Experimental Allergic Encephalomyelitis in the Rat: Response to Encephalitogenic Proteins and Peptides

Abstract. *Lewis rats were used to determine the encephalitogenic activity of myelin basic protein of different species and of 45-residue fragments of basic protein. Basic protein from guinea pigs was more active than that from rats, and the fragments from the two species showed the same order of activity. Bovine basic protein was the least active of the intact proteins, and the respective fragment was inactive. Studies of serum-binding capacity did not support the hypothesis that blocking antibody played a role in this biological variation, whereas consideration of the amino acid sequences of the three fragments suggested that differences in primary structure, operating either at the sensitization or the effector phase of the immune response, could account for the variation.*

Experimental allergic encephalomyelitis (EAE) can be produced in several species of animals by the injection of tissue from the central nervous system (CNS) in complete Freund's adjuvant (*1*). The autoimmune nature of the disorder has been established by the transfer of the disease with sensitized immune cells (*2*). There is considerable variation in the capacity of different species of animals to develop EAE (*3*). For example, the guinea pig is quite sensitive to induction of the disease, whereas the mouse is relatively resistant (*4*). There is also variation in the capacity of a single strain of animal to develop the disease in response to challenge with CNS tissue from different species. For example, Paterson *et al.* (*5*) found, in the highly inbred Lewis strain of rat, that whole CNS tissue from a xenogeneic animal (guinea pig) was more encephalitogenic than was tissue from an allogeneic animal (Wistar rat); the latter tissue was, in turn, more active than syngeneic tissue (from the Lewis rat). This was not due simply to the degree of "foreignness" of the material injected, because another xenogeneic CNS tissue (bovine) was less active than the above-mentioned tissues (*6*). We report here the initial phase of a study to elucidate the mechanisms responsible for such variations in the Lewis rat.

Because most, if not all, of the encephalitogenic activity of whole CNS tissue resides in the basic protein of myelin (*7*), Lewis rats were challenged with the basic protein from four sources: bovine, guinea pig, Wistar rat, and Lewis rat. Highly purified basic protein was prepared from the CNS tissue by a modification (*8*) of the method of Martenson *et al.* (*9*). By this procedure the smaller of the two basic proteins of rat myelin is purified and obtained in good yield. Because the two proteins are equally encephalitogenic (*10*), the smaller basic protein from rat was used.

Female Lewis rats (Microbiological Associates), between 10 to 12 weeks of age, were studied. Each rat received 0.1 ml of a water-oil emulsion containing the appropriate antigen and 250 μg of *Mycobacterium butyricum*; half was injected into each hind foot pad. Animals were assessed daily by an observer who did not know what antigen treatment had been given. The severity of clinical signs was graded on a scale from 1 to 6; 1 indicated mild weakness of hind legs on two successive days, and 6 denoted a moribund state. Animals were killed at grade 6 or when they had been assigned the same clinical grade for three successive days. Surviving animals were killed after 3 weeks, and sections of the cerebrum, cerebellum, brainstem, and spinal cord were stained with hematoxylin and eosin. The severity of pathological changes was graded on a scale from 1 to 8 by two independent observers who were unaware of the treatments given.

The results are given in experiment A, Table 1. Each basic protein was given at three doses from 25×10^{-4} to 25×10^{-6} μmole per rat. The highest dose of protein, 50 μg, produced EAE in 100 percent of animals regardless of its source. However, significant differences in day of disease onset and in clinical and pathological grades were seen at this dose. By these criteria, guinea pig protein was the most active, the syngeneic and allogeneic rat proteins were intermediate in activity, and the bovine protein was the least active. When 5 μg of protein was given, the differences were apparent by all criteria. The guinea pig protein again produced EAE in 100 percent of animals; encephalitogenic activity, as judged by the other criteria, was only slightly less than that seen at the higher dose. In contrast, only one of five rats that received the syngeneic (Lewis) rat protein developed EAE, and the allogeneic rat and bovine proteins were inactive. None of the basic proteins was active at a dose of 0.5 μg. This entire experiment was repeated, with essentially identical results. The results obtained with basic protein from guinea pig and Lewis rat parallel those of Paterson *et al.* (*5*), in which whole CNS tissue was used. They also found that CNS tissue from the Wistar rat was more active than that from Lewis rat and less active than that from guinea pig. We did not find any difference in the activity of the basic protein from the two rat sources, but emphasize that only the smaller of the two basic proteins from rat was compared (*11*).

