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The role of serum factors in the suppression of experimental allergic encephalomyelitis: evidence for immunoregulation by antibody to the encephalitogenic peptide

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SUMMARY

Lewis rats immunized with myelin basic protein (MBP) in Freund's complete adjuvant (FCA) suffer from a single episode of paralysis from which they recover spontaneously. Subsequent to recovery, further episodes of paralysis cannot normally be induced by reimmunization with MBP in FCA. It is well established that serum, obtained from rats in the refractory state, can suppress the induction of experimental allergic encephalomyelitis (EAE) when given to animals from the time of immunization with MBP in FCA. Here it is shown that treatment with some such sera from Day 7 after immunization also suppressed the disease. However, not all convalescent sera were suppressive, indicating that rats immunized with MBP in FCA could become refractory to EAE without assayable levels of suppressive activity in their sera. In the context of this result it was notable that a correlation was found between the level of antibody specific for the encephalitogenic peptide in sera and the ability to suppress EAE. An inverse relationship was also shown between the amount of anti-encephalitogenic peptide antibody produced after immunization and the severity of EAE induced. Spleen cells from animals treated with Lewis anti-MBP serum after immunization with MBP in FCA could be activated to transfer EAE by *in vitro* culture with MBP despite the absence of any clinical signs in the donor animals, i.e. the serum inhibited the expansion or differentiation of these cells rather than preventing their priming or bringing about clonal deletion.

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) can be induced in Lewis strain rats by immunization with myelin basic protein (MBP) in Freund's complete adjuvant (FCA). A single episode of paralysis is induced, which is secondary to vascular permeability changes effected by $CD4^+$ T lymphocytes in the central nervous system (CNS) (Sedgwick, Brostoff & Mason, 1987). The animals recover spontaneously from this episode of paralysis (Hinrichs, Roberts & Waxman, 1981); however, a second mild episode of paralysis occurs 5–10 days later in approximately one-third of animals (McFarlin, Blank & Kibler, 1974). Subsequent to this spontaneous recovery, further episodes of paralysis cannot normally be induced by reimmunization with MBP in FCA (Willenborg, 1979; Hinrichs *et al.*, 1981; MacPhee & Mason, 1990). The spontaneous recovery of rats from EAE is mediated by an increase in endogenous corticosteroid production (Levine, Sowinski & Steinetz, 1980; MacPhee, Antoni & Mason, 1989), but the mechanisms responsible for the subsequent refractory state have not been clearly defined.

It is well established that serum obtained from animals that have recovered from EAE is able to inhibit EAE when given to recipient animals from the time of immunization with MBP or CNS tissue in FCA (Paterson & Harwin, 1963; Nakao & Roboz-Einstein, 1965; Hughes, 1974; Willenborg, 1981; Killen & Swanborg, 1982). Suppressor cells have been described in animals convalescent from EAE and there is some evidence that these are B lymphocytes (Welch, Holda & Swanborg, 1980; Killen & Swanborg, 1982; Pesoa, Hayosh & Swanborg, 1984). $CD8^+$ T lymphocytes are known to play no essential role in the maintenance of the refractory phase of EAE (Sedgwick, 1988). A suppressor T-lymphocyte line prepared from the spleens of animals in the refractory phase of EAE has been described, but no effect was seen when the cell line was given *in vivo*, so the relevance of this observation is not clear (Ellerman, Powers & Brostoff, 1988). More recently, it has been proposed that an immune response against the antigen receptor of encephalito-

Abbreviations: BSA, bovine serum albumin; CNS, central nervous system; EAE, experimental allergic encephalomyelitis; FCA, Freund's complete adjuvant; FCS, fetal calf serum; MBP, guinea-pig myelin basic protein; NLS, normal Lewis serum; OVA, ovalbumin; PBS, phosphate-buffered saline; Pep, synthetic peptide 70–86 of guinea-pig MBP; Pep-BSA, synthetic peptide 70–86 of guinea-pig MBP conjugated to BSA.

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genic T lymphocytes may be the mechanism involved (Howell *et al.*, 1989; Vandenbark, Hashim & Offner, 1989).

Refractoriness to EAE can be abrogated by mild immunosuppression with total lymphoid irradiation (Willenborg, 1982), cyclophosphamide (Minagawa *et al.*, 1987) or cyclosporin A (Polman *et al.*, 1988), which suggests that this process has an immunological basis. These treatments do not prevent the spontaneous recovery of animals from EAE, therefore the spontaneous recovery and subsequent refractoriness to reinduction of disease are likely to be mediated by distinct mechanisms.

