Targeting Autoantigen to B Cells Prevents the Induction of a Cell-mediated Autoimmune Disease in Rats

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Summary

Immunization protocols that induce high levels of delayed-type hypersensitivity are often associated with low levels of antibody production, whereas alternative immunization strategies can produce the opposite effect. This reciprocal relationship appears to depend, at least in part, on the fact that T cell–derived lymphokines that are predominantly involved in one type of response inhibit the development of those T cells that promote the alternative one. Such a regulatory mechanism is likely to be bistable in that whenever one form of response is established, spontaneous development of the alternative one will be inhibited. We have applied this concept to the control of a cell-mediated autoimmune disease in rats. By covalently linking the autoantigen to anti-IgD antibody, we have targeted it to B cells for presentation to antigen-specific T cells. This form of presentation favors antibody production and may be expected to antagonize the cell-mediated disease-inducing response to the same antigen. To test this hypothesis, use was made of the fact that experimental allergic encephalomyelitis (EAE), when induced with the encephalitogenic peptide of guinea pig myelin basic protein, is purely a cell-mediated disease. The experiments show that Lewis rats, immunized with the peptide in its encephalitogenic form, were protected from disease when simultaneously injected with the peptide coupled to anti-IgD monoclonal antibodies. Control experiments showed that neither peptide nor anti-IgD alone were protective, and the peptide covalently coupled to irrelevant antibodies also failed to protect. Spleen cells from animals protected from disease by the anti-IgD-peptide conjugate, when activated in vitro with the encephalitogen, were able to transfer EAE to naive recipients. The results demonstrate that a cell-mediated immune response can be controlled by appropriate targeting of the specific antigen without inducing T cell anergy and suggest a potential strategy for preventing autoimmune diseases that are essentially cell-mediated in type.

E xperimental allergic encephalomyelitis (EAE) is an inflammatory disease of the central nervous system (CNS)[1] that can be induced in a number of species of experimental animals by the injection of antigenic material obtained from brain or spinal cord (1–3). In Lewis strain rats immunized with guinea pig myelin basic protein (MBP), or with a peptide consisting of amino acid residues 70–86 (4) of the mature protein sequence, a focal mononuclear cell infiltration develops in the CNS white matter 10–11 d after immunization. This reaction is associated with an ascending paralysis that first affects the tail and then the hind limbs. Incontinence develops in ~50% of the animals. Remarkably, the disease remits spontaneously so that within 5–6 d of the first

sign of paralysis, the animals recover completely (5). A single episode of relapse is observed in a few percent of animals, but in all cases convalescent rats become completely refractory to further attempts to reinduce the disease.

Studies on the effect of adrenalectomy at different stages of the disease suggest that, while the spontaneous recovery of rats from EAE is a consequence of the immediate immunosuppressive effects of corticosterone released from the adrenal glands in response to the stress of the disease (5, 6), the refractory phase may represent a stable corticosterone-induced change in the balance of lymphokine production towards those that inhibit cell-mediated immunity and favor antibody synthesis (7–9). This interpretation of the experimental results rests on the finding that the lymphokines involved in cell-mediated immunity and humoral immunity are agonists for the response with which they are associated,

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[1] *Abbreviations used in this paper:* CNS, central nervous system; MBP, myelin basic protein.

but are antagonists for the alternative one (10, 11). This mutual antagonism provides an explanation for the observation that there appears to be a reciprocal relationship between the two types of immune reaction (12). From these considerations it might be anticipated that immunization protocols that induce those lymphokines involved in humoral immunity would antagonize the development of EAE. There is evidence that antigen presentation by B cells has such an inductive effect (13, 14).

To promote the presentation to T cells of the encephalitogenic peptide of MBP by B cells in the experiments to be described, it was covalently coupled to mAbs that recognize rat IgD. Anti-IgD targeting antibodies were chosen since serum levels of IgD are negligible while a high proportion of rat B cells express surface IgD (S. V. Hunt, personal communication; and D. W. Mason, unpublished results).

Materials and Methods

Animals and the Induction of EAE. Lewis strain rats, from our own specific pathogen-free facility, were used throughout. To induce EAE, adult animals of either sex were injected in the hind footpads with 50 μg MBP emulsified in CFA (5). In individual experiments all animals were of the same sex.

