Species Restrictions of a Monoclonal Antibody Reacting with Residues 130 to 137 in Encephalitogenic Myelin Basic Protein

Abstract. *A monoclonal antibody (immunoglobulin G1) has been produced that reacts against myelin basic protein present in or extracted from the brains of many mammals—with certain important exceptions. Because of known species differences in amino acid sequences of basic protein and of certain peptide fragments, the binding site for this particular antibody appeared likely to include residues 130 to 137. Confirmation of this hypothesis was obtained by amino acid composition of the major immunoreactive peptides produced by thermolysin digestion of human basic protein and isolated by high-performance liquid chromatography.*

Polyclonal antibodies to encephalitogenic myelin basic protein (BP) have been recognized as being directed against at least three antigenic determinants situated in the amino, mid, and carboxyl regions of the molecule (*1*), but more specific identification of these locations has not been possible with such heterogeneous reagents. We have used the hybridoma technique of Kohler and Milstein (*2*) to obtain monoclonal antibodies for locating antigenic determinants in BP. Since the amino acid sequence of BP is known completely for a few and at least partially for many species (*3*) and since selected peptide fragments are readily available for comparative purposes (*4*), we have been able to localize the antibody-combining site of one of these monoclonal antibodies to BP within a short region near the carboxyl end of BP.

Hybridomas were produced and tested as follows. A BALB/c mouse was given an intraperitoneal injection of 0.1 ml of monkey BP (0.5 mg) in Freund's adjuvants every 2 weeks, for a total of three injections. The first injection contained killed mycobacteria (0.2 mg); the subsequent ones did not. Spleen cells taken 4 days after the last immunization were hybridized to NS-1 myeloma cells, with the use of polyethylene glycol, thymic lymphocytes as a feeder layer, and selective hypoxanthine-aminopterin-thymidine medium (*5*). Supernatants were tested for antibodies to monkey BP. One of the antibody-producing hybridomas was cloned by limiting dilutions, and the supernatants of the clones were retested extensively against BP or BP peptides from various species. Portions of the original positive hybridomas and the clones were stored frozen.

The BP was prepared from the brains or spinal cords of monkeys (*Macaca nemestrina*), humans, pigs, cattle, NIH guinea pigs, New Zealand rabbits, chickens, chimpanzees, and Lewis rats by a batch technique (*6*). Peptide fragments of BP from monkeys, guinea pigs, and rabbits were prepared by limited peptic digestion and molecular sieve or ion-exchange chromatography (*4*). These were identified as monkey residues 1 to 90, 91 to 172, and 118 to 172; guinea pig residues 38 to 120 and 121 to 172; and rabbit residues 1 to 155 and 156 to 172 (*3*). In addition to unfractionated rat BP, which consists of a mixture (1:2) of large and small molecular forms of BP, we used the small BP (rat S) purified from rat brain. In rat-S, residues 119 to 159 are deleted.

