Peptides of Myelin Basic Protein Are Encephalitogenic in Rats without the Aid of Emulsions (43150)

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Abstract. **Immunization with peptides is usually done with the aid of Freund's adjuvant. Using peptides derived from myelin basic protein, we show that aqueous solutions can be antigenic (encephalitogenic in this instance) in Lewis rats. The first procedure involved multiple doses of aqueous peptide, increased absorption into the lymphatic system from the peritoneal cavity in the postinflammatory state, and the use of pertussis vaccine. Three different peptides containing the major encephalitogenic site were active in this system, with the activity somewhat proportional to the size of the fragment. The second procedure, the direct delivery of peptide to lymph nodes by percutaneous inoculation, was equally successful and did not require the use of pertussis vaccine.**
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Many biologically active peptides are antigenic. Peptides are usually incorporated into Freund's adjuvant as a water in oil emulsion when their immunologic properties are under study. Antigens are more effective when emulsified in mineral oil because they are widely disseminated in lymph nodes and are protected from excessively rapid catabolism (1). Unfortunately, oily adjuvants introduce problems of emulsion stability, an undesired inflammatory focus at the inoculation site, antigenic competition, and sometimes disseminated arthritis if mycobacteria are used, as in Freund's complete adjuvant (FCA).

Recently, we reported that the antigenicity (in this instance, encephalitogenicity) of the intact myelin basic protein (MBP) could be demonstrated without employing FCA (2). This advance was made possible by a new method for increasing absorption of inoculum into draining lymph nodes (3) or by direct inoculation into lymph nodes (4). In view of the importance of peptide research in many areas of biomedical science, we report extension of the work on MBP to its constituent peptides. Like MBP, aqueous solutions of the peptides are antigenic (encephalitogenic) without the need for oily adjuvants.

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Materials and Methods

Male Lewis rats, 8–10 weeks old, from Harlan Sprague-Dawley, Indianapolis, IN, were kept in hanging metal cages with wire mesh floors with Purina Rodent Chow 5001 and tap water available *ad libitum*. All procedures conformed to governmental and institutional requirements. One week before immunization, sodium hypochlorite (NaOCl, 0.026%, a 1/200 dilution of household bleach, 50 ml/kg) was injected intraperitoneally to produce a sterile chemical peritonitis. Although it did not produce any indisposition, this procedure is known to increase absorption of subsequent inocula by inactivating the scavenger function of the greater omentum. This assures greater contact of the inoculum with the lymphatics in the diaphragm (5), the stomata of which are widened (6).

The antigens were MBP from Hartley guinea pig brain and its constituent peptides, the preparation and characterization of which have been reported previously (7–10). For the first series of experiments, 11 nmol of MBP (0.2 mg) or peptides were dissolved in sterile saline and injected intraperitoneally always in a volume of 1 ml. Subsequently, lower and higher doses were studied to the extent permitted by the limited supply of these materials. Inoculations of NaOCl and antigen were preceded by an overnight fast to avoid accidental penetration of the gastrointestinal tract. In most instances, intraperitoneal inoculation of antigen was repeated after 1, 2, and 3 days, but without prior fast. Several hours after the second injection of antigen, the rats were

given 1 ml of a 1/10 dilution of a pertussis vaccine concentrate (2×10^{10} organisms) intraperitoneally and another 1 ml intravenously to increase their susceptibility (2).

For direct inoculation of lymph nodes, the popliteal node was enlarged by subplantar injection of 200 mg of metallic tin powder suspended in 0.5 ml of saline in the right hindfoot. Three weeks later injections into the node were easily done percutaneously (4).

Immunologic effects were monitored by development of experimental allergic encephalomyelitis (EAE). Clinical signs of EAE were scored daily: 1+ for weak, flaccid tail; 2+ for limb weakness or ataxia; and 3+ for paralysis. Histologic evaluation was done on spinal cords fixed in Bouin's fluid and removed from vertebral column as described previously (11). Longitudinal segments of the entire cord and draining mediastinal lymph nodes were embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Perivascular mononuclear infiltrates were scored from 1+ to 4+ according to their number and severity, without knowledge of the treatments.