The refractory phase of EAE develops despite the continued presence of MBP-reactive T lymphocytes. The presence of these cells can be demonstrated by the adoptive transfer of EAE, from convalescent animals to naive syngeneic recipients, by the intravenous injection of donor spleen cells after their *in vitro* culture with MBP (Holda, Welch & Swanborg, 1980). In all of the studies described above, where serum or cells were used to suppress EAE, they were given to animals from the time of immunization with MBP in FCA and may therefore have been interfering with the initial priming of lymphocytes. In contrast, the refractory phase of EAE develops in animals that have already generated EAE-inducing effector cells. For this reason, a protocol was tested where treatment with serum from convalescent animals was delayed until 7 days after immunization with MBP in FCA, by which time T-lymphocyte priming would be expected to have occurred.

There is controversy as to the immunogen required to induce the production of the serum suppressor factor (Nakao & Roboz-Einstein, 1965; Hughes, 1974; Willenborg, 1981; Killen & Swanborg, 1982) and little is known about the mechanism of this serum-mediated suppression. These questions will be addressed here.

MATERIALS AND METHODS

Animals

Eight- to 12-week-old Lewis strain (RT.1^l) rats of either sex were used, and these were bred in the specific pathogen-free animal house of the MRC Cellular Immunology Unit, Sir William Dunn School of Pathology. Animals were age- and sex-matched within each experiment. All procedures were performed under ether or halothane anaesthesia.

Induction of EAE

Animals were immunized with 50 μg guinea-pig MBP [prepared as by Brostoff & Mason (1984), but without the cation exchange chromatography step], emulsified in 100 μl FCA [10 mg/ml; *Mycobacterium tuberculosis* H37 Ra in Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI)] and given subcutaneously into the hind footpads (active EAE). Alternatively, the disease was transferred to naive syngeneic recipients by spleen cells obtained from these animals. The spleens were removed from animals that had recovered from EAE and single cell suspensions were prepared. The cells were cultured at a concentration of 2×10^6 viable leucocytes per ml in RPMI-1640 medium (Gibco Ltd, Paisley, Renfrewshire) containing 5% heat-inactivated fetal calf serum (FCS), $2{\cdot}5 \times 10^{-5}$ M 2-mercaptoethanol and 2 μg/ml MBP, for 72 hr in 5% CO_2 in air at 37°. The cells were harvested and washed three times with phosphate-buffered saline (PBS) containing 0·2% bovine serum albumin (BSA). 5×10^7 viable leucocytes were then injected intravenously (Richert *et al.*, 1979). 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 paresis; 3, unilateral hind limb paralysis; 4, bilateral hind limb paralysis; and 5, bilateral hind limb paralysis and incontinence.

Preparation of sera

Lewis strain rats were immunized with 50 μg MBP or 4 μg synthetic peptide 70–86 (Pep) of guinea-pig MBP (the encephalitogenic peptide; amino acid sequence GSLPQKSQ RSQDENPVV; Chou *et al.*, 1979) or 100 μg of the same peptide coupled to BSA (Pep-BSA), in FCA as described for the induction of active EAE. The peptide was synthesized by the Merrifield solid-phase method (Merrifield, 1963) and was coupled to BSA using the bifunctional coupling reagent maleimidobenzoyl-*N*-hydroxysuccinimide (Liu *et al.*, 1979). Pep and Pep-BSA were a gift from Dr N. P. Groome (Department of Biology, Oxford Polytechnic). To prepare control sera, animals were immunized with 100 μg BSA or 100 μg ovalbumin (OVA; Sigma Chemical Co., St Louis, MO) in FCA, or were left unimmunized. The antisera were usually prepared by serially bleeding groups of animals during various periods from Days 20 to 100 after immunization and pooling the serum, which was prepared by allowing the blood to clot for 1 hr at room temperature followed by incubation for several hours at 4° prior to centrifugation at 750 ***g*** to remove the clot and cells. Some antisera were prepared from blood collected at specific times after immunization and details are given in the results section.

Treatment of animals with the antisera

On Days 7, 9, 11 and 13 after immunization with MBP in FCA, the animals were given 2 ml of serum intravenously in the tail vein. Alternatively, 2 ml serum were given intraperitoneally on Days 6, 7, 8, 9 and 10 after immunization.

