Treatment of Experimental Allergic Encephalomyelitis with Encephalitogenic Basic Proteins[1] (36174)

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Successful treatment of experimental allergic encephalomyelitis (EAE) after development of clinical signs and histologic lesions has been reported. The immunosuppressive drugs, ACTH (1), methotrexate (2), cyclophosphamide (3), melengestrol and hydrocortisone (4), as well as anti-lymphocytic serum (5–8), facilitated recovery of paralyzed animals. Treatment of EAE by immunologically specific methods would avoid the systemic resistance-lowering effects of these drugs. Immunologically specific prevention and suppression of EAE by brain basic protein have already received considerable attention but treatment of established disease with this agent has been reported only by Alvord *et al.* (9). This subject warrants intensive study because the possibility (as yet unproven) of a relationship between EAE and multiple sclerosis has stimulated therapeutic trials of brain basic protein in human patients (10). The present work concerns basic protein therapy in rats with EAE produced either by active or passive immunization. The relationship between the species of origin of the therapeutic basic protein and the species of the antigenic neural tissue used to elicit EAE has been studied.

Methods. Male rats, from Microbiological Associates Inc., 200–275 g in weight, were of the Lewis strain except for one experiment on the Fisher 344 strain. EAE was induced by inoculation of 0.05 ml of an encephalitogenic emulsion into one of the sole pads of the right hind foot. The emulsion was prepared by cycling equal volumes of a 40% spinal cord homogenate and Freund's complete adjuvant (8.5 parts Bayol F mineral oil, 1.5 parts Arlacel A emulsifying agent, 4 mg/ml killed human tubercle bacilli) between two syringes. The emulsion was heated to 60° for one hour to reduce bacterial contamination. In the experiment with monkey spinal cord, a relatively weak antigen in our system, 0.1 ml of a pertussis vaccine concentrate was injected into the dorsum of the right hind foot as an ancillary adjuvant, to insure a disease of sufficient severity for clinical evaluation. Pertussis was not used with the stronger antigens because it would have caused a rapidly fatal form of EAE (11).

The rats were scored daily for clinical signs of EAE: 1+, limpness of the tail; 2+, limb weakness; 3+, paralysis. Dragging of the tail, without flaccidity, was not considered a sign of EAE. Reproducibility of scores between two observers was good; differences of one grade occurred in only 6 of 60 observations. These minor differences would have affected group averages very little or not at all. Nevertheless, all data in Table I are derived from a single observer's scores and these were highly reproducible. Rats that developed signs too late for inclusion in the experiment, or not at all, were deleted from the study. On the day treatment was started, usually 10 or 11 days after inoculation and 1 or 2 days after onset of clinical signs, the rats were graded clinically, arranged in order of severity, and assigned alternately to the basic protein and control groups. Rats that had the same score were assigned in numerical order, the animals having been numbered at the time of immunization. This procedure ensured that the average scores were almost equal at initiation of therapy, while it ex-

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TABLE I. Treatment of EAE in Rats with Basic Proteins.

Exp.	Encephalitogenic antigen[a]	Basic protein therapy[b]	Severity of clinical signs[c] Days after start of therapy 0	2	4	7	Deaths[d]	Histologic severity[e] (survivors)
1	G. pig	G. pig ×1	1.7	1.9			0/9	2.9
		Saline ×1	1.5	2.3			0/10	3.9
2	G. pig	G. pig ×2	1.5	1.5	0.8		0/6	2.7
		Saline ×2	1.3	2.0	1.8		1/6	4.0
3	G. pig	G. pig ×3	1.6	1.2	0.3	0	1/7	2.2
		Saline ×3	1.6	2.0	2.4	0	4/7	3.9
4	G. pig	G. pig ×3	1.9	2.0	0.6	0	3/8	2.0
		Saline ×3	1.8	2.6	2.0	0	7/8	4.0
		Histone ×3	1.8	2.9	3.0	0	7/8	4.0
5	Rat	G. pig ×3	1.9	1.8	1.1	0	0/8	2.4
		Rat ×3	1.9	2.0	0.8	0	2/8	2.3
		Monkey ×3	1.9	2.4	1.7	0	2/8	3.3
		Saline ×3	2.0	2.8	2.1	0	7/11	3.0
6	Monkey	Monkey ×3	1.9	1.7	0	0	2/9	0.7
		Saline ×3	2.0	2.1	1.1	0	3/8	2.3
7, 8	G. pig[f]	G. pig[g] ×1	1.1	0.9			0/12	1.2
		Saline ×1	1.2	0.8			0/13	2.6

[a] Species of origin of spinal cord tissue used for active immunization.

