Immunologic Studies of Acetylated Encephalitogenic Myelin Basic Protein[1] (36616)

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Our previous attempts to study the immunologic properties of the encephalitogenic myelin basic protein (BP) have been severely complicated owing to the highly charged nature of the BP molecule. BP, like basic proteins in general, is poorly immunogenic (1–3). Moreover, the BP molecule interacts nonspecifically with serum proteins and erythrocyte membranes. Our attempts to employ BP in passive hemagglutination tests have been unsuccessful owing to nonspecific clumping of the erythrocytes. In a previous study we increased the immunogenicity of BP by rendering it less basic through conjugation with diazotized arsanilic and sulfanilic acid groups (3). Serum samples from rabbits immunized with these conjugated BP preparations were analyzed by passive hemagglutination employing as test antigen a nonbasic brain extract which contained BP, but which did not cause nonspecific clumping of tannic acid-treated erythrocytes (3). However, the obvious disadvantages included the use of a test antigen different from the one employed for immunization, and possible changes in the antigenicity of the BP molecule employed for immunization as a result of diazotization with haptenic groups.

In the present paper we describe a passive hemagglutination method which utilizes BP as the test antigen but does not result in nonspecific clumping of the erythrocytes. The nonspecific clumping can be easily prevented by the simple procedure of formalin-treating the cells prior to tanning. In addition, the formalinized erythrocytes are quite stable, and one large batch of cells can be employed for numerous tests over a long period of time, thus minimizing variability of results due to differences in sheep cells.

Furthermore, we have compared unmodified BP and BP rendered less basic by acetylation of free amino groups in order to study the effect of alteration of molecular charge on immunogenicity and antibody specificity. Acetylated BP (AcBP) was previously shown capable of producing experimental allergic encephalomyelitis (EAE) in guinea pigs (4).

Materials and Methods. BP was obtained by acid extraction of chloroform–methanol pretreated bovine brain (4–6) and acetylated with acetic anhydride as previously described (4). Briefly, 30 mg BP were dissolved in 6 ml distilled water and cooled in an ice bath. While maintaining the pH at 8.0 with 1 *N* NaOH, 1.5 ml of acetic anhydride was slowly added dropwise, with stirring. This solution was then dialyzed overnight against distilled water. The percentage of remaining free amino groups was determined by the Ninhydrin test (4, 7).

Bovine serum albumin (A grade), and bovine gamma globulins (Fr II) were obtained from Pentex, and acetylated in the same manner.

Three New Zealand white rabbits were immunized with BP, and three others received acetylated BP (AcBP). The first injection consisted of 2 mg of antigen emulsified in Freund's complete adjuvant containing 8 mg/ml of *Mycobacterium butyricum*, administered intradermally. This was followed by

[1] Supported by research grants 649-A-1 from the National Multiple Sclerosis Society and NS-06985 from the National Institute of Neurological Diseases and Stroke, U.S. Public Health Service.

[2] Submitted in partial fulfillment of the requirement for the M.S. degree.

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weekly injections of 4 mg of antigen emulsified in adjuvant containing 6, 4, 2 and 2 mg/ml of *M. butyricum,* respectively. Five injections were given. A total of 18 mg of antigen was injected into each rabbit.

Rabbits were bled before the initial injection and then on days 3, 7, 10, 14, 17, 21, 24, 28, 31, 36, 38, 45, 63 and 90 respectively. The sera used for hemagglutination studies were heat inactivated at 56° for 30 min.

Sheep red blood cells were treated with formalin according to the method of Csizmas (8) and used for passive hemagglutination (3, 5). A 100 ml quantity of fresh sheep blood was centrifuged at 600*g*, the supernatant discarded and the packed cells washed 5–7 times in 500 ml of cold saline (0.15 *M* NaCl). The washed packed cells were then suspended in 500 ml of phosphate buffered saline (0.009 *M* Na_2HPO_4, 0.006 *M* KH_2PO_4, 0.135 *M* NaCl) at a pH of 6.8. One hundred milliliters of formaldehyde (40% U.S.P.) at a pH of 5.5–6.0 was poured into a dialysis tube (2.5 cm diameter). This tube was placed in the bottom of a one liter erlenmeyer flask and the suspended blood cells poured over it. The flask was carefully agitated at room temperature on a mechanically operated orbital shaker for approximately 2–4 hr during which time the cells turned a chocolate brown. The dialysis tube was then removed, and the contents, *i.e.*, formaldehyde, added directly back to the flask. Shaking was continued for an additional 12–18 hr. The cells were filtered through surgical gauze to remove clotted cell debris. The filtered cells were suspended in 300 ml of saline, then washed six times using 120 ml of saline for each wash. After the final washing, the cells were suspended in an equal volume of saline and stored as a 50% stock suspension at 4°.