Monoclonal Antibodies and the Preparation of Antibody-Peptide Conjugates. mAb MARD-3 against rat IgD was purchased from the Experimental Immunology Unit, University of Louvain, Belgium. The mAb MRC OX-60, which was derived from a BALB/c mouse immunized with rat thoracic duct lymphocytes, has been shown to react with IgD on ~50% of rat IgD$^+$ B cells. The reason it does not react with all IgD$^+$ B cells is not known at present. Immunoprecipitation studies using MRC OX-60 and MARD-3, and inhibition of binding of MRC OX-60 mAb with IgD myeloma protein, show that this mAb reacts with rat IgD with high affinity but with no other rat Ig isotype (15). MRC OX-21 reacts with a component of human complement but not rat tissues (16). All three mAbs are mouse IgG1 Igs and were prepared from ascitic fluid by sodium sulphate precipitation and ion exchange chromatography. The F(ab')$_2$ fragment of OX-60 was prepared by pepsin digestion, for 14 h at 37°C, of purified OX-60 IgG at pH 4.0, using 0.2 mg of enzyme per 1 mg Ig and at a protein concentration of 5 mg/ml.

The mAbs MARD-3, OX-60, OX-60 F(ab')$_2$, and BSA (Sigma Chemical Co., Poole, UK) were individually covalently linked to the encephalitogenic peptide of MBP by the sulpho-SMCC reagent (Pierce Chemical Co., Rockford, IL). The encephalitogenic peptide (4) was synthesized by the Fmoc HOBt/NMP method (17) but with a Cys residue added to its NH$_2$ terminus to facilitate coupling by the sulpho-SMCC reagent (full sequence: CGSLPQ-KSQRSQDENPVV).

Transfer of EAE by In Vitro T Cell Activation. EAE was transferred from immunized donors to naive recipients as described (18). Briefly, splenocytes from rats immunized with MBP in CFA, or with CFA alone, were stimulated in vitro for 3 d with MBP or with the encephalitogenic peptide thereof, and then injected intravenously into syngeneic recipients. The cell doses used for transfer are given in Results.

Immunochemistry. Portions of the thoracic and lumbar spinal cord were removed from rats in experimental and control groups on day 27 after immunization. 5-μm cryostat sections were fixed in ethanol at 4°C and stained with mAbs to detect infiltrating leukocytes using OX-1 and OX-30 mAbs that recognize all isoforms of the leukocyte-common antigen (rat CD45) (19). Bound antibody was detected by an immunoperoxidase technique as described (20). Specificity of staining was established by using OX-21 mAb in place of the anti-CD45 mAbs.

Results and Discussion

Rats were immunized subcutaneously with MBP in CFA and injected intravenously on the day of immunization and again 7 d later, with either a control preparation or the encephalitogenic peptide of MBP conjugated to mouse anti–rat IgD mAb. As Table 1 shows, the control injections (Exp. 1, rows 4 and 5: Exp. 3, rows 2 and 3; Exp. 4, Row 2) had little effect on the severity of disease, although the onset of disease was a little delayed in some cases. On the other hand, intravenous injection of the anti-IgD-peptide conjugates produced a dose-dependent suppression of EAE such that most animals given two 100-μg doses developed no detectable signs of disease (Exp. 2, row 3; Exp. 5, rows 2, 3, and 4). Animals were also protected if a single 100-μg dose was given 7 d before immunization with MBP in adjuvant (data not shown), but more detailed injection protocols, such as more frequent lower doses, have not been examined. Conjugates of the peptide with anti-IgD F(ab')$_2$ were also protective (Exp. 1, rows 2 and 3).

Contrary to expectation, antipeptide antibody levels, assayed by solid phase RIA (8), were not elevated in anti-IgD-peptide conjugate–protected rats compared with those in the control animals (data not shown). Sera were assayed from bleeds made on days 7, 10, 14, 21, and 25 after immunization, that is, throughout the period of disease development in the controls. In principle, any IgD-positive B cell has the potential to act as an APC for T cell activation in this system (21), whereas only peptide-specific B cells are capable of giving rise to antipeptide antibody-secreting plasma cells. Consequently, it is possible that the induction of T cells with the ability to antagonize cell-mediated immunity can occur without the concomitant production of high levels of specific antibody. In systems where B cell–targeted antigen has been used to evoke high levels of antibody synthesis, much higher doses of antigen were used (13, 14).