Results

The first experiment compared the complete MBP molecule to peptide 1–88 which contains the major encephalitogenic site and to peptide 89–167 which does not have that site, all at a 11-nmol dose. At this dose level, peptide 1–88 was equal to MBP in encephalitogenicity (Table I). Omission of the NaOCl pretreatment reduced the activity of peptide 1–88 to the same degree as MBP, thus extending to peptides our previous reports on advantages of immunization in the postinflammatory state (2, 3, 12–14). Peptide 89–167 was nonencephalitogenic.

Further comparison of MBP with peptide 1–88 was done by injecting the 11-nmol dose only twice instead of four times. With this suboptimal schedule, the superior encephalitogenicity of the whole MBP molecule became evident (Table I). Interestingly, the addition of separate but simultaneous intraperitoneal injections of 11 nmol of nonencephalitogenic peptide 89–167 to 11 nmol of peptide 1–88 did not increase the activity of 1–88 (Table I).

The next group of experiments was done with the full schedule of four intraperitoneal injections as well as NaOCl and pertussis treatments. Peptide 1–88 and several smaller fragments were encephalitogenic at various dose levels. MBP was several times more potent than peptide 1–88 (Table II), confirming the conclusion of the previous experiment. Fragment 45–88 was slightly more active than 63–88, and both were less encephalitogenic than 1–88 (Table II). Fragments 45–62 and 1–44 were not encephalitogenic even at very high dose levels.

In the last experiment, peptide 1–88 was demonstrated to be encephalitogenic even when injected without the benefit of NaOCl or pertussis vaccine treatments. This was accomplished by direct inoculation into lymph nodes. The right popliteal nodes of eight rats were enlarged by injections of tin powder into the hindfoot 3 weeks in advance. Four of these rats were given four successive daily percutaneous injections of 11 nmol of peptide 1–88 directly into the popliteal node. Three of the four rats developed clinical signs of EAE 9 or 10 days after the first dose and all had

Table I. EAE Produced by Aqueous Solutions of MBP or Peptide Is Increased in Postinflammatory State

Pretreat[a]	Antigen[b]	No. of 11-nmol doses	EAE clinical signs		EAE lesions[e]
			Incidence[c]	Severity[d]	
NaOCl	MBP	4	4/4	2.5	3.9
NaOCl	1–88	4	4/4	2.8	3.8
NaOCl	89–167	4	0/4	0.0	0.0
None	MBP	4	2/4	0.5	2.6
None	1–88	4	1/4	0.3	1.6
None	89–167	4	0/4	0.0	0.0
NaOCl	MBP	2	4/4	1.0	ND
NaOCl	1–88	2	0/4	0.0	0.4
NaOCl	1–88 + 89–167	2 + 2	0/4	0.0	0.6

[a] One week before immunization, intraperitoneal.
[b] Peptides designated by inclusive numbers; intraperitoneal. Rats also received pertussis vaccine intraperitoneally and intravenously 1 day after starting immunization.
[c] Numerator, number of rats with clinical signs. Denominator, total number of rats.
[d] Average scores, scale of 0 to 3+.
[e] Average scores, scale of 0 to 4+. ND, not done.

Table II. Dose-Response Comparison of MBP and Peptides

Antigen	Dose (nmol)[a]	EAE clinical signs		EAE lesions
		Incidence	Severity	
MBP	5.5	14/14	2.7	3.9
MBP	1.1	10/10	2.5	3.6
MBP	0.55	10/12	2.3	3.1
MBP	0.11	2/8	0.5	1.0
MBP	0.055	0/4	0.0	0.0
1–88	11	3/4	2.3	2.6
1–88	5.5	4/4	2.0	2.8
1–88	0.55	0/4	0.0	0.3
45–88	28	3/4	2.0	3.1
45–88	11	2/4	0.5	2.0
63–88	28	1/4	0.8	1.4
63–88	11	1/4	0.3	0.9
45–62	55	0/4	0.0	0.0
1–44	28	0/4	0.0	0.0
Saline	—	0/10	0.0	0.0

[a] Repeated on four consecutive days, intraperitoneal. All rats also received NaOCl intraperitoneally 1 week before first dose and pertussis vaccine intraperitoneally and intravenously 1 day after first dose. Collected results from five experiments.

