Resistance to Proteolytic Degradation of Myelin Basic Protein Adsorbed on Resin[1] (37405)

RUBEN GRUENEWALD, SEYMOUR LEVINE, AND LORENZO FORBES

Pathology Department, New York Medical College, Center for Chronic Disease, Bird S. Coler Hospital, Welfare Island, New York, New York 10017

A basic protein (BP) from myelin is the antigen responsible for elicitation of experimental allergic encephalomyelitis (EAE). Its encephalitogenic capacity is not expressed when aqueous solutions are injected (1) but it is detected when BP is incorporated in oily emulsions (Freund's adjuvant) or adsorbed on certain particulate materials (2). Protection of antigen from enzymatic degradation is one of the mechanisms proposed to explain the action of immunologic adjuvants (3). The employment of aqueous suspensions of particulate adjuvants has enabled us to test this hypothesis *in vitro*.

Methods. BP was prepared from rat spinal cord (4). It was bound to a washed suspension of cation exchange resin with known adjuvant properties (2) (cation exchange form of polystyrene–divinylbenzene resin No. 6B, 3.5 μm spherical particles, Particle Information Service, Los Altos, CA). The resin was saturated by slowly shaking 100 or 200 mg resin with 5 or 10 mg BP in 10 or 20 ml distilled water at room temperature for 2 hr. The particles were washed twice with water to remove soluble BP. Under these conditions, 80% of the BP was adsorbed, as determined by spectrophotometric assay of the supernatant (5).

Resin-bound BP suspended in 6–9 ml water was adjusted to pH 8.0 with 3% ammonium hydroxide. The suspension was shaken slowly for 3 hr at 37° with enzymes dissolved in 0.5 *M* Tris buffer (pH 8.0), or with Tris buffer alone. Pronase (B grade) and alpha chymotrypsin (A grade from bovine pancreas, both from Calbiochem, Los Angeles, CA) were used as representative proteolytic enzymes, known to be capable of degrading BP to nonencephalitogenic products (6). The reaction products, containing resin-bound BP and residual enzymes, were bioassayed by intraperitoneal injection of 2 ml aliquots (20 mg resin and 0.8 mg original BP) into female Lewis rats (150–200 g, Microbiological Associates, Inc., Walkersville, MD). The rats were observed for clinical signs and their entire spinal cords were studied histologically for EAE lesions (2).

The potency of the enzymes was confirmed by reacting them with soluble BP under identical conditions of pH, temperature and time. In this instance, however, residual BP was bioassayed after dilution in saline to 1 mg/ml of original basic protein and emulsification in an equal volume of Freund's complete adjuvant. The dose of 0.05 ml, representing 25 μg of original BP, was injected into a left foot pad.

Results. The encephalitogenicity of soluble BP was completely destroyed by pronase at 1:200 enzyme:basic protein ratio, and by chymotrypsin at 1:25 (Table I). In contrast, BP bound to resin was unaffected by these enzymes at the same concentration or even when the enzymes were deliberately employed at concentrations 6.7 and four times higher than the concentrations that were fully effective for soluble BP (Table I). The remarkable ability of resin to protect BP from enzymatic attack is further highlighted by the fact that the resin is a relatively weak adjuvant (less than 0.2 mg rat BP is usually ineffective), so that the observed *retention* of encephalitogenicity excludes even a modest degradation and loss of bound BP. On the other hand, the powerful adjuvant (Freund's) used for bioassay of soluble BP ensures that

[1] Supported wholly by National Multiple Sclerosis Society Grant 536C12.

TABLE I. Enzymes Destroy Encephalitogenicity of Soluble But Not Resin-Bound Basic Protein.

Encephalitogen	Enzyme added	Enzyme/BP ratio (w/w)	Clinical signs, incidence[a]	Clinical signs, severity[b]	Histological lesions, incidence
Soluble BP	None	—	17/17	2.8	17/17
	Pronase	1/1250	1/3	2.0	3/3
		1/200	0/13	—	0/13
	Chymotrypsin	1/125	3/6	2.7	6/6
		1/25	0/5	—	0/5
Resin BP	None	—	4/10	1.5	9/10
	Pronase	1/200	1/5	1.0	4/5
		1/30	2/5	2.0	5/5
	Chymotrypsin	1/6	2/5	1.0	5/5

[a] Numerator: number of rats with clinical signs; denominator: total number of rats.

[b] Average severity, graded from zero to 3 plus. Only rats with clinical signs were used for calculation of mean.

the observed *loss* of encephalitogenicity represents virtually complete enzymatic destruction of soluble BP.

In agreement with the bioassay, no elution of BP from resin by enzymes was found spectrophotometrically. Furthermore, BP could not be eluted from resin by HCl or NaOH in the pH range of 0 to 10.5.