Immunohistochemical staining of cryostat sections of spinal cords from rats in Exp. 5 of Table 1 showed, in the controls, heavy focal infiltrates of mononuclear cells that persisted into the recovery phase of the disease (day 27) but in the rats receiving anti-IgD-peptide conjugates, results were more variable. Some animals that showed no clinical signs of paralysis had few if any focal infiltrates in the spinal cord whereas others that remained free from disease and those that developed minimal signs had focal infiltrates not strikingly less marked than control rats with severe paralysis (data not shown). This result resembles that seen in rats protected from EAE by anti-CD4 mAb treatment (18). In these experiments also, marked leukocyte infiltration of the CNS was not incompatible with near normal neurological function. It is not known in what qualitative way disease-inducing infiltrates differ from those that are apparently innocuous.

Table 1. *Protection from Paralysis by Targeting of the Encephalitogenic Peptide to B Cells*

Exp.	Treatment	Peak disease score (mean score)		Statistics vs. controls
	Nil	4, 4, 4, 5, 5	(4.4)	–
1	50 μg F(ab')$_2$ OX-60-peptide	0.5, 1, 2	(1.2)	<0.02
	100 μg F(ab')$_2$ OX-60-peptide	0, 0.5, 1, 1, 2	(0.9)	<0.004
	100 μg BSA-peptide	2, 4, 5, 5, 5	(4.2)	NS
	20 μg peptide	5, 5, 5, 5, 5	(5.0)	NS
	Nil	5, 5, 5, 5, 5	(5.0)	–
2	50 μg OX-60-peptide	0, 0.5, 1, 1, 1	(0.7)	<0.004
	100 μg OX-60-peptide	0, 0, 0, 0, 1	(0.2)	<0.004
	Nil	2, 2, 5, 5, 5	(3.8)	–
3	50 μg OX-60 mAb	2, 4, 5, 5, 5	(4.2)	NS
	100 μg OX-60 mAb	4, 4, 4, 5, 5	(4.4)	NS
	Nil	5, 5, 5	(5.0)	–
4	100 μg OX-21-peptide	4, 4, 4, 4, 4	(4.0)	<0.02*
	Nil	4, 4, 5, 5, 5	(4.6)	–
5	100 μg OX-60-peptide	0, 0, 0, 0, 1	(0.2)	<0.004
	100 μg MARD-3-peptide	0, 0, 0, 0, 1	(0.2)	<0.004
	50 μg OX-60-peptide + 50 μg MARD-3-peptide	0, 0, 0, 0, 0	(0.0)	<0.004

All rats were immunized in the hind footpads with 50 μg guinea pig MBP in CFA on day 0 of the experiments. In some cases, control rats received injections of PBS intravenously on the day of immunization and again 7 d later, while others received no PBS injections. Injection of PBS had no effect and the results of controls given, or not given, PBS have been pooled. All other groups received the stated doses of peptide conjugates intravenously on days 0 and 7. Paralysis was scored on a scale of 1–5 as follows. 1, limp tail; 2, hindlimb weakness; 3, unilateral hindlimb paralysis; 4, bilateral hindlimb paralysis; 5, bilateral hindlimb paralysis plus urinary incontinence (female) or urinary retention (male). The severity of paralysis was assessed daily. Disease reached maximum severity between days 12 and 14 after immunization in all control groups. In those rats treated with OX60 peptide that developed low levels of paralysis, this reached its peak on days 15–16. In the table, the peak disease score is recorded for each rat within an experimental group and the mean score for the group as a whole is given in parentheses. As indicated, there were five rats in each group except in two instances. Statistical significance was evaluated by the Wilcoxon rank-sum test.
* Note that although there is a statistically signficant difference in the severity of disease between rats injected with OX-21-peptide and controls (Exp. 4), the effect is very weak and does not reach significance when compared with, for example, the controls in Exps. 1 and 5.

In principle, it was possible that presenting the encephalitogenic peptide to CD4$^+$ T cells by targeting it to B cells resulted in the induction of anergy in the peptide-specific T cells (22, 23). Such an effect was not seen in mice injected with a polyclonal anti-IgD antibody; instead, this treatment induced the synthesis of IL-4 (13), which, as mentioned in the introduction, would be expected to antagonize any cell-mediated response to the immunogen. However, the possibility that an anti-IgD mAb might induce T cell anergy has not been studied. To examine the question of anergy in the present experiments, a test was made of the ability of splenocytes from anti-IgD-peptide–treated rats to passively transfer EAE. Two groups of eight rats were immunized with MBP in CFA but one of these two groups was also injected with 100 μg OX60-peptide conjugate on days 0 and 7. A control group of four rats was injected with adjuvant alone. Four rats in each of the first two groups were observed throughout the experiment for signs of EAE and the remaining two sets of four were used, on day 13 after immunization, as spleen cell donors for the passive transfer of EAE.