One possible explanation for the difference in the encephalitogenic activity of basic proteins of different origin—namely, the more foreign the protein, the greater the degree of encephalitogenic activity—seems not to be the case in these studies in the Lewis rat. Although the basic protein from guinea pig was the most active, the bovine protein was the least active. Another possibility is that one basic protein is less active than another because it contains, in addition to the encephalitogenic site, an area of the molecule which induces formation of antibody with blocking activity. Such an explanation was postulated by Paterson and Harwin (*12*) to explain the difference in capacity of Wistar and Lewis rats to develop EAE following challenge with CNS tissue from guinea pig; Wistar rats develop

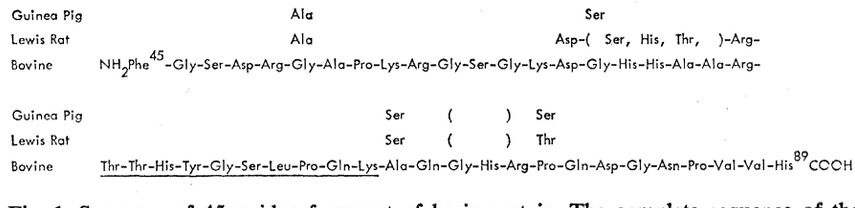

Fig. 1. Sequence of 45-residue fragment of basic protein. The complete sequence of the bovine fragment is shown; substitutions and deletions (parentheses) are indicated for the fragments from guinea pig and Lewis rat. The tryptic peptide T-8 is underlined. Amino acid abbreviations: Phe, phenylalanine, Gly, glycine; Ser, serine; Asp, aspartic acid; Arg, arginine; Ala, alanine; Pro, proline; Lys, lysine; His, hisidine; Thr, threonine; Tyr, tyrosine; Leu, leucine; Gln, glutamine; Asn, asparagine; and Val, valine.

milder and more transient disease which is paralleled by complement-fixing antibody against antigens in guinea pig CNS. Using radioimmunoassay for the detection of antibodies (*13*), we found that the serums from rats challenged with bovine basic protein had the highest capacity to bind ^{125}I-labeled basic protein, but serums from rats challenged with basic protein from guinea pig and Lewis rat also bound labeled antigen. Of the two latter groups, rats challenged with basic protein from guinea pig had the higher antigen-binding capacity. We have not yet characterized by immunochemical techniques the serum factors to which the basic protein binds.

Finally, differences in primary structure of the encephalitogenic regions of the basic proteins might account for variations in their activity in Lewis rats. The portions of the basic protein molecules responsible for encephalitogenicity in the rat have not been characterized. Several active sites have been determined, however, for other species. We showed previously that a fragment of bovine basic protein, consisting of the 45 amino acids from residues 44 to 89, is highly encephalitogenic in rabbits (*14*) and monkeys (*15*). The amino acid sequences of the guinea pig and bovine fragments have been reported (*16*); these, together with the sequence for the Lewis rat, are shown in Fig. 1. Because the sequence of these fragments is known and the relatively small size of the fragments simplifies the problem of possible multiple encephalitogenic or antigenic sites, they were used in the following studies. Fragments were prepared from CNS tissue from guinea pig, Lewis rat, and cows by methods developed in this laboratory (*15*). A single band on disk gel electrophoresis was obtained with each fragment. Tryptic peptide maps, stained first with ninhydrin and then with Ehrlich's reagent, showed no contamination, particularly by the peptide containing tryptophan (residue 116); this peptide is encephalitogenic in the guinea pig (*17*). The dose was identical in micromoles to the amount of the respective basic protein administered in the previous experiment; the molecular weight of the fragment is approximately 5,000 compared to 18,000 for the parent proteins of most species and 14,000 for the rat protein. The intact basic protein of the guinea pig was tested at the same time.