Table III. EAE Produced by Inoculation of Aqueous MBP or Peptide into Lymph Nodes

Antigen[a]	Day[a]	Inoculated lymph node[b]	EAE clinical signs		EAE lesions
			Incidence	Severity	
MBP	0,1,2,3	Right popliteal	4/4	1.5	4.0
1–88	0,1,2,3	Right popliteal	3/4	1.5	3.8
MBP	0,1,2,3	Popliteals, elbows	2/2	1.0	3.5
1–88	0,1,2,3	Popliteals, elbows	1/2	1.0	3.0
MBP	0,0,0,0	Popliteals, elbows	1/2	0.5	2.5
1–88	0,0,0,0	Popliteals, elbows	0/2	0.0	1.0

[a] Each of four doses of 11 nmol in aqueous solution was injected on consecutive days or on the same day. Unlike the experiments of Tables I and II, neither pertussis vaccine nor NaOCl were administered.
[b] Tin powder inoculated 3 weeks beforehand in the right hindfoot (or in all four feet) enlarged the right popliteal lymph node (or both popliteal and both elbow nodes), so that most of the MBP or peptide inoculations could be done percutaneously.

histologic lesions (Table III). The other four rats were given MBP in the same manner and they developed equally severe EAE, thereby confirming the previous report (2). Four pairs of rats were pretreated with tin powder in *all four feet*. When the four successive peptide or MBP percutaneous injections were rotated on a daily basis among the four sets of enlarged lymph nodes, all of these rats developed EAE. When all four peptide or MBP doses were given *on the same day* (distributed among the four sets of enlarged nodes), the EAE signs and lesions were distinctly less despite the injection of the same total dose (Table III).

Discussion

By injecting four doses of antigen in rats whose susceptibility was enhanced by the postinflammatory state and by pertussis vaccine, we were able to demonstrate encephalitogenicity, not only in MBP, but in all of the peptides that contained the major encephalitogenic epitope. However, neither the postinflammatory state nor pertussis vaccine were essential parts of the immunizing regimen, as peptides were encephalitogenic without their assistance when delivered directly to lymph nodes by percutaneous inoculation. EAE has not been produced previously by aqueous solutions of peptides without FCA. We have not yet tried another way to circumvent the need for FCA, that is to adsorb peptides on the surface of finely divided particulate materials. This method was effective with the whole molecule of MBP (15) and it was shown that adsorbed MBP was protected from enzymatic degradation (16). In the present work, enhanced absorption into lymph nodes or direct delivery into lymph nodes may have served the same purpose by minimizing or completely avoiding enzymatic degradation at inoculation sites. In addition, NaOCl pretreatment or direct delivery into lymph nodes may have augmented the essential steps of antigen processing and presentation because of activation or increased numbers of macrophages in the lymph nodes, or increased contact between antigen and macrophages.

Peptide 1–88 contains the major encephalitogenic epitope (72–84) of guinea pig MBP for Lewis rats (17–19). Nevertheless, the whole MBP was more potent on an equimolar basis than peptide 1–88 (Table II). This might be explained by a secondary site in 89–167, and 1–88 might be more effective than 45–88 because of a secondary site in 1–44. Weak, secondary epitopes in guinea pig MBP (89–115) have been suggested but not proven (9, 10, 20). More likely, smaller peptides may be less antigenic simply because of their small size.

Many authors have suggested that presentation of endogenous myelin antigens to lymphocytes *in situ* in the nervous system might lead to EAE (and multiple sclerosis?) by autosensitization (21, 22). The demonstration in the present work that FCA is not essential for the encephalitogenicity of peptides adds an element of credibility to the concept of autosensitization. However, our work puts emphasis on access of peptide antigens to lymph nodes rather than presentation of endogenous central nervous system (CNS) antigen to immunocompetent lymphoid cells that circulate through the CNS. Other methods of producing EAE with MBP but without conventional adjuvants have also relied on lymphoid tissue (23, 24). Perhaps more study should be devoted to the lymphatic drainage of the CNS. CNS lymphatics, despite their topographic restriction (25), might be significant for autoimmunization.

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