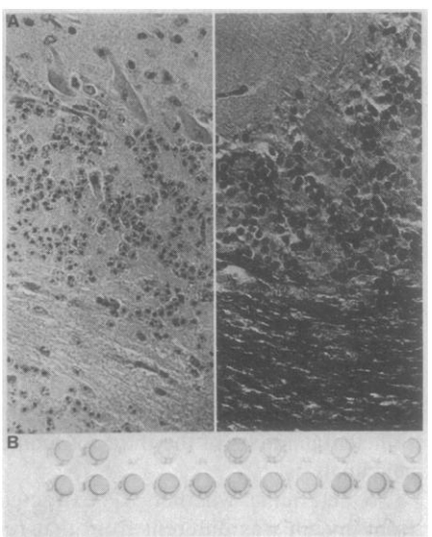

Fig. 1. (A) Immunoperoxidase staining with hybridoma supernatant showing the specific brown reaction with monkey (right) but not guinea pig (left) cerebellar myelin that was fixed, embedded in the same paraffin block, and stained simultaneously on the same slide. The prominent nucleoli (stained red with the Kernechtrot counterstain) are characteristic of guinea pig cerebellar granule cells. (B) Comparable ELISA reactions with the BP-specific monoclonal hybridoma supernatant (top) and the positive control polyclonal hyperimmune rabbit antiserum to BP (middle) in wells coated from left to right as follows: no antigen, monkey BP, bovine BP, guinea pig BP, rabbit BP, pig BP, chimpanzee BP, monkey BP peptide 91 to 172, monkey BP peptide 1 to 90, rat BP, rat-S BP (residues 119 to 159 deleted), and human BP. Negative controls (bottom) include wells without antigen coating, antigen-coated wells without antibodies to BP but with buffer, hybridoma medium or normal mouse serum containing the same concentration of IgG1 (0.1 mg/ml) as present in the hybridoma supernatant.

We detected and quantified antibodies specific for BP using an adaptation of the solid-phase enzyme-linked immunosorbent assay (ELISA) (*7*). Four different mouse monoclonal antibodies to human melanomas (*5*), namely, 3.1 (IgG1), 3.2 (IgG2a), 3.3 (IgG2b), and 4.1 (IgG1), and other mouse monoclonal antibodies currently under investigation were used as negative controls for nonspecific binding of antibody to the BP-coated wells. Other negative controls routinely included BP-coated plates without the putative primary antibody, but with the other reagents, and noncoated plates with the primary antibody as well as the other reagents (*7*). A positive control with a high-titered polyclonal rabbit antiserum to monkey BP (*8*) was also routinely included to ascertain that the wells were coated with specific BP antigens.

Of the 480 wells initially seeded, 95 contained viable hybridomas on day 9, and six of these contained antibodies to monkey BP. From one of these wells, a hybridoma line was established after initial cloning at five cells per well and then formal cloning three times at one cell per well. From this line, 12 subclones have been intensively studied. Each produced only IgG1, as defined by immunodiffusion against specific antiserums to mouse globulin subclasses (*9*). Supernatants of each consistently reacted, in dilutions of 1:1500 to 1:8000, with BP extracted from human, chimpanzee, monkey, rabbit, bovine, and rat brain; but none of the antibodies, even undiluted, reacted with BP extracted from guinea pig, chicken, or pig brain (Fig. 1). None of the antibodies reacted with other unrelated but also highly basic antigens, such as lysozyme (Worthington), calf thymus lysine-rich histone (Schwarz/Mann), cytochrome c (Sigma), or the gray-band contaminants of some BP preparations (*10*). All antibodies reacted with monkey BP peptides containing residues 91 to 172 and 118 to 172 and rabbit peptide containing residues 1 to 155, whereas none of them reacted with monkey peptide containing residues 1 to 90, guinea pig peptides containing residues 38 to 120 or 121 to 172, or with rabbit peptide containing residues 156 to 172 or rat-S (in which residues 119 to 159 are deleted). By contrast, the polyclonal rabbit antiserum to BP reacted with all of the species and fragments of BP (as well as the gray-band contaminant) but not with the other unrelated antigens.

Certain quantitative differences in the amount of antibody bound, already apparent in Fig. 1, became more obvious on inhibition studies (Fig. 2). Rabbit BP and rabbit peptide 1 to 155 are only about

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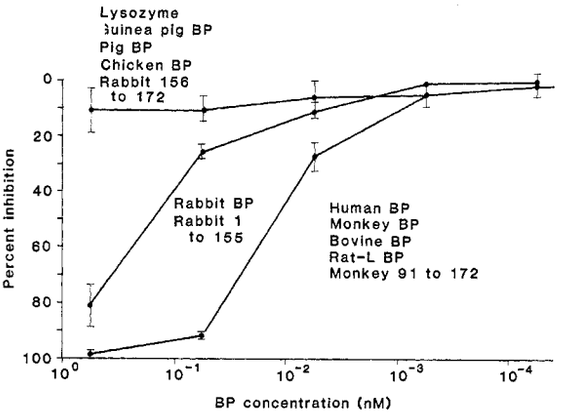

Fig. 2. Inhibition of a standard binding of monkey BP and hybridoma supernatant by various concentrations of whole BP, peptide fragments of BP extracted from different species, and an unrelated antigen, lysozyme (*11*). Each antigen was assayed one to four times at each concentration. Three degrees of inhibition were apparent: none, intermediate, and complete. The two to five antigens that overlapped were grouped together, as indicated. Points represent means and bars, the standard error.

