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The Development of Experimental Allergic Encephalomyelitis with Immunizing Doses of Myelin Basic Protein (38871)

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Experimental allergic encephalomyelitis (EAE) is an autoimmune neurological disease of the central nervous system (CNS) characterized by lymphocytic inflammation and demyelination of the white matter (1–3). The disease is caused by a delayed hypersensitivity reaction to myelin basic protein (BP) (4), or to specific peptide regions derived from the BP (5, 6) and has proved to be an excellent model for cell-mediated immunity (7), similar in many respects to multiple sclerosis, postinfectious encephalomyelitis, and postrabies vaccinal encephalomyelitis (7).

Prevention of EAE was accomplished in rats and guinea pigs by pretreatment of the animals with whole CNS tissue (8–10) or with emulsions of BP in incomplete Freund's adjuvant (IFA) (11, 12). Antibodies induced by BP-complete Freund's adjuvant (CFA) emulsions were reported to play an insignificant role in induction (13), reversal (14, 15), or severity of EAE (16). In contrast, suppression of EAE was accomplished by passive transfer of serum from immunized animals (17). Thus, the role of encephalitogen-induced antibodies in inhibiting subsequent onset of EAE remains unclear, due to the small number of rabbits (14) and the large amounts of CFA which have been used for immunization and booster injections (14, 18). Neither have early detection of delayed-type hypersensitivity (DTH) and the development of EAE after the administration of large doses of the BP from the CNS tissue of different species been reported.

In view of the contemplated use of the BP for the treatment of human demyelinating diseases (19), rabbit experiments have been designed to evaluate the appearance of humoral antibodies, DTH, and EAE in response to the administration of relatively large amounts of BP.

Materials and Methods. Animals. White male New Zealand rabbits (Buckberg Lab Animals, Tomkins Cove, NY) weighing 5–6 lb were used in all experiments. They were housed in wire cages and provided with food and water ad lib.

Source of basic protein. The BP was prepared from fresh frozen rat, bovine, and guinea pig spinal cords and from human, monkey, and rabbit brains by previously described procedures (20, 21).

Immunization schedule. Individual rabbits were bled before immunization with the BP. The initial low and high doses totaled 4.5 and 23 mg BP per rabbit, respectively, and were administered in three separate simultaneous injections. These injections were made up of 1.125 or 5.75 mg (25% of the total BP) emulsified with CFA (50 μg *M. butyricum*) injected in the hind foot pad; 2.25 or 11.50 mg BP (50% of total BP) in IFA (total volume 0.3 ml) injected subcutaneously; and 1.125 or 5.75 mg (25% of total BP) dissolved in sterile saline (0.5 ml) injected intravenously via the peripheral ear vein. All animals received three booster injections consisting of 2.25 or 11.50 mg (50% of the initial dose of BP) emulsified in IFA injected subcutaneously at days 7, 14, and 21 after the initial immunization. The total amount of protein injected into each rabbit was 11.25 and 57.50 mg BP for low and high doses, respectively. Immunized rabbits were bled successively at intervals of 7 days after immunization and the serum was separated from clotted blood and frozen at $-70°$ before use.

Clinical and histological signs of disease. The immunized rabbits were inspected daily for clinical signs of EAE. These included loss of weight, ataxia, lethargy, tremors, incontinence, and hind leg paralysis. Rabbits showing initial signs of EAE such as loss of weight were not sacrificed; those with leth-