Measurement of anti-MBP antibody

Antibody in serum was measured by a solid-phase trace radioimmunoassay by a method similar to that described by Price *et al.* (1986). All incubations were at 4°. Soft plastic 96-well microtitre plates (Flow Laboratories Ltd, Irvine, Ayrshire) were coated with guinea-pig MBP by incubating 75 μl MBP at 20 μg/ml in PBS in each well for 4 hr, followed by two washes with distilled water. The plates were air-dried and stored desiccated at 4°. No loss of activity of the plates was observed after storage for several weeks. Sera to be assayed were diluted serially in PBS/0·05% Tween 20/10 mM NaN_3, and 40 μl were added to triplicate wells. As a standard, a known amount of a rat anti-MBP monoclonal antibody (Clone 12; Groome *et al.*, 1986), which was a gift from Dr N. P. Groome, was diluted in normal Lewis serum (NLS) and titrated on each plate. NLS alone was included as a negative control. After incubation for 24 hr, the sera were removed and the plates washed twice with distilled water. Immunoglobulin binding to the plates was detected by adding to each well 50 μl affinity-purified rabbit anti-rat immunoglobulin, labelled with ^{125}I by the chloramine T method (Byrt & Adda, 1969; at approximately 0·07 μg/ml; 150,000 c.p.m.), in PBS/0·05% Tween 20/10 mM NaN_3/10% normal rabbit serum, and incubating for 1 hr at 4°. This labelled reagent was then removed; the plates were washed twice in distilled water and allowed to air-dry before cutting off the individual wells with a hot wire. The amount of ^{125}I bound to the wells was

determined by counting for 15 seconds on a gamma counter (LKB Rackgamma, Pharmacia, Uppsala, Sweden). The amount of anti-MBP antibody present was estimated from the displacement of the titration curve relative to the Clone 12 standard.

Measurement of anti-encephalitogenic peptide antibody

Synthetic peptide 70–86 of MBP was coupled to soft plastic 96-well plates with glutaraldehyde, by a method modified from Groome, Harland & Dawkes (1985). Each well was incubated with 75 μl of 1·25% (w/v) glutaraldehyde (Koch-Light Ltd, Haverhill, Sussex) in 0·1 M phosphate buffer, pH 5·0, for 24 hr. The plates were then washed twice and 75 μl peptide at 5 μg/ml in 0·1 M phosphate buffer, pH 8·0, were added to each well for 24 hr. After washing twice in distilled water, the remaining active glutaraldehyde-binding sites were blocked by incubating for 3 hr with 100 μl of 0·1% (v/v) ethanolamine (BDH Chemicals Ltd, Poole, Dorset). The plates were again washed twice in distilled water and stored desiccated at 4°. Sera were assayed essentially as for anti-MBP, but the normal rabbit serum was omitted from the diluent for the labelling antibody and an antiserum prepared from four Lewis rats immunized with 50 μg MBP in FCA 100 days previously was used as a standard. The titration of this antiserum defined 1 unit of anti-peptide antibody.

Some assays of both anti-MBP and anti-peptide antibody were performed using an enzyme-linked immunosorbent assay with essentially the same protocol.

RESULTS

Some anti-MBP sera inhibited the induction of clinical signs of EAE

Lewis rats were immunized with 50 μg MBP in FCA on Day 0 and were given 2 ml Lewis anti-MBP serum intravenously on Days 7, 9, 11 and 13 after immunization. Depending on the batch of antiserum used, this protocol gave results varying from complete prevention of clinical signs of EAE (Fig. 1a), to no observed suppression (Fig. 1d). Of six batches of antiserum raised against MBP, three were inhibitory and three were not. Three batches of antiserum given intraperitoneally on Days 6, 7, 8, 9 and 10 were not inhibitory (one of these batches of antiserum was also not inhibitory when given intravenously on Days 7, 9, 11 and 13). There were no differences in the clinical course of EAE between the different groups of serum-donor animals; and there were no clear differences in the period of serum collection between suppressive and non-suppressive antisera. Other possible reasons for this heterogeneity will be discussed later. When one of the antisera was diluted 1/10, a slight suppression of disease was apparent (Fig. 1b) but this was not statistically significant. Antisera raised against OVA or BSA were never found to be inhibitory.

Antiserum raised to a synthetic encephalitogenic peptide inhibited EAE

Rats immunized with peptide 70–86 of MBP develop a single episode of paralysis from which they recover spontaneously and become refractory to further disease induction, in the same way as animals immunized with MBP in FCA. In attempting to define the antigen required to induce the suppressive activity in serum, immunization of animals with synthetic peptide 70–86 of MBP would rule out the possibility of contamination of MBP with other neural components.