[b] Species of origin of basic protein used for therapy, or nature of control material, and number of treatments.

[c] Numbers are group averages of individual clinical scores, scale 0 to 3+. Scores on days 1, 3, 5, 6 omitted for brevity. Rats were killed on last day for which data are entered.

[d] Number that died/total number treated.

[e] Average score for EAE lesions in *survivors*, scale 0 to 4+.

[f] Passive transfer experiments: species of origin of spinal cord tissue used for active immunization of donors of lymph node cells.

[g] Passive transfer experiments: treatment given to recipients of immunized lymph node cells. Two experiments gave similar results and are combined.

cluded any possibility of subjective bias in making assignments. Subsequent clinical scores were determined without knowledge of the animal's treatment group or number.

Basic proteins were prepared from guinea pig brain, rat spinal cord, and African Green monkey brain (12, 13). Each protein was dissolved in saline, 0.2 mg/ml, and the dose of 1 ml was given intravenously. The controls were given 1 ml of calf thymus histone of the same concentration, or 1 ml of saline. In Experiments 1, 7 and 8, a single treatment was given and the rats were killed 2 days later. In Experiment 2, treatment was repeated after 2 days and the rats were killed 2 days later. In Experiments 3–6, treatment was repeated 2 and again 4 days after initiation of therapy and survivors were sacrificed after an additional 3 days. The 2-day interval between treatments was chosen because suppressive effects of 0.2 mg basic protein last 3–4 days at most (14, 15).

Rats were sacrificed 2 or 3 days after the last treatment by ether anesthesia and exsanguination. Hindbrain and spinal cord were fixed in Bouin's fluid, embedded in entirety in paraffin, sectioned and stained with hematoxylin–eosin. Slides were randomized and

scored without knowledge of group of origin: 1+, up to 4 or 5 lesions on the entire slide; 2+, a few lesions in each of several different low-power fields; 3+, lesions in many different fields; 4+, many lesions in all or almost all fields.

Results. In Experiments 1–4, the spinal cord used for induction of EAE and the basic protein used for therapy were both derived from the guinea pig (Table I). One, two or three treatments were given to rats after they developed clinical signs. In each experiment, the basic protein-treated rats had less progression of signs and earlier regression than the saline or histone treated controls. Beneficial effects on clinical scores were most marked at *intermediate* times (4 days after beginning treatment). Observations at earlier times revealed that clinical signs occasionally worsened one or even 2 days after start of basic protein treatment in individual rats, even when this was not reflected in the group averages and even when the affected rat eventually improved. Late observations (7 days) were of no value because of complete clinical recovery of remaining rats in both treated and control groups.

In each experiment where deaths occurred, mortality was reduced by basic protein therapy. Among all groups treated with three injections of guinea pig basic protein only 4 out of 23 rats died (17%), while the figures for the corresponding controls were 25 out of 34 (74%). The 4 deaths in the treated groups all occurrred in rats that were paralyzed (3+ score) when treatment was initiated. It may be that brain damage and/or urinary tract complications were already too severe in these few animals to permit recovery.

Histological lesions among survivors were reduced by basic protein in each experiment. Among all groups treated with 1, 2 or 3 injections of guinea pig basic protein (46 survivors), the average histologic score was 2.1, while the average for the 37 corresponding controls was 3.4. It should be noted that rats that died before the end of experiment were lost to the histological part of the study. This introduced a selective factor because the high mortality in the control groups caused them to lose severely afflicted members that would have contributed high histological scores. Were it not for this selective factor, some of the control groups would have reached even higher scores, which would have emphasized the beneficial effect of basic protein treatment even more.