10 percent as effective as human, chimpanzee, monkey, bovine, or rat-L BP or monkey peptide 91 to 172 in inhibiting the binding of monkey BP and hybridoma supernatant (*11*). There are thus three classes of proteins or peptides: nonreactive (guinea pig, pig, chicken, and rat-S BP; rabbit peptide 156 to 172 and monkey peptide 1 to 90), weakly reactive (rabbit BP and rabbit peptide 1 to 155), and strongly reactive (all other species of BP tested and monkey peptides 91 to 172 and 118 to 172).

From the fact that rat-L BP and monkey peptide 118 to 172 react well with our antibody, whereas rat-S does not, we conclude that the antibody-binding site is within residues 119 to 159 (the ones deleted from rat-S). Within this region the amino acid sequences of human, bovine, and rabbit BP are completely known, and the sequences of chimpanzee, chicken, rat-L, pig, and guinea pig partially known. There are four regions where species-specific differences are known: 119 to 124, 130 to 137, 142 to 150, and 153 to 160. Two of these can be definitely eliminated, since the weakly reactive rabbit BP has the same sequence as the strongly reactive bovine BP at residues 119 to 124 and differs from bovine BP in residues 142 to 150 only at the one site (residue 145), where it is the same as the strongly reactive rat-L BP. The sequence 153 to 160 can probably be eliminated since the nonreactive guinea pig BP is probably the same as the strongly reactive bovine BP (*4*, *10*). There remains, therefore, only one region, residues 130 to 137, where all of the strongly reactive BP's are identical, where the weakly reactive rabbit BP has a single substitution at residue 133, and where two of the three nonreactive BP's are reported to have two or more substitutions at residues 130 to 131, 133 to 134, and 137. The sequence of the third, guinea pig BP, is only partially known, but since guinea pig BP is known to be missing at least one serine in this carboxyl terminal region (*10*), we postulate that either serine-133 or serine-137 is the missing one.

Since thermolysin degrades BP into relatively large peptides that should spare not only residues 130 to 137 but also 119 to 124 and most of 153 to 160 (*12*), we digested human BP with thermolysin (*13*), separated the resulting peptides by gradient elution (*14*) on high-performance liquid chromatography (HPLC), and tested each of the 34 peaks for inhibition as measured by ELISA (*11*). Only three peaks, those eluting at 11, 12, and 15 percent acetonitrile, were inhibiting. The same three peaks were obtained by elution from an immunoabsorbent column (*15*). After desalting with trifluoroaectic acid and an acetonitrile gradient (*16*), we lyophilized the three peaks and determined that the amino acid compositions and subsequent sequence determinations of two of them were the same as those of residues 125 to 137 and 127 to 137. We did not obtain sufficient amounts of the third for amino acid analysis.

To determine whether BP extracted from myelin was different from that remaining in myelin, we used immunoperoxidase for the simultaneous staining of monkey and guinea pig cerebellum fixed in Formalin and embedded in the same paraffin block. Figure 1 confirmed the predicted positive reaction with monkey and negative reaction with guinea pig myelin; the prominent nucleoli of the guinea pig granule cells served as an internal control, definitively identifying this species. The high-titered polyclonal rabbit antiserum to BP (*8*) stained the myelin of both species well.

We conclude that the monoclonal antibody technique, coupled with highly sensitive assays such as the ELISA and HPLC, should continue to be useful in defining other antibody-combining sites in BP (*1*). We suspect that species restrictions demonstrable at the other antigenic sites will be comparable to those at encephalitogenic sites (*17*) and that the old concept of species nonspecificity of certain protein antigens, which was based on analyses with polyclonal hyperimmune antiserums, will have to be modified. These techniques should help in the search not only for particular antibodies to BP, but for BP or its peptides present in experimental allergic encephalomyelitis and in human diseases such as multiple sclerosis.