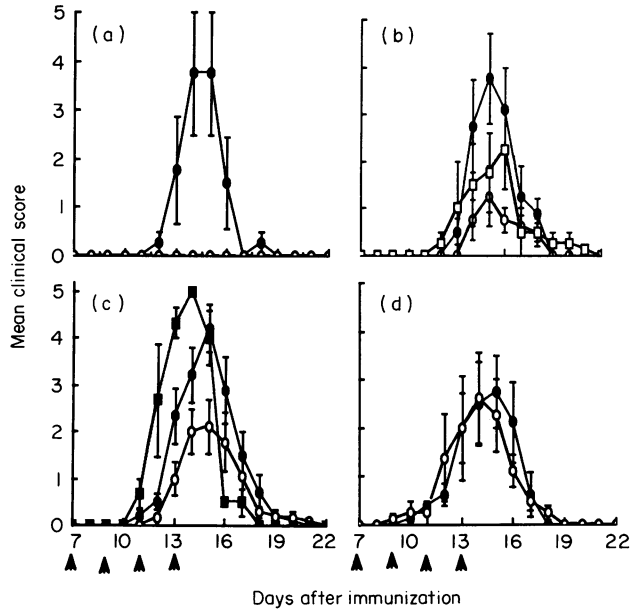

Figure 1. Suppression of EAE with Lewis anti-MBP serum. The results shown represent the mean clinical score plus or minus the standard error. (a), (b) and (d) represent data from single experiments. In (c) the data for NLS- and Lewis anti-MBP-treated animals were pooled from two identical experiments and the data for Lewis anti-OVA-treated rats were from a single experiment. Each graph represents results for treatment with a different batch of Lewis anti-MBP serum. Lewis rats were immunized with 50 μg MBP in FCA on Day 0 and given 2 ml of the specified serum intravenously (i.v.) on Days 7, 9, 11 and 13 after immunization. The data from the inhibitory batches of Lewis anti-MBP (a), (b), and (c) were pooled and the difference in aggregate clinical score (the sum of the clinical scores for each day when the animal was paralysed) between the Lewis anti-MBP-treated and the NLS-treated group was tested for statistical significance by Wilcoxon's rank sum test. The difference was highly significant ($P < 0{\cdot}001$). (●) NLS treated ($n = 4$ in a, b and d, $n = 9$ in c); (○) Lewis anti-MBP treated ($n = 4$ in a, b and d, $n = 9$ in c); (□) Lewis anti-MBP (1:10) treated ($n = 4$); (■) Lewis anti-OVA treated ($n = 3$); arrow, 2 ml serum given i.v.

Serum from peptide 70–86-immunized rats that was collected on the first day of full recovery from paralysis (Days 16–18 after immunization) did not suppress EAE (Fig. 2a). However, a pooled antiserum prepared from peptide 70–86-immunized animals, serially bled on Days 15, 19, 22, 27, 32, 36, 40, 43, 47 and 56 (2 ml blood were taken on each occasion) after immunization, was protective (Fig. 2b). The antiserum collected at recovery from EAE contained very little anti-peptide 70–86 antibody (0·08 units), whereas the serum prepared from later bleeds contained 1 unit of anti-peptide antibody. An antiserum prepared in the same way from BSA-immunized animals was not suppressive.

Transfer of anti-MBP serum elevates the level of antibody specific for the encephalitogenic peptide in the serum of recipient animals

Previous studies of serum suppression have suggested that antibody to a CNS antigen might be the factor involved;

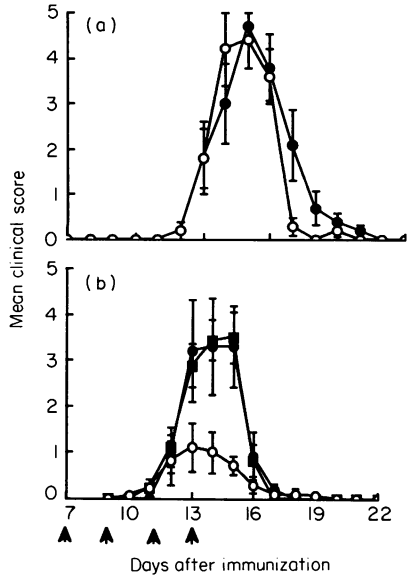

Figure 2. Suppression of EAE with Lewis antiserum against peptide 70–86 of MBP. The results represent the mean clinical score plus or minus the standard error. The results in (a) are from a single experiment, and the results in (b) are pooled data from two identical experiments. Lewis rats were immunized with 50 μg MBP in FCA on Day 0 and given 2 ml of the specified serum i.v. on Days 7, 9, 11 and 13 after immunization. The anti-peptide antiserum used in (a) was prepared by bleeding animals, immunized with 4 μg peptide 70–86 in FCA, by cardiac puncture on the first day that they had completely recovered from EAE (Day 16–18 after immunization). The antisera used in (b) were prepared by serially bleeding animals immunized with 4 μg peptide 70–86 or 100 μg BSA, in FCA from Days 15–56 after immunization. There was no significant difference between the aggregate clinical scores (the sum of the clinical scores for each day when an animal was paralysed) of the anti-peptide 70–86-treated animals and the NLS-treated controls in (a). In (b) the aggregate clinical scores for the anti-peptide 70–86-treated animals were statistically different from the controls by Wilcoxon's rank sum test ($P < 0.005$). (●) NLS treated ($n = 5$); (○) Lewis anti-peptide 70–86 treated ($n = 5$ in a, $n = 9$ in b); (■) Lewis anti-BSA treated ($n = 9$); arrow, 2 ml serum given i.v.