A 4% suspension of formalinized cells was tanned by mixing for 1/2 hr at room temperature with an equal volume of a 1/20,000 dilution of tannic acid in saline. The cells were centrifuged and resuspended in saline three times and finally diluted to a 4% suspension. Cells were coated with antigen by mixing the 4% cell suspension with the same volume of antigen in saline, for 1/2 hr at room temperature. The cells were again washed three times and finally diluted to make a 1.5% suspension. The diluent was 4% normal rabbit serum in 0.15 *M* barbital–saline buffer at pH 8.0.

The microtiter method was employed for the hemagglutination tests. Preliminary experiments revealed that anti-BP antibody titers were the same whether the cells were coated with BP at 50 or 100 μg/ml, and the latter concentration was chosen for routine testing. Anti-AcBP titers were similar when 25 or 50 μg of AcBP/ml were employed to coat the cells. However, AcBP occasionally caused nonspecific clumping at concentrations above 50 μg/ml, and we routinely employed a concentration of 25 μg/ml in our studies.

Gel diffusion studies were carried out according to the method of Ouchterlony (9), employing 0.8% agarose (Sea Kem) in 0.1 *M* sodium acetate, pH 7.4 (Dr. Marian W. Kies, personal communication). Serum was used undiluted, and the optimal concentration of each antigen was found to be 250-500 μg/ml.

Results. Since sheep erythrocytes agglutinated nonspecifically when tanned and coated with BP, our first objective was to develop a suitable modification of the passive hemagglutination procedure. We achieved considerable success by formalin-treating the sheep cells prior to tanning. The formalinized erythrocytes were stable for long periods of time, and more important, when coated with BP, even at a concentration of 100 μg/ml, failed to exhibit nonspecific clumping.

As illustrated in Table I, each of the three rabbits immunized with bovine BP produced antibodies detectable by the passive hemagglutination method employing BP as the test antigen coated on the erythrocytes. These antibodies first appeared about three weeks after the start of immunization, and persisted at a titer of 1/16–1/64 until the final bleeding on day 90. Similar antibody titers were observed when AcBP was employed as test antigen in the hemagglutination test (Table I). Regardless of test antigen, there was no evidence of nonspecific agglutination. Controls, which included formalin-treated tanned

TABLE I. Passive Hemagglutination. Antibody response following immunization with BP and AcBP.

Bleeding (day)	Hemagglutinin titers											
	Immunized with BP						Immunized with AcBP					
	Rabbit R 25		Rabbit R 26		Rabbit R 27		Rabbit R 22		Rabbit R 23		Rabbit R 24	
	BP[a]	AcBP[a]	BP	AcBP	BP	AcBP	BP	AcBP	BP	AcBP	BP	AcBP
0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	4	0	0	0	8
14	2	2	0	0	0	0	0	4	0	0	2	16
17	2	2	2	2	2	0	0	8	2	0	2	8
21	4	8	2	2	32	32	0	2	2	8	4	8
24	8	8	4	8	16	8	0	8	2	8	16	16
28	32	32	8	8	—[b]	8	4	32	2	8	8	32
31	—	4	8	32	8	8	2	16	2	16	4	64
36	32	32	16	16	8	8	16	32	2	16	16	128
38	32	32	2	2	8	8	2	16	2	8	32	128
45	32	—	8	4	4	16	4	16	2	16	32	128
63	64	64	32	32	8	16	2	4	4	32	16	128
90	64	64	16	16	16	16	4	16	4	32	16	128

[a] Test antigen.
[b] Not determined.

cells coated with BP and added to normal rabbit serum (NRS), and formalinized tanned uncoated cells added to anti-BP serum, were uniformly negative.

Modification of 70% of the free amino groups with acetic anhydride rendered BP less basic, as demonstrated electrophoretically. Each of the three rabbits immunized with AcBP responded with the production of hemagglutinating antibodies specific for this antigen (Table I). These were detected in the serum of two of the rabbits 10 days after the start of immunization, at a time when the rabbits immunized with unaltered BP had not produced detectable anti-BP hemagglutinins.