TABLE I. THE RELATIONSHIP BETWEEN ANTIBODY RESPONSE AND DISEASE INDUCTION

Source of (BP)	Rabbit number	Dose	Ab	EAE	
				Clinical	Histological
Human	111	L	—	35	++
	112	L	—	—	—
	168	H	64	—	+
	169	H	64	—	—
Bovine	105	L	—	—	—
	106	L	—	—	—
	119	H	—	21	++
	120	H	29	—	—
	150	H	—	28	++
	151	H	29	—	—
	164	H	60	—	—
	165	H	60	—	+
Monkey	109	L	—	—	—
	110	L	—	—	+
	177	L	21	—	—
	178	L	28	—	++++
	179	L	—	12	+++
	189	L	—	—	++++
	190	L	—	—	++++
	121	H	29	—	—
	122	H	—	14	+++
	156	H	34	—	—
	157	H	34	—	—
	180	H	21	—	—
	181	H	21	—	—
	182	H	—	12	+++
	191	H	28	—	+
	192	H	28	—	—
Guinea pig	113	L	—	35	+++
	114	L	—	35	++
	166	H	—	—	+
	167	H	—	—	+
Rat	107	L	—	21	++
	108	L	—	—	—
	183	L	50	—	—
	184	L	50	—	—
	185	L	50	46	++
	193	L	—	—	+++
	194	L	—	12	++
	173	H	15	150	++
	186	H	21	—	+
	187	H	21	—	++
	188	H	21	—	—
	195	H	—	27	++
	196	H	30	—	—

[a] The course of development of immunological response to the encephalitogenic myelin basic protein derived from the central nervous system tissue of five species are shown. Two different doses are given. Rabbits injected with low doses (L) and high dose (H) are shown. The day at which the antibody (Ab) and disease (experimental allergic encephalomyelitis) were first detected are indicated by the number of days after immunization. The (−) sign under symptomatic EAE refers to the absence of hind leg paralysis and incontinence; however, it does not preclude loss of weight or lethargy observed in 90% of the immunized rabbits. Histological changes of the brain and spinal cord are reported negative (−) indicating the absence of any lesion characteristic of experimental allergic encephalomyelitis or as (+). The highest score is designated by 4+ characteristic of extensive perivascular infiltration usually observed in paralyzed animals.

argy and hind leg paralysis were sacrificed 2–3 days after appearance of these symptoms.

The skin test assay. The DTH assay was performed as early as 5 days after immunization. There was no difference in the magnitude of the skin response to 25 μg of BP derived from five different species. For this reason the skin test was routinely done with BP from bovine CNS tissue. The assay, which is routine in our laboratory (5), was administered by one individual and the erythema recorded at 1, 3, 4, and 24 hr independently by two other individuals. Rabbits showing a negative response at 1, 3, and 4 hr were considered positive DTH respondents when the erythema began to develop after 4 hr, reaching a maximum response by 24 hr. Controls and rabbits immunized with CFA in saline emulsions gave negative response to the BP.

The antibody assay. The initial antibody screening was performed by the immuno-, double-diffusion, Ouchterlony technique. The serum was placed in the center well with four serial dilutions of antigen in the outer wells. Each serum was tested against a range of 1.25–10.0 μg BP antigen per well. Sera showing immunoprecipitating lines were confirmed by immunoelectrophoresis.

Results. The clinical and laboratory findings in rabbits immunized with BP isolated from the CNS tissue of five different species are shown in Tables I–III. Regardless of the amount of BP injected, the majority (90%) of the rabbits lost 10–25% of their body weight within 15 days after immunization. Circulating anti-BP antibodies appeared after weight loss. At this time, the rabbits

TABLE II. THE DELAYED-TYPE HYPERSENSITIVITY RESPONSE IN RABBITS IMMUNIZED AND SKIN TESTED WITH THE BASIC PROTEIN.[a]

	Rat Low Dose			Rat High Dose			Monkey Low Dose			Monkey High Dose		
Days	183	184	185	186	187	188	177	178	179	180	181	182
5	5	13	8	8	ND	5	15	12	13	13	14	10
14	9	15	9	11	ND	5	15	20		22	14	
21	11	10	10	9	ND	7	17	15		14	14	

[a] Rabbits were skin tested with the basic protein from bovine myelin at indicated days after immunization with low and high doses of the basic protein derived from rat and monkey CNS tissue. The antigen, 25 μg, was dissolved in 0.1 ml sterile saline and injected intracutaneously. The numbers shown are the average cross-sectional diameters (in mm) of the erythematous area recorded 24 hr after the test. Because of the severity of the disease, rabbits 179 and 182 were sacrificed before the second skin test was performed. Identical skin test results were obtained when the above immunized rabbits were skin tested with basic protein derived from rabbit CNS tissue.