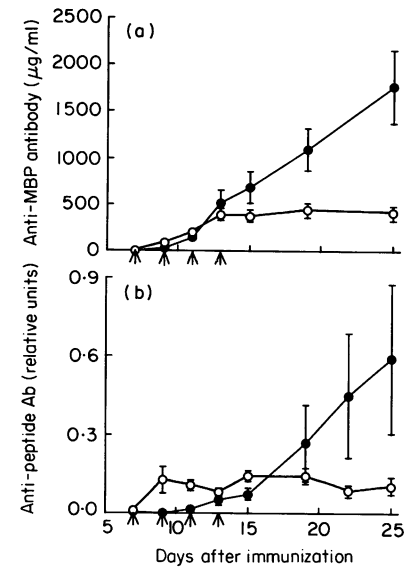

Figure 3. Antibody levels in Lewis anti-MBP-treated animals. The data shown are the mean plus or minus the standard error. The data in (a) were pooled from three experiments (for clinical scores see Fig. 1 a–c), and in (b) the data were pooled from two experiments (for clinical scores see Fig. 1 a, b). Lewis rats were immunized with 50 μg MBP in FCA on Day 0, and given 2 ml of NLS or Lewis anti-MBP i.v. on Days 7, 9, 11 and 13 after immunization. The animals were bled from a cut in the tail on the days indicated (prior to serum transfer on Days 7, 9, 11 and 13) and the levels of serum antibody (Ab) specific for MBP or peptide 70–86 of MBP were measured by solid-phase radioimmunoassay. (●) NLS treated ($n = 13$ in a, $n = 8$ in b); (○) Lewis anti-MBP treated ($n = 13$ in a, $n = 8$ in b); arrow, 2 ml serum given i.v.

however, there have been little supporting data. The antibody titres achieved in recipients of serum from animals that had recovered from EAE were measured in only one previous study (Paterson & Harwin, 1963), but these levels were not compared to those developing spontaneously in control animals injected with encephalitogen but not given convalescent serum.

The levels of antibody to MBP and the encephalitogenic peptide were examined in the serum recipients, in order to determine whether these antiserum transfers elevated antibody levels above those found in recipients of control sera. Anti-MBP antibody levels were marginally higher in anti-MBP-treated animals on Day 9 after immunization, but by Day 13 the antibody levels in treated animals stopped increasing while the level continued to rise in NLS-treated controls (Fig. 3a). By Day 25 after immunization, Lewis anti-MBP-treated animals had four times less anti-MBP antibody in serum than the NLS-treated controls. Adoptive transfer of anti-MBP resulted in a significant increase in the amount of anti-peptide 70–86 antibody from Day 7 until Day 15 after immunization (Fig. 3b). Transfer of antiserum therefore had the effect of 'accelerating' the anti-peptide 70–86 antibody response. Suppression of antibody production in the recipient was again observed at times later than this. The suppression of antibody synthesis by preformed antibody is a well-established phenomenon (Uhr & Moller, 1968).

Suppressive sera contained more antibody to the encephalitogenic peptide than non-suppressive sera

The above observations suggested that anti-peptide antibody was a candidate for the factor responsible for the suppression. Because there was heterogeneity in the suppressive activity of different pools of antiserum, the levels of anti-MBP and anti-peptide 70–86 antibody in inhibitory and non-inhibitory sera were compared. Sera that gave a reduction of 50% or greater in the aggregate clinical score of recipient animals, when compared to NLS-treated controls, were arbitrarily classified as 'suppressive'. Sera that were less inhibitory than this were classified as 'non-suppressive'. There was no significant difference in the level of anti-MBP antibody (as determined using MBP as the target antigen in the solid-phase assay) in suppressive and non-suppressive sera. Suppressive sera, however, all contained more anti-peptide 70–86 antibody than did non-suppressive sera, this difference being statistically highly significant (Fig. 4). However, within the group of suppressive sera there was no relationship between the amount of anti-peptide antibody and the degree of suppression observed.