The results are shown in experiment B, Table 1. The activity of the guinea pig fragment was identical to that of the parent protein. The activity of the Lewis rat fragment was also comparable to that of the parent protein (experiment A); and, like the latter, was less than the activity of the guinea pig counterpart. The bovine fragment, in contrast to the parent protein (experiment A), was inactive at all doses. The capacity of serums from these rats to bind the respective ^{125}I-labeled basic protein was not quantitatively different from that of normal serums.

One of the tryptic peptides of the bovine fragment, T-8 (Fig. 1), was synthesized in this laboratory and shown to possess moderate activity in the rabbit (*18*). This peptide is identical in all three species but, as would be predicted from the studies reported here, this peptide was inactive in the Lewis rat even at a dose of 100 μg per rat. Therefore, more than these ten amino acids are required for the production of EAE in the rat. The differences in primary structure of the remaining portions of the three fragments could account for the differences in activity in the Lewis rat. The portions of the guinea pig and rat fragments adjacent to the COOH-terminal end of T-8 are almost identical but differ appreciably from the corresponding region in the bovine fragment (Fig. 1). However, the portions of the guinea pig and rat fragments adjacent to the NH_2-terminal end of T-8 are different, and this portion of the guinea pig sequence resembles more closely that of the bovine sequence. The remaining portions of the three fragments are not significantly different. The absence of activity of the bovine fragment could be due to the marked difference in the sequence adjacent to the COOH-terminal end of T-8. The intermediate activity of the Lewis fragment could be explained by the fact that, although the sequence near the COOH-terminal end of T-8 is almost identical to that of the guinea pig, the sequence near the NH_2-terminal end of T-8 is quite different. These considerations could also account for differences in activity of the intact basic proteins from guinea pig and rat. Since the intact bovine basic protein was moderately active at a dose of 25×10^{-4} μmole, this molecule may contain a region, encephalitogenic in the Lewis rat, which is different from the 45-residue fragment. Alternatively, the intact protein may contain determinants that facilitate the encephalitogenicity of the fragment. The bovine fragment does have mild activity at a dose of 250×10^{-4} μmole.

The variation in response of Lewis rats to CNS tissue from different sources is duplicated, for the most part, in responses to the respective basic proteins. The results obtained with the fragments suggest that this variation is caused by sequence differences among the proteins and not to the production of blocking antibody. The effect of such sequence differences could reflect immunological specificity operating at two different phases of the immune response. These are (i) the sensitization phase, when lymphocytes recognize a determinant and undergo proliferation; and (ii) the effector phase, when such sensitized lymphocytes recognize this determinant in the target tissue and produce disease. One explanation of our results, therefore, is that a larger population of Lewis rat lymphocytes recognize the sequence of the guinea pig fragment than recognize the Lewis rat sequence, and that few if any lymphocytes recognize the sequence of the bovine fragment. Alternatively,

Table 1. Experimental allergic encephalomyelitis produced in the Lewis rat by basic proteins (experiment A) and by basic protein fragments (experiment B) from different animal sources. Disease occurrence is given by *N*, the number of diseased rats divided by the number of rats injected. Degrees of clinical severity (C, graded from 1 to 6) and pathological severity (P, graded from 1 to 8) are given as averages for diseased animals. Other abbreviations: GP, guinea pig; BP, basic protein (tested along with the guinea pig fragment in experiment B).

Source	Dose (10^{-4} μmole)	*N*	Onset (day)	Severity	
				C	P
		Experiment A			
GP	25	5/5	10.8	6.0	4.5
Bovine	25	5/5	14.4	3.3	2.1
Wistar	25	5/5	12.8	5.0	3.9
Lewis	25	5/5	13.0	4.8	4.3
GP	2.5	5/5	11.0	5.6	3.6
Bovine	2.5	0/5			
Wistar	2.5	0/5			
Lewis	2.5	1/5	20.0	1.0	0.5
GP	0.25	0/5			
Bovine	0.25	0/5			
Wistar	0.25	0/5			
Lewis	0.25	0/5			
		Experiment B			
GP	25	5/5	11.0	5.6	5.7
GP (BP)	25	5/5	10.8	5.4	5.1
Bovine	25	0/5			
Lewis	25	5/5	13.0	5.0	3.1
GP	2.5	5/5	11.8	5.0	4.6
GP (BP)	2.5	5/5	12.8	5.4	4.2
Bovine	2.5	0/5			
Lewis	2.5	1/5	20.0	2.0	0.8
GP	0.25	1/5	15.0	1.0	2.5
GP (BP)	0.25	0/5			