TABLE III. RELATIONSHIP BETWEEN THE DEVELOPMENT OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS AND THE PRESENCE OF CIRCULATING ANTIBODIES.[a]

				+Ab		−Ab	
Dose	No. of rabbits	EAE	+Ab	+EAE	−EAE	+EAE	−EAE
Low	20	12	5	2	3	10	5
High	25	13	18	6	12	7	0
Total	45	25	23	8	15	17	5

[a] The rabbits were immunized with low or high doses of the basic protein as described in the text. The presence (+Ab) or absence (−Ab) of circulating anti-BP antibodies is shown in relationship to the development of experimental allergic encephalomyelitis (EAE) evidenced by clinical and histological criteria.

started to gain weight without force feeding or else developed frank clinical signs of the disease.

Our data show that onset of the disease occurred as early as day 12 after immunization (Table I). At this time, the symptoms were mild, including loss of weight and appetite as well as lethargy from which the rabbits could recover. In addition to loss of weight, certain rabbits (Nos. 179, 122, 182, 194) developed hind leg paralysis from which they never recovered. Rabbits that survived the initial loss of weight might at a later date develop clinical signs of disease. In these rabbits, the onset of EAE could be as late as 150 days (Table I), and the symptoms included hind leg paralysis, followed rarely by death.

Plasma-cell infiltration around blood vessels of the CNS was demonstrated clearly in sections of the brain and spinal cord. However, the absence of hind leg paralysis did not predict negative pathology (Table I). Rabbits surviving the first EAE episode clearly showed cellular infiltration of the CNS tissue, a finding not reflected in the clinical course of the disease (Table I). The pathologic changes in these animals varied in severity; the highest score, 4+, is generally observed in diseased animals showing hind leg paralysis and incontinence.

The DTH response was the first immunological expression observed in the animals of this study. The skin hypersensitivity was specific for BP, regardless of its origin, and was detectable as early as 5 days after immunization (Table II). The response began to appear 4 hr after the skin test, and was maximal by 24 hr. The erythematous area was round, indurated, and occasionally showed central necrosis. At 48 hr the erythema faded, and disappeared by 72 hr. Hyperimmunized animals with detectable circulating antibody titers gave skin test

values consistent with antibody-mediated skin response. In these animals the skin response appeared within 1 hr after the skin test, reaching a maximum within 24 hr, and the erythema persisted for more than 3 days after the test. Histological examination of the skin test site of antibody-mediated response revealed limited mononuclear cell infiltration of the skin when compared to the extensive cellular infiltration characteristic of skin test sites of DTH-respondent animals.

Twenty-five percent (5/20) and 72% (18/25) of the animals immunized with low and high doses of the BP, respectively, developed specific anti-BP antibodies (Table I). Although the BP derived from guinea pig CNS tissue did not elicit antibody response in the four rabbits tested, two of the rabbits developed pathological signs of EAE. The relationship between anti-BP antibodies and the development of signs of EAE is shown in Table III. Two of the low-dose rabbits developed disease as did six of the high-dose animals in the presence of circulating anti-BP antibodies. In contrast, 10 of the low-dose and 7 of the high-dose rabbits developed EAE without demonstrable humoral antibodies specific for BP during the time they became diseased. Fifty-one percent (23/45) of immunized rabbits (Table III) developed circulating antibodies, regardless of the dose. The prevalence of EAE in these rabbits was 34.8% (8/23) compared to 77.3% (17/22) of the rabbits without detectable antibodies.