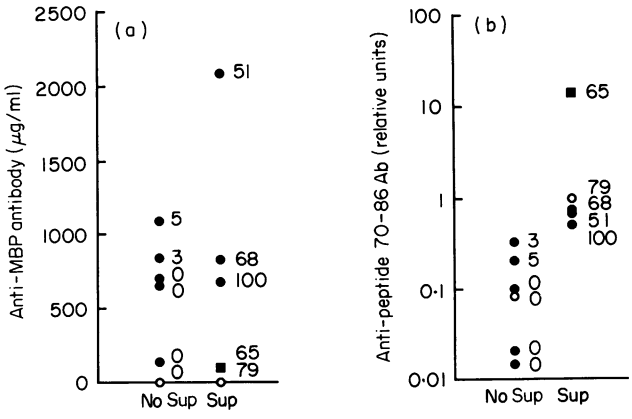

Figure 4. The relationship between the amount of anti-MBP or anti-peptide 70–86 antibody in sera and their capacity to inhibit EAE. Each point on the graphs represents a single experiment. Sera were defined as suppressive (Sup) or non-suppressive (No Sup) based on the percentage suppression of the mean aggregate clinical score (the sum of the clinical scores for each day when an animal was paralysed) in groups of three to five animals compared with normal Lewis serum-treated controls. Lewis rats were immunized with 50 μg MBP in FCA on Day 0 and were given 2 ml of serum i.v. on Days 7, 9, 11 and 13 after immunization (or in the case of two anti-MBP sera, intraperitoneally on Days 6, 7, 8, 9 and 10 after immunization). Sera that suppressed EAE by 50% or more were classed as suppressive, and sera that gave less than 50% suppression were classified as non-suppressive. The numbers next to the points on the graphs represent the percentage suppression of EAE by each antiserum. The sera tested were from animals immunized with MBP ($n=8$), peptide 70–86 ($n=2$), and Pep-BSA ($n=1$). The serum antibody (Ab) levels were determined by solid phase radioimmunoassay. By Wilcoxon's rank sum test the anti-MBP antibody levels did not differ significantly between suppressive and non-suppressive sera but anti-peptide 70–86 antibody levels were significantly higher in suppressive sera ($P<0·005$). (●) Anti-MBP; (○) anti-peptide 70–86; (■) anti-Pep-BSA.

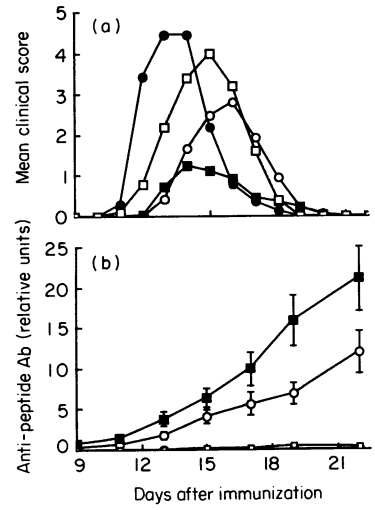

Figure 5. Severity of clinical EAE correlates inversely with the level of antibody against the encephalitogenic peptide produced. The data represent the mean (plus or minus the standard error in b). The data were pooled from three separate experiments which gave the same result. In (a) the error bars are omitted for clarity. The animals were immunized as follows with the antigens in 50 μl FCA (100 μl FCA in total): Group 1 (●) peptide/BSA ($n=7$), 4 μg peptide 70–86 in the left hind footpad, 100 μg BSA in the right hind footpad; Group 2 (■) Pep-BSA ($n=13$), 4 μg BSA in the left hind footpad, 100 μg Pep-BSA in the right hind footpad; Group 3 (○) peptide/Pep-BSA ($n=9$), 4 μg peptide 70–86 in the left hind footpad, 100 μg Pep-BSA in the right hind footpad; Group 4 (□) peptide/Pep + BSA ($n=5$), 4 μg peptide 70–86 in the left hind footpad, 4 μg peptide 70–86 mixed with 100 μg BSA in the right hind footpad. The aggregate clinical scores of the groups (the sum of the clinical scores for each day of paralysis) were compared by Wilcoxon's rank sum test. For 1 versus 2, $P<0·001$; 1 versus 3, $0·01<P<0·025$; 1 versus 4, not significant; 2 versus 3, $0·025<P<0·05$.