Of considerable interest was the finding that, unlike the antibodies produced in response to BP, the antibodies elicited in response to AcBP reacted better with erythrocytes coated with AcBP at 25 μg/ml than with unmodified BP at 100 μg/ml (Table I). This is illustrated graphically in Fig. 1, which shows the relative hemagglutinin responses of antiserum R-24 and R-25, anti-AcBP and anti/BP sera respectively, with formalinized, tannic acid treated sheep erythrocytes coated with BP or AcBP. Anti-BP serum reacted equally well with BP and

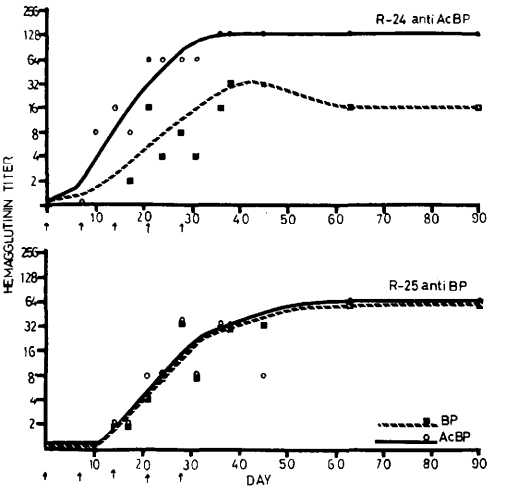

FIG. 1. Passive hemagglutination. Comparison of hemagglutinin titers of anti AcBP (R-24) and anti BP (R-25) serum with formalinized, tannic acid-treated sheep erythrocytes coated with BP (dashed line) or AcBP (solid line).

AcBP, whereas anti-AcBP serum reacted better with AcBP, than with BP.

To further investigate this apparent difference in immunologic specificity we carried out immunodiffusion studies to simultaneously compare sera from the rabbits immunized with each antigen and the respective antigenic preparations. As shown in Fig. 2, serum from rabbit R-25, which was immunized with BP, produced a faint line of precipitation near the antiserum well when reacted with BP. This serum also reacted with AcBP, forming a precipitin band which merged in a reaction of identity with the band formed by reaction with BP (Fig. 2). In contrast, serum from rabbit R-24, which was immunized with AcBP, contained antibodies that reacted equally well with AcBP and BP, as evidenced by the reaction of identity with these two antigens (Fig. 2), and in addition, antibodies specific only for acetylated BP. These precipitin bands were much stronger than the reactions observed with anti-BP serum. In these studies the concentration of each antigen was 500 μg/ml. The precipitating antibodies were produced by two rabbits immunized with each antigen, and appeared 24–31 days after the start of immunization.

On further examination, (Fig. 3a) it was observed that anti-AcBP serum reacted with acetylated bovine gamma globulin (AcBGG) and acetylated bovine serum albumin (AcBSA). An additional precipitin band appears to be specific for AcBP (Fig. 3a wells 1