As Table 2 shows, most of the rats immunized with MBP in CFA developed severe EAE and spleen cells from them, when activated in vitro with either MBP or the encephalitogenic peptide-induced EAE in recipient animals. Significantly, the group of rats protected from EAE by the injection of anti-IgD peptide conjugate, while developing little or no disease themselves, also served as donors of transferred EAE. As the data show, the incidence and severity of the disease in the recipients was no less than that observed when splenocytes from nonprotected donors were used to transfer EAE, and this was equally true when either whole MBP or the encephalitogenic peptide was used in the in vitro activation step of the transfer. The third group of rats, immunized with adjuvant alone, did not develop EAE, and their splenocytes failed to transfer disease after in vitro stimulation with MBP or peptide. Evidently the in vivo priming of donors was an

Table 2. *T Cells from Rats Protected from EAE by Injection of Anti-IgD-Peptide Conjugates Are Not Anergic to the Encephalitogen*

Treatment of donors	Peak disease score in donors (mean score)	Antigen used in vitro*	Peak disease score in recipients (mean score)	Statistics vs. controls
MBP/CFA	0‡, 4‡, 4‡, 4‡, 0, 4, 4, 4	MBP	1, 1, 1 (1)	–
	(3)	Peptide	1, 1, 1 (1)	–
MBP/CFA + OX-60-peptide	0‡, 0‡, 0‡, 0‡, 0, 0, 1, 1 (0)	MBP	1, 1, 2 (1.3)	NS
		Peptide	1, 1, 4 (2)	NS
CFA alone	0‡, 0‡, 0‡, 0‡ (0)	MBP	0, 0, 0 (0)	0.05
		Peptide	0, 0, 0 (0)	0.05

Spleen cells from rats protected from EAE by the injection of anti-IgD-peptide conjugate were assayed for their ability to transfer EAE after in vitro activation with either MBP or the encepthalitogenic peptide. There were three rats in each recipient group. Splenocytes from unprotected animals or from rats injected with CFA alone served as positive and negative controls, respectively. The statistical data were calculated with respect to the positive controls. The majority of recipients that developed signs of paralysis did so 5 d after cell transfer and had recovered by day 9. The two exceptions occurred in the group of three rats that had received cells that had been obtained from unprotected MBP/CFA-immunized donors and that had been stimulated in vitro with the encephalitogenic peptide. These rats developed disease on days 6 and 7, respectively, and had both recovered by day 9.
* Spleen cells from donor rats were stimulated in vitro, at 10^6 cells/ml, with either 2 µg/ml MBP or 2 µg/ml of the encephalitogenic peptide. After 3 d in culture, 5 × 10^7 cells were injected into each naive recipient.
‡ Animals used as donors of spleen cells for adoptive transfer of EAE.

essential step in the adoptive transfer. These results, therefore give no indication that the anti-IgD-peptide conjugate mediated its protective effect by the induction of T cell anergy but, instead, allowed the in vivo priming of the donor splenocytes to proceed normally. As with the histological finding reported in the previous section, these results are also similar to those obtained in rats in which EAE was prevented by anti-CD4 mAb treatment in that splenocytes from animals so treated were also found to be fully competent to transfer disease after in vitro activation (18).

EAE can be inhibited in a number of ways, including the use of mAbs to CD4 and class II MHC antigens (24, 25). These protocols require the use of relatively large amounts of antibody and lack antigen specificity. Preimmunization with liposomes containing MBP (26), or with large amounts of encephalitogen in IFA (27, 28), also induces refractoriness to EAE, possibly by essentially the same mechanism as that described herein involving protection by the B cell-targeted peptide. Treatment of rats with mAb to the V_β determinant of the TCR or immunizing them with synthetic peptides whose amino acid sequences are found in the appropriate V_β chain have also been reported to prevent EAE (29–31), but it is uncertain how general such approaches might be in other autoimmune diseases where more than one V_β family may be involved. The method of disease control reported here does not require the use of adjuvant, is antigen specific, and is apparently without side effects. It remains to be established whether it has broader applications.

The data also raise certain possibilities. In at least some instances, are suppressor T cells simply those that promote the reciprocal type of immune response to that which is being assayed by the experimenter, and can rheumatoid factor B cells, by taking up and processing immune complexes (32), influence the lymphokine repertoire of T cells responding to the antigenic component of these complexes?

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