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3. We use the alignment of amino acid residues originally prepared by M. O. Dayhoff, Ed. [*Atlas of Protein Sequence and Structure* (National Biomedical Research Foundation, Silver Spring, Md., 1972), vol. 5], as corrected for an unnecessary deletion at residue 3 [*ibid.*, Suppl. 2 (1976)], and as amplified by other investigators [P. R. Dunkley and P. R. Carnegie, *Biochem. J.* **141**, 243 (1974); R. E. Martenson, V. Luthy, G. E. Deibler, *J. Neurochem.* **36**, 58 (1981); F. C. Westall, M. Thompson, S. S. Kalter, *Life Sci.* **17**, 219 (1975)]. Since a deletion must be inserted at residue 147 to accommodate the glutamine added in chimpanzee and pig BP, the original numbering of residues 142 to 172 remains correct, whereas those from residues 3 to 146 must be decreased by one. Thus, important landmarks for certain peptide fragments include pairs of phenylalanine residues at 44 to 45 and 90 to 91, the single tryptophan at 117, and the rat-S deletion of residues 119 to 159.
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were washed with 0.02*M* phosphate-buffered saline containing 0.05 percent Tween 20 (*p*H 8.0) between subsequent incubations at 25°C with hybridoma supernatant (2 hours) and rabbit antiserum to mouse IgG, IgM, and IgA (Calbiochem, La Jolla, Calif.), followed by peroxidase-conjugated goat gamma globulin directed against rabbit IgG (Fc fragment, heavy chain–specific) (Cappel Laboratories, Cochranville, Pa.). Incubations were for 2 hours each. The final color reaction was developed by adding *o*-phenylenediamine in dilute hydrogen peroxide; the enzymatic reaction was stopped with 8*N* H_2SO_4; and the plates were read within the hour by means of a titer plate reader (Flow Laboratories, Rockville, Md.) at 492 nm.

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11. Various concentrations of selected proteins or peptides were added to hybridoma supernatant (diluted 1:100), incubated for 6 hours at room temperature, and then portions were tested in triplicate with the ELISA technique for residual ability to bind 200 ng of monkey BP adsorbed to each well.
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13. Human BP and twice crystallized thermolysin (100:1) were incubated in 50 m*M* tris-HCl, 5 m*M* CaCl, *p*H 7.5, for 30 minutes at 37°C. The reaction was stopped with 1*N* HCl (0.05 ml per milliliter of digest), centrifuged 20 minutes at 1000*g*, and the supernatant was lyophilized.
14. A liquid chromatograph (Varian model 5000, Palo Alto, Calif.) was used with an octadecylsilane, 10-μm silica, monomeric coverage, reverse-phase column and UV-50 detector. The mobile phase was 0.48 percent phosphoric acid, *p*H 3.0, and a gradient from 0 to 30 percent acetonitrile was established over 60 minutes.
15. Purified IgG1 from ascites fluid monoclonal antibody was covalently bound at 10 mg/g to cyanogen bromide–activated Sepharose 4B (Pharmacia, Piscataway, N.J.); 5 mg of thermolysin-digested human BP was added in 10 ml of 0.02*M* phosphate-buffered saline (*p*H 8.0) containing 0.05 percent Tween 20 to minimize nonspecific binding. After incubation for 30 minutes at 25°C, the unbound peptides were washed off with 20 ml of phosphate-buffered saline with Tween, and the Tween was washed off with 20 ml of phosphate-buffered saline without Tween in order not to interfere with the subsequent HPLC analysis. The specifically bound peptides were then eluted with 0.2*M* glycine HCl and 4*M* NaCl, *p*H 2.9.
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18. We thank B. Jones, L. Katzenberger, and I. Laszlo for important technical assistance, J. D. Meyers for loaning us the Flow reader, C. J. Gibbs for a fresh-frozen chimpanzee brain, and E. H. Fischer for biochemical advice and assistance as well as the thermolysin. This research was supported in part by National Multiple Sclerosis Society research grant RG-805-D19, American Cancer Society grant RD-69, and National Institutes of Health grants CA-19148, CA-25558, and CA-26584.