An accelerated anti-encephalitogenic peptide immune response was associated with poor induction of EAE

Given that the adoptive transfer of anti-peptide 70–86 antibody appeared to suppress the development of clinical signs of EAE, animals were immunized with the peptide coupled to the foreign carrier protein BSA (Pep-BSA) in an attempt to generate enhanced antibody production, as has been described for other MBP peptides (Groome *et al.*, 1985). Pep-BSA was poorly encephalitogenic, unlike the free peptide which was highly encephalitogenic (Fig. 5). There was little antibody production in animals immunized with the peptide, but high titres of antibody against the encephalitogenic peptide were produced after immunization with Pep-BSA. There were at least two possible explanations for this phenomenon. One possibility was that the peptide coupled to BSA was presented in such a way that B-lymphocyte responses were favoured and the EAE effector T lymphocytes were poorly stimulated. An alternative hypothesis was that the increased levels of anti-peptide antibody were associated with inhibition of the disease process. To test these hypotheses, animals were immunized with a dose of peptide 70–86 known to be encephalitogenic in one hind footpad and Pep-BSA in the contralateral footpad. By this immunization protocol, the disease was much less severe than that induced by the peptide alone ($0·01<P<0·025$) and this was associated with high levels of anti-peptide antibody production. The disease was, however, more severe than when Pep-BSA was given alone ($0·025<P<0·05$) and, for reasons not understood, the level of antibody production was less. If the peptide was mixed with the BSA rather than covalently coupled to it, there was a slight delay in the onset of clinical signs, but the disease was no less severe than that induced by the peptide alone. This was associated with marginally elevated levels of antibody production. Therefore an accelerated anti-peptide 70–86 antibody response was associated with suppression of clinical signs of EAE.

Further evidence that an accelerated anti-peptide 70–86 antibody response results in suppression of clinical EAE comes from the dose–response for immunization with the peptide. Optimal disease induction was found at doses of 1–6 μg peptide. When 50 μg peptide were given, less severe disease was induced in association with an increase in antibody production (Table 1). Only one animal out of 68 immunized with an optimal dose of the peptide (4 μg) failed to show any clinical signs of EAE. This animal made an uncharacteristically accelerated antibody response against the peptide, with 4·6 units on Day 11, 14·7 units on Day 13, and 6·9 units on Day 15 after immunization (compare with the data in Fig. 5).

Table 1. Immunization with a high dose of the encephalitogenic peptide results in mild disease in association with increased production of anti-peptide antibody

Dose of peptide (μg)	Mean aggregate clinical score (±SE)	Mean anti-peptide antibody on Day 20 (±SE)
5	14·4±0·9 (n=9)	0·001±0·001 (n=6)
50	7·6±2·1 (n=14)	2·13±1·08 (n=6)

The number of animals indicated were immunized with the given dose of peptide 70–86 of guinea-pig MBP in 100 μl FCA split between the hind footpads. The results shown represent the mean aggregate clinical score (the sum of the clinical scores for each day when an animal was paralysed), and the level of antibody against peptide 70–86 measured in the sera of six animals from each group collected 20 days after immunization, expressed in arbitrary units as defined in the Materials and Methods.

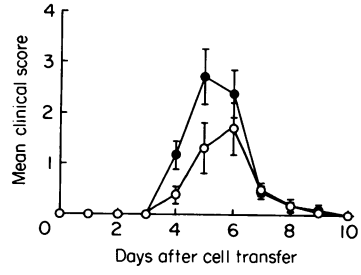

Figure 6. Adoptive transfer of EAE with spleen cells from animals where EAE was completely suppressed by Lewis anti-MBP. The results shown are from two separate experiments and represent the mean clinical score plus or minus the standard error. Spleen cells were prepared from animals treated with NLS or Lewis anti-MBP on Days 7, 9, 11 and 13 after immunization with 50 μg MBP in FCA. In the Lewis anti-MBP-treated group, only animals that showed no clinical signs of EAE were used. In the first experiment the spleens were removed 108 days after initial immunization and in the second experiment 59 days after immunization. The spleen cells were cultured *in vitro* with MBP, and 5 × 10^7 viable leucocytes were injected intravenously into each naive syngeneic recipient. The clinical scores for recipients of Lewis anti-MBP-treated donors were significantly lower than the control group by Wilcoxon's rank sum test on Days 4 and 5 after cell transfer (P<0·05). However, the difference in aggregate clinical scores between the groups was not statistically significant. (●) Cells from NLS-treated donors (n=9); (○) cells from Lewis anti-MBP-treated donors (n=8).

Antiserum treatment did not prevent the priming of EAE effector cells

A feature of the refractory state of EAE is the presence of primed memory cells in the spleens of convalescent animals that can be reactivated *in vitro* to transfer disease to naive syngeneic recipients (Holda *et al.*, 1980). Spleen cells from animals where EAE was completely prevented by treatment with Lewis anti-MBP serum were cultured *in vitro* with MBP and then injected intravenously into naive syngeneic recipients. There was no significant difference in the disease transferred when it was compared to that transferred by spleen cells from NLS-treated controls (Fig. 6). This indicates that the antiserum did not block the priming of the effector cells that cause EAE, but blocked their differentiation or expansion. Therefore the state induced by antiserum treatment is similar to the refractory state induced by immunization with MBP in FCA.