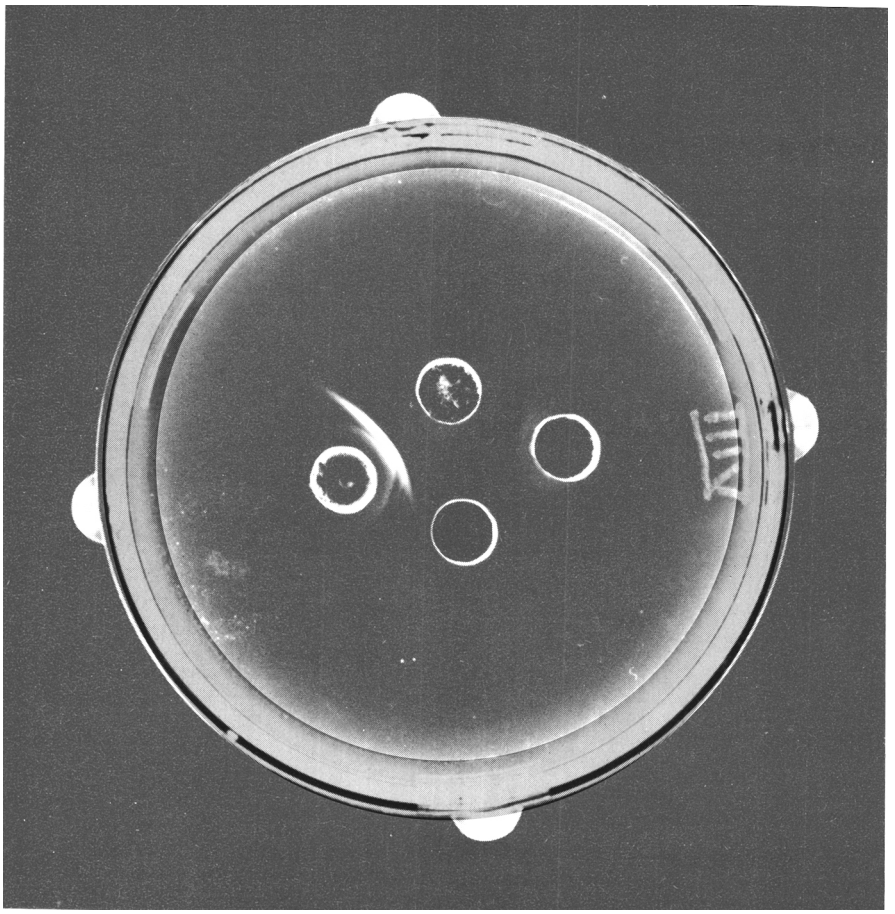

FIG. 2. Immunodiffusion analysis of anti BP serum R-25 (right well) and anti AcBP serum R-24 (left well) reacted against BP (bottom well) and AcBP (top well). The concentration of each antigen was 500 μg/ml.

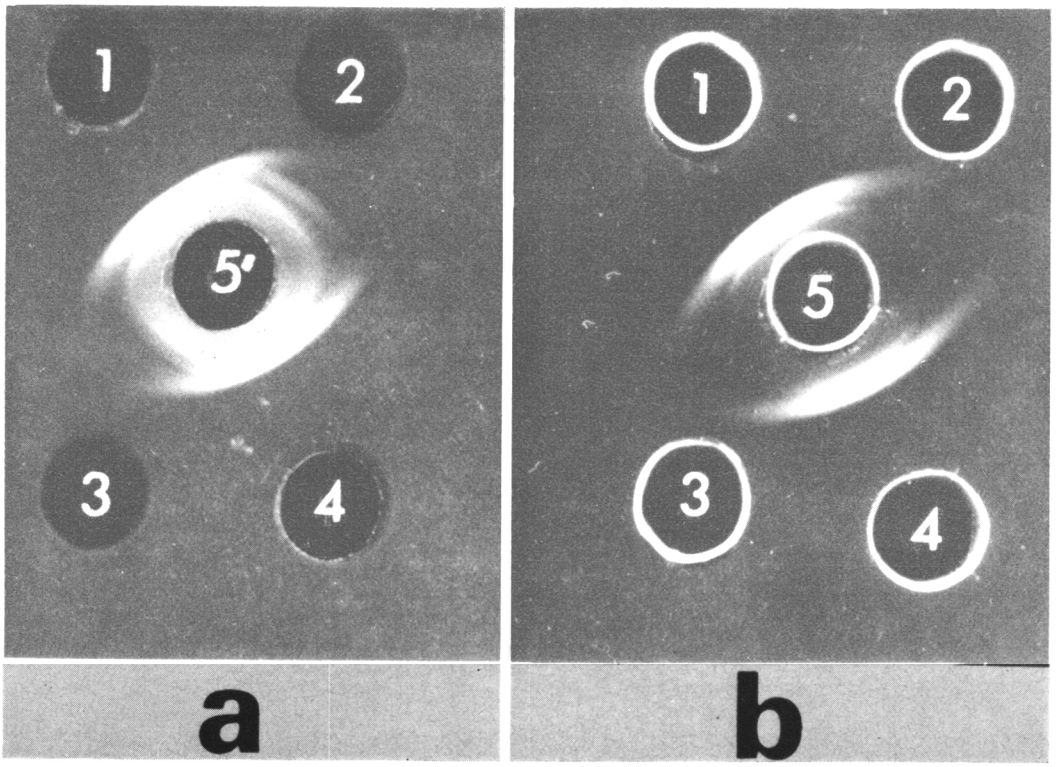

FIG. 3. Immunodiffusion analysis of anti AcBP serum R-24. (a) Well: (1) AcBP, (2) AcBGG, (3) AcBSA, (4) AcBP, (5) antiserum R-24. The concentration of each antigen was 500 μg/ml. (b) Well: (1) AcBP, (2) BGG, (3) BSA, (4) AcBP, (5) antiserum R-24. The concentration of each antigen was 400 μg/ml.

and 4).

Figure 3b illustrates the reaction pattern seen when unmodified BGG and BSA (wells 2 and 3 respectively) were reacted against anti-AcBP serum. There was no reaction with these proteins, whereas the characteristic homologous reaction with AcBP was seen.