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Affective Behavior in Patients with Localized Cortical Excisions: Role of Lesion Site and Side

Abstract. *The perception of emotion in verbal and facial expression, and the spontaneous production of conversational speech were studied in patients with unilateral focal excisions of frontal, temporal, or parieto-occipital cortex. Lesions of the left hemisphere impaired the matching of verbal descriptions to appropriate verbal categories of emotional states, whereas with lesions of the right hemisphere, the matching of different faces displaying similar emotional states was impaired. The effects of lesions of both left and right hemisphere occurred regardless of the locus of the lesion. On the other hand, frontal-lobe lesions had differential effects upon unsolicited talking; lesions of the left frontal lobe virtually abolished this behavior, whereas lesions of the right frontal lobe produced excessive talking. These data suggest that the nature of the behavioral stimulus as well as the locus and side of damage must be considered in the study of the neural basis of affective behavior.*

Although there has been extensive study of the role of the left and right hemispheres in the production of cognitive processes (*1*), there have been relatively few studies specifically designed to consider the complementary roles of the two hemispheres in the production of affective behavior. An understanding of the neural control of human affective behavior might be improved by studying those stimuli that seem most important in human social interaction, namely facial expression and language. We thus examined the effects of cortical excision upon the perception of emotion in facial expression, the perception of emotion in verbal expression, and the spontaneous production of conversational speech, by making use of experimental designs modeled on previous studies of affective behavior of nonhuman species (*2*). In addition, we compared these data to our previous investigation of the effects of cortical lesions on spontaneous facial expression (*3*).

The subjects were 20 right-handed normal control subjects and 58 patients at the Montreal Neurological Hospital who had undergone a unilateral excision of cortical tissue of the frontal (7 left, 14 right), temporal (13 left, 17 right), or parieto-occipital (4 left, 4 right) regions for the relief of epilepsy (*4*). When the temporal lobe was removed, the amygdala was partially or completely removed, and the hippocampus was partially removed.

The perception of emotion in facial and verbal expression was measured by a nonverbal photograph-matching test and a sentence-matching test, respectively. In the photograph-matching test, the subject was first shown seven key photographs, each of which depicted one of the verbally categorizable emotions described by Ekman, Friesen, and Ellsworth (*5*). These have been characterized as sadness, fear or terror, happiness or amusement, anger, disgust or contempt, surprise, and interest or attention. The subject was then shown a series of 24 photographs of faces taken from *Life* magazine and was asked to match each of them with the key photograph that most closely expressed the same emotion (*6*). In the sentence-matching test, the subject was given the verbal categories of emotion listed above and was asked to describe the emotion of a person described in each of 48 sentences describing an event illustrated in a photograph from *Life*. Expressive behavior in the form of unsolicited talking was measured by noting each time a patient interrupted testing with comments about the test or with extraneous remarks.

Patients with lesions of the right hemisphere, irrespective of the lesion site, were significantly impaired on the photograph-matching test (Fig. 1A) whereas patients with lesions of the left hemisphere were significantly impaired on the verbal test (Fig. 1B) (*7*). Patients with left frontal-lobe lesions seldom interrupted testing by spontaneously talking, whereas those with right frontal-lobe lesions frequently did so (Fig. 2) [$F(5, 44) = 5.1$, $P < .05$].

In a previous study (*3*) we recorded the frequency of spontaneous facial expressions in a similar patient population, finding a large reduction in spontaneous facial expression in patients with left or right frontal-lobe lesions, compared with patients with temporal- or parietal-lobe lesions. Thus the increased spontaneous talking of the right frontal-lobe lesion patients in the present study stands in marked contrast to their relatively infrequent changes in facial expression.

The extremely limited spontaneous talking of our patients with left frontal-lobe lesions supports a number of previous reports describing a striking impoverishment of the spontaneous narrative speech produced by patients with left frontal lesions (*8*), an observation that has been quantified with the Thurstone Word Fluency Test (*9*, *10*) in either written or oral form. The frequent spontaneous talking of the right frontal-lobe patients can probably be ascribed to the tendency of right frontal lesions to pro-

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