When rat spinal cord was used to elicit EAE, basic proteins from rat or guinea pig were effective therapy (Experiment 5). Monkey basic protein reduced mortality but had no effect on histologic scores. However, the selective effect of excess deaths among controls may have been at work here, so that a small therapeutic effect of monkey basic protein cannot be excluded. In any event, a beneficial effect of monkey basic protein was clearly demonstrable when the eliciting spinal cord antigen was of monkey origin (Experiment 6).

Passive transfer of EAE. This technique permits the separation of immunologic events at the site of inoculation and its draining lymph nodes from what transpires after sensitized lymphoid cells enter the blood stream. In brief, donor Lewis rats were inoculated with guinea pig cord, Freund's adjuvant and pertussis vaccine to achieve an intense immunization. Seven days later, the lymph nodes draining the site of inoculation were harvested, processed into a cell suspension and injected intravenously into normal recipients (15). Most recipients developed clinical signs of EAE after 3 to 7 days and they were treated either 5 or 7 days after passive transfer. As in the active sensitization experiments, guinea pig basic protein had a definite therapeutic effect (Experiments 7, 8). Although the short duration of these two experiments and the relatively mild signs did not allow detection of clinical improvement, the histological evaluation revealed a clear effect with almost no overlap of values with the controls.

Statistical analysis. This was based on standard error of the differences in means between all rats represented in Table I that were treated with guinea pig basic protein and all the corresponding saline and histone controls. Clinical signs 2 days after start of therapy averaged 1.5 for 49 basic protein-treated rats and 2.1 for 63 controls; differ-

ence of means significant at 5% level. Signs 4 days after start of therapy averaged 0.8 for 25 basic protein-treated rats and 2.2 for 23 controls; difference significant at 1% level. Histological lesions among survivors averaged 2.1 for 46 basic protein-treated rats and 3.4 for 37 controls; difference significant at 1% level.

Discussion. One or more injections of 0.2 mg basic protein had a consistent beneficial effect on EAE in 8 experiments even though clinical signs and histological lesions were already well established. This effect was manifest in the histological scores 2 or more days after the start of treatment and in the obvious clinical improvement after 4 days. The occurrence of a therapeutic effect in so short a time as 2 days makes it unlikely that the basic protein injection had stimulated production of blocking antibodies, a suggestion made by Campbell (16) to explain the results of Alvord *et al.* (9).

In the passive transfer experiments the effect of basic protein could not be at the site of inoculation nor on the production of sensitized cells. In recipients of passively transferred cells the basic protein could have influenced the ability of sensitized lymphoid cells to multiply or to recruit other cells, or it could have influenced their migration to, or interaction with, the central nervous system.

The relationships between species of origin of the therapeutic basic protein and species of spinal cord used for elicitation of EAE were in excellent accord with data reported for suppression of disease before lesions are formed (9, 15). Even after clinical signs of EAE develop, it is possible that histological lesions are constantly being formed anew as well as resolved. Therefore, the so-called "therapeutic" effect of basic protein may really represent a "suppressive" effect on new lesion formation, while resolution, continuing at its natural pace, is responsible for the lowering of scores.

The explanation for the partially species-specific relationship between induction of EAE and therapeutic effectiveness is not apparent. Neither the amino acid sequence nor the chemical basis for encephalitogencity in the rat have been defined for the basic proteins used in the present experiments. The marked difference between the specific activity in the rat of rat and guinea pig myelin basic proteins, on the one hand, and monkey, human and bovine basic proteins on the other (15) suggests that there are important structural differences.

Summary. The therapeutic effect of myelin basic protein on established EAE, reported for actively immunized guinea pigs by Alvord *et al.*, has been extended to rats with clinical signs of EAE produced by active sensitization or passive transfer. Furthermore, there was evidence of a limited degree of species specificity inasmuch as rat or guinea pig basic proteins were more effective than monkey basic protein in treating EAE produced with rat spinal cord and adjuvants. However, monkey basic protein appeared to be a fully effective therapeutic agent when EAE was produced with monkey spinal cord. The present work derives importance from current interest in treating patients with multiple sclerosis with basic protein or substances derived from basic protein. The experiments described herein provide a basis for further investigation, in which therapeutic gains can be measured by clinical signs, mortality and histological scoring after only 1 to 3 treatments and in as short a time as 2 days.

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