The anti-BP sera did not react with BGG, BSA, their acetylated derivatives, or NRS.

Discussion. The present findings reveal that the highly cationic myelin basic protein will coat tannic-acid treated, formalinized sheep erythrocytes without producing nonspecific hemagglutination, even at a concentration of 100 μg/ml. We employed this system to demonstrate anti-BP hemagglutinins in the serum of rabbits hyperimmunized with BP. Previously Hruby *et al.* (2) detected hemagglutinating anti-BP antibodies in hyperimmune rabbit serum with erythrocytes to which BP had been coupled covalently with bis-diazotized benzidine (BDB). However, the merits of the tannic acid procedure over the BDB-method include ease of preparation and relative stability of the former, as discussed by Stavitsky and Arquilla (10). Moreover, the formalinized tanned cells are stable on storage and will not hemolyze in hypotonic salt solution or in the presence of complement and specific antibody (8). Thus, the same batch of cells can be used for numerous tests over a long period of time, resulting in increased uniformity of results. A passive hemagglutination method employing tanned chicken erythrocytes has also been reported, although extremely low concentrations of BP were required for coating in order to avoid clumping of the cells (11).

Each of the three rabbits immunized with unmodified BP produced antibodies which presumably were specific for BP, although the possibility that these antibodies were di-

rected against trace contaminants (12) cannot be excluded. From the hemagglutination and gel diffusion studies with anti-BP serum it would appear that the antibodies react equally well with both test antigens, BP and AcBP, suggesting that acetylation did not alter the immunogenicity of the native BP molecule. That AcBP is structurally similar to unmodified BP was suggested earlier by the finding that AcBP causes EAE in guinea pigs (4). However, the present studies with anti-AcBP serum revealed that, indeed, acetylation does result in altered immunogenicity of the BP molecule. The anti-AcBP sera reacted better with formalinized, tannic acid-treated sheep erythrocytes coated with AcBP than with cells coated with unaltered BP. This might suggest that additional antigenic determinants are present on the AcBP molecule. However, the possibility that the two test antigens differ in their ability to coat the erythrocytes cannot be ruled out, especially in view of the fact that different concentrations of AcBP and BP were found optimal for coating the cells.

The tentative conclusion that new determinant groups were added to BP by acetylation was confirmed by gel diffusion studies. Anti-BP serum reacted equally well with BP and AcBP, forming a single faint precipitin band which merged in a reaction of identity. With respect to anti-AcBP serum, a common band of precipitation was also seen in comparative studies with BP and AcBP. This reaction was considerably stronger than the comparable reaction attained with anti-BP serum. Perhaps the relative increase in intensity of this reaction in the former system might suggest enhanced immunogenicity of AcBP relative to unmodified BP. However, by gel diffusion analysis it was apparent that anti-AcBP serum contained antibodies which also reacted with determinant groups present on AcBSA and AcBGG, but not with unmodified BSA and BGG (Fig. 3). The additional spur seen when AcBP and AcBGG were compared with anti-AcBP (Fig. 3a) might reflect different immunoglobulin classes in the BGG preparation. The present findings would seem to be in agreement with the observations of Sri Ram and Maurer (13), that antibodies produced in response to one acetylated protein cross react with unrelated acetylated proteins, and that part of the specificity of these antibodies is directed against sites on the protein which contain the acetyl group.

Thus, it appears that immunization of rabbits with AcBP gives rise to antibodies directed against native determinants on the BP molecule, as demonstrated by their reactivity with unmodified BP. In addition, antibodies are also produced which are directed toward the acetyl group or a region of the molecule structurally changed by acetylation of the protein. These latter antibodies cross-react with unrelated acetylated proteins, but not with their respective unmodified proteins.

None of the rabbits used in the present investigation developed EAE, which is agreement with previous reports that repeated injections of BP result in suppression of the disease (14, 15).

Summary. Rabbits were hyperimmunized with bovine myelin basic protein (BP) or its acetylated derivative (AcBP). The sera were studied by means of passive hemagglutination with formalinized, tannic acid-treated sheep erythrocytes coated with BP or AcBP. Formalin treatment eliminates the nonspecific clumping of BP-coated sheep cells, which we previously encountered. Sera were also analyzed by immunodiffusion in agarose. The rabbits immunized with BP produced antibodies which reacted equally well with BP and AcBP. However, the rabbits immunized with AcBP formed antibodies which were apparently directed against the acetyl group, in addition to anti-BP antibodies.

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Received Mar. 24, 1972. P.S.E.B.M., 1972, Vol. 140.