DISCUSSION

We have re-examined the role of serum suppressor factors in the maintenance of the refractory phase of EAE. In all previously reported studies animals were treated with the suppressive antisera from the time of immunization with the encephalitogen. Objections to this protocol were discussed in the introduction. A different treatment regimen was employed in the present work in an attempt to create a situation more closely resembling the refractory phase of EAE. When treatment with anti-MBP was begun on Day 7 after immunization with MBP in FCA, only 5/11 convalescent sera were found to suppress EAE. These sera had higher anti-peptide 70–86 antibody titres than the sera that were ineffective, but it is unclear at this time whether the anti-peptide antibody itself is responsible for the observed suppression or is instead a marker for some other process modulating the immune response. Determination of the specificity of the suppression is clearly indicated, and experiments are currently being performed to determine the precise nature of the suppressive factor. In animals where treatment with convalescent serum prevented the development of EAE, the priming of the encephalitogenic T lymphocytes was not prevented (as is also the case in the refractory phase of EAE; Holda *et al.*, 1980).

The observation that antiserum raised against MBP is suppressive (Nakao & Roboz-Einstein, 1965; Willenborg, 1981; Killen & Swanborg, 1982) has been extended here by the finding that serum obtained from animals immunized with the synthetic encephalitogenic peptide (peptide 70–86 of MBP) suppressed EAE. This suggests that the antisera used in earlier studies were not suppressive merely by virtue of immunization with another component of nervous tissue present as a contaminant.

Suppression of the disease was found in animals where there was little demonstrable increase in the amount of anti-MBP antibody in the serum at any point (Fig. 3a). If anti-MBP antibody were the important factor it would have to be argued that a particular isotype that developed late in the immune response to MBP was responsible for the suppression rather than the total amount of antibody present. Furthermore, there

was no correlation between the amount of anti-MBP antibody in sera and the amount of suppression observed, again indicating that antibody against whole MBP is a poor candidate for the suppressive factor.

Anti-peptide 70–86 antibody levels were, however, significantly elevated in recipients of Lewis anti-MBP serum during the time that EAE would normally have developed. Data presented in Fig. 5 indicate that immunization with Pep-BSA led to accelerated production of anti-peptide 70–86 antibody and was poorly encephalitogenic. Its effect was dominant in that its presence suppressed induction of disease by an immunization that would normally be encephalitogenic. Similarly, Raziuddin, Kibler & Morrison (1981) found that MBP coupled to lipopolysaccharide, which is a potent B-lymphocyte stimulator, was more effective in preventing the reinduction of EAE than MBP alone, and it is possible that this was associated with increased antibody production, but this was not measured. Sera that were suppressive contained more anti-peptide 70–86 antibody than did non-suppressive sera, and the hypothesis can be advanced that anti-peptide antibody is responsible for the suppression. A precedent for the regulation of a tissue-damaging cell-mediated immune response *in vivo* by antibody against the immunizing antigen is found in the passive enhancement of allografts by alloantisera (Morris, 1980) and the suppression of an anti-viral cytotoxic T-lymphocyte response by monoclonal antibodies to the virus (Bangham, 1986). There are several reports which indirectly suggest a role for antibody in the regulation of EAE. Treatment of rats with an irradiation protocol that inhibited antibody production allowed the reinduction of EAE in animals in the refractory phase of the disease (Willenborg, 1982). The offspring of mothers in the refractory phase of EAE were protected from the induction of EAE by a factor in milk. This protection was MBP-specific, correlated with the level of anti-MBP antibody transferred to the neonate and declined with the same kinetics as maternal immunoglobulin levels in the animal (Smith & Rumjanek, 1984; Brenner *et al.*, 1986).

In vitro it has been demonstrated that monoclonal antibodies specific for MBP can inhibit the proliferation and cytotoxicity of MBP-specific T-lymphocyte clones (Jingwu *et al.*, 1989), a phenomenon that has been described previously for other antigens (Corradin & Engers, 1984; Lamb *et al.*, 1984). The association between the antibody response to the epitope of MBP recognized by encephalitogenic T lymphocytes and suppression of the clinical disease suggests that, *in vivo*, the humoral response to MBP can down-regulate the T-lymphocyte-mediated immune response. It would be of interest to determine whether anti-encephalitogenic peptide monoclonal antibodies could be used in the suppression of EAE.

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