of T cell help for antibody synthesis.

The alternative suggested explanation for the prevention of EAE by anti-IgD-peptide pretreatment, namely the modification of the cytokine response to MBP by this conjugate, derives from the observations that when nonresting B cells serve as APCs, the T cells activated in this manner synthesize IL-4 (7–9) and have, more broadly, a Th2-type cytokine repertoire (7–9, 32). Because the cytokines IL-4, IL-10, and IL-13 produced by Th2 cells are known to antagonize cell-mediated immune responses (11–13, 33–37), it could be anticipated that targeting of the encephalitogenic peptide of MBP to B cells would induce T cells whose cytokines would inhibit EAE (7, 11, 14, 15, 38). Consistent with these published findings, T cells from pretreated rats differed from those of controls. They produced consistently less IFN- γ on activation *in vitro* (Fig. 4) and were much inferior in their capacity to transfer EAE to naive recipients (Fig. 2). These two observations are compatible since it has been shown previously that MBP-specific T cell lines and clones that induce EAE in recipient rats are IFN- γ producers (15, 16, 38) and EAE is regarded as a cell-mediated autoimmune disease (2, 14, 39). In contrast to this apparent deficiency in cell-mediated

immune responses in T cells from pretreated rats, the mRNA levels for IL-4 and IL-13 were ~5–10 times higher than those from controls (Fig. 5). As noted, these cytokines are known to antagonize cell-mediated immune responses so that these observations are consistent with the failure of pretreated rats to develop EAE. The ability of injections of neutralizing anti-rat IL-4 mAb to attenuate the protective effect of the anti-IgD-peptide pretreatment (Table 2) also indicates a role for IL-4 in this protection. However, these findings do not account for the diminished cell infiltration into the CNS that resulted from the pretreatment unless it can be demonstrated that cells with a Th2-type cytokine repertoire, unlike Th1 cells, either do not migrate into the noninflamed CNS or do not recruit other leukocytes into tissues from the circula-

tion. Relevant to this interpretation of our data, it has been reported that Th1 but not Th2 clones mediate delayed-type hypersensitivity (40) and that IFN- γ plays an essential role in the recruitment of lymphocytes into delayed-type hypersensitivity lesions (41).

Combining these published data with the data in this paper makes it possible to account for the protective effects of the anti-IgD-peptide conjugate and for the histological findings in our experiments. We conclude that the conjugate pretreatment induced a Th2-type T cell response to the encephalitogen and that the cells induced in this manner have an intrinsic inability to infiltrate the noninflamed CNS. The results also suggest that further studies on the expression of adhesion molecules on subsets of T cells would be valuable.

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