The adsorption of *enzymes* to resin was measured spectrophotometrically and it was found that both pronase and chymotrypsin were adsorbed to free but not to BP-saturated resin. However, the possibility remained that enzymes were somehow inactivated by resin. It was important to determine whether the protection of BP from enzymatic attack was due to interaction of BP with resin, and therefore a durable effect, or whether it merely represented inactivation of available enzyme due to interaction of enzyme with resin.

For this purpose an experiment was designed to test the activity of enzymes after they had been in contact with resin. Resin (200 mg) was saturated with BP at 37° and it was found that at that temperature, 20 mg BP was adsorbed. An additional equal amount of BP was then added so as to ensure the presence of substrate in the liquid phase (supernatant). Next, pronase or chymotrypsin were added and the mixture incubated for 3 hr at 37°. To ensure an ample supply of enzyme, a second aliquot of enzyme was added at the midpoint of the incubation. Resin and liquid phase were separated by centrifugation and each was bioassayed separately (Table II). In order to maintain similar conditions as in the previous experiments, the separated resin sediments were resuspended in solutions of enzymes that had been incubated separately under identical conditions except for the absence of resin and BP. Despite the exposure of the resin-bound BP to a double attack by enzyme, the resin retained its encephalitogenicity, thus confirming the results of Table I. More important, however, the separated supernatants had lost their encephalitogenicity despite the large amount of BP originally introduced and despite the use of the powerful Freund's adjuvant for the assay. Clearly, the enzymes had retained their ability to destroy the encephalitogenicity of soluble BP while they were unable to degrade resin-bound BP in the same tubes. Even when a fresh supply of BP was added to aliquots of the separated supernatants, they were still able to inactivate BP, indicating the persistence of active enzymes (Table II).

Discussion. The adjuvant effect of resin probably depends on its ability to carry BP to regional lymph nodes and to place it in an intracellular environment as well as to protect it from degradation. Our *in vitro* model for enzymatic degradation differs from the situation *in vivo* in the choice of enzymes and incubation conditions. Nevertheless, this

TABLE II. Enzymes Destroy Encephalitogenicity of Soluble Basic Protein But Not That of Resin-Bound Basic Protein in the Same Tube.

Tube	Enzyme added	Enzyme/BP ratio (w/w)[a]	Encephalitogen	Clinical signs, incidence[b]	Clinical signs, severity[c]	Histological lesions, incidence
1	None	—	Soluble BP (supernatant)[d]	5/5	2.8	5/5
			Resin BP (sediment)[e]	3/5	1.3	5/5
2	Pronase	1/123	Soluble BP (supernatant)[d]	0/5	—	0/5
			Resin BP (sediment)[e]	2/5	1.0	5/5
3	Chymotrypsin	1/1.8	Soluble BP (supernatant)[d]	0/5	—	0/5
			Resin BP (sediment)[e]	3/5	1.0	5/5
4	None	1/74	Supernatant from tube 2 incubated with fresh soluble BP	0/5	—	0/5
5	None	1/1.2	Supernatant from tube 3 incubated with fresh soluble BP	0/5	—	0/5

[a] Refers to total (soluble and resin-bound) enzyme/BP ratios except in the case of Tubes 4 and 5 where only the amount of fresh BP is considered.

[b] Numerator: numbers of rats with clinical signs; denominator: total number of rats.

[c] Average severity, graded from zero to 3 plus. Only rats with clinical signs were used for calculation of mean.

[d] Twenty-five micrograms original BP in Freund's adjuvant for each rat.

[e] Twenty milligrams resin and 2.0 mg adsorbed BP for each rat.

model offers concrete evidence that a particulate adjuvant does protect antigen against enzymatic degradation, a hypothesis heretofore based only on conjecture. In addition, the results suggest that BP is so strongly bound to resin as to raise intriguing questions as to whether, or how, the antigen manages to escape from the resin in order to enter those intracellular pathways that eventually lead to development of cell-mediated immunity. The easy visualization of resin suggests that morphological studies of this system may yield further data on adjuvant mechanisms.

Summary. Myelin basic protein (BP) in aqueous solution is degraded by pronase or chymotrypsin to nonencephalitogenic products. The encephalitogenicity of BP is resistant to these proteolytic enzymes when BP is adsorbed on a cation exchange resin known to be an immunologic adjuvant. The protection of BP by resin is not due to inactivation of enzymes.

1. Levine, S., Wenk, E. J., Kies, M. W., and Alvord, E. C., Jr., Neurology **15**, 560 (1965).

2. Levine, S., Sowinski, R., Gruenewald, R., and Kies, M. W., Immunology **23**, 609 (1972).

3. Freund, J., Amer. J. Clin. Pathol. **21**, 645 (1951).

4. Deibler, G. E., Martenson, R. E., and Kies, M. W., Prep. Biochem. **2**, 139 (1972).

5. Murphy, J. B., and Kies, M. W., Biochim. Biophys. Acta **45**, 382 (1960).

6. Hashim, G. O., and Eylar, E. H., Arch. Biochem. Biophys. **129**, 635 (1969).

Received Dec. 7, 1972. P.S.E.B.M., 1973, Vol. 143.