Discussion. The main finding described in this report is the development of EAE after immunization with large amounts of BP derived from human, bovine, monkey, guinea pig, and rat CNS tissues. More than 55% (25/45) of the immunized rabbits developed disease as early as 12 and as late as 150 days postimmunization. These results are in agreement with those of others (14, 18) who showed that a single or multiple doses of BP induce EAE regardless of the amount of BP, the amount of adjuvant, or the frequency of BP injections.

The first apparent immunological response to large doses of BP was induction of BP-specific DTH regardless of the source of the BP used for immunization or skin testing. These results are consistent with the conclusion that cellular sensitization responsible for EAE occurs at an early stage during the development of an immunological response to the BP antigen. The delayed skin reactivity, which correlates with subsequent development of disease (4), appears as early as 5 days postimmunization. The magnitude of the delayed response increases after the first booster injection (14 days), and by day 35 the delayed response shifts to immediate hypersensitivity, in which the response appears shortly after the introduction of the antigen into the skin and develops into a large, diffuse, and reddish area within 24 hr. The shift from delayed to immediate hypersensitivity correlates with the appearance of circulating anti-BP antibodies.

With respect to EAE induction in the presence of humoral antibodies, our results confirm previously published reports (14, 18) and further reveal an inverse relation between the development of antibodies and EAE. The number of rabbits developing EAE in the absence of antibodies (17 rabbits) was more than double the number of diseased (eight rabbits) in the presence of antibodies. Specific mechanisms for antibody-mediated suppression of EAE can not be elucidated by this study; however, experiments have shown that EAE may be suppressed by passive transfer of serum from immunized animals (17). Moreover, animals immunized with BP in IFA, which generally gives rise to humoral antibodies, were found refractory to the development of EAE when they were subsequently challenged with encephalitogenic emulsions of BP (22).

The immune response to the BP appears to proceed in stepwise fashion. Initially cell-mediated immunity appears, as demonstrated by the early detection of delayed skin hypersensitivity prior to antibody or disease onset. It is possible, however, that cell-mediated and humoral immunity develop concurrently, both of which influence disease development. Repeated administration of the BP may induce a sponging effect and thus suppress the level of available circulating antibodies. That such a reaction may occur is suggested by the immediate skin hypersensitivity response of immunized

rabbits to small amounts of the BP. The same concept could be applied to describe the fate of sensitized cells. The repeated administration of large amounts of BP and its dissociation from the injection site may induce "immune paralysis" as a result of avid recognition between the antigen and the sensitized cell. Evidence to support this argument is found in the fact that prolonged administation of the BP leads to p rotection and suppression of EAE in monkeys (23), and treatment of recipients before or after transfer of lymph node cells from sensitized animals abolishes the transfer of EAE (24).

It is, therefore, difficult to ascribe the suppression of EAE to a single factor such as encephalitogen-induced paralysis of sensitized cells or to antibody–antigen interaction which sponges out the antigen and prevents its interaction with receptor cells. It is clear, however, that large amounts of BP injected into rabbits give rise to cell-mediated immunity responsible for EAE. In view of the finding that more than 55% of the immunized rabbits developed EAE, the danger stemming from the use of the BP for the treatment of demyelinating diseases lies in the encephalitogenic property of the antigen and in the possible onset of EAE during or after the cessation of treatment with the encephalitogen.

Summary. Rabbits immunized with low (11.25 mg) and high (57.50 mg) doses of myelin basic protein from several species develop antibasic protein antibodies, delayed type hypersensitivity, and clinical and pathological signs of experimental allergic encephalomyelitis. More than 55% of rabbits immunized with relatively high doses of basic protein develop disease. The absence of circulating antibasic protein antibodies in immunorespondent animals is associated with the appearance of clinical or histological signs of experimental allergic encephalomyelitis; however, the presence of humoral antibodies did not prevent completely the development of disease. Delayed-type hypersensitivity, specific for the basic protein, appears as early as 5 days after immunization and is maintained in nondiseased and surviving animals. Neither excess encephalitogen nor encephalitogen-induced antibody is sufficient to suppress completely the eventual development of clinical or histological manifestations of disease.

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