Lewis rat lymphocytes may recognize all three fragments equally well, but those sensitized by the bovine fragment may lack the capacity to interact with basic protein in the CNS because the sequence comprising the immunological determinant against which the lymphocytes are sensitized is not sufficiently similar to the sequence present in Lewis rat CNS. This latter concept is supported by the demonstration by Bergstrand and Kallén (*19*) that portions of xenogeneic (bovine) basic protein do not produce EAE in the guinea pig but have the capacity to induce delayed hypersensitivity. If the latter explanation were the only factor, however, one would expect the Lewis rat fragment to be at least as active as the guinea pig fragment. To account for the greater activity of the latter, the first explanation must be valid, and the possiblity of another variable must be considered. For instance, guinea pig fragment may contain a helper determinant that results in the stimulation of more lymphocytes than does the equivalent dose of the Lewis rat fragment. Further characterization and elucidation of the mechanisms responsible for the variation in the production of EAE are important, particularly because immunological mechanisms have been postulated to be operative in human demyelinating disease.

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Lower pH Limit for the Existence of Blue-Green Algae: Evolutionary and Ecological Implications

Abstract. *Observations on a wide variety of acidic environments, both natural and man-made, reveal that blue-green algae (Cyanophyta) are completely absent from habitats in which the* p*H is less than 4 or 5, whereas eukaryotic algae flourish. By using enrichment cultures with inocula from habitats of various* p*H values, the absence of blue-green algae at low* p*H was confirmed.*

The blue-green algae are of evolutionary interest, as they are the only prokaryotic organisms carrying out an oxygen-evolving photosynthesis. Their origin in the Precambrian is well substantiated, and it is likely that these algae were responsible for the initial rise in the oxygen level of the atmosphere near the end of the Precambrian (*1*). Eukaryotic algae, which arose later than the blue-green algae, differ in that their photosynthetic apparatus is present within a distinct cellular organelle, the chloroplast, whereas in the blue-green algae the photosynthetic apparatus exists as a series of cytoplasmic membrane systems perhaps connected to the plasma membrane (*2*). The blue-green algae are of considerable ecological interest since they often form massive blooms in polluted waters and since a number of species of blue-green algae produce toxins active against fish and mammals (*3*).

It is widely assumed that the blue-green algae are highly adaptable and are very tolerant of environmental extremes (*4*). However, the environmental tolerance of blue-green algae is, in many respects, not well substantiated. Only with respect to high temperatures is it clear that these algae are considerably more tolerant and adaptable than eukaryotic algae (*5*). In this report evidence is presented that in very acidic environments blue-green algae are completely absent, whereas eukaryotic algae often proliferate exceedingly well.

The data presented here are based on extensive observations of acidic habitats, both natural and man-made, throughout the world; detailed studies of acidic habitats in Yellowstone National Park; observations of algal distributions in natural *p*H gradients; and enrichment culture studies with media of various *p*H values and inocula from habitats of various *p*H values. The *p*H values were determined either directly at the site or soon after collection, by using an Orion battery-operated *p*H meter and combination glass electrode. The *p*H meter was always standardized with a buffer of *p*H near that of the sample.

Both thermal and nonthermal habitats were studied. Thermal habitats are particularly favorable for determining the lowest *p*H limit for blue-green algae because at temperatures above about 56° to 60°C eukaryotic organisms (both photosynthetic and nonphotosynthetic) are always absent and only prokaryotic organisms are present (*6*). Since some species of blue-green algae are able to grow at temperatures up to 70° to 73°C (*7*), the only oxygen-producing photosynthetic organisms at temperatures above 60°C would be blue-green algae (*8*). Observations in over 200 habitats of *p*H less than 4 throughout the world revealed that at temperatures above 56°C no photosynthetic organisms at all are found, and at temperatures from 40° to 56°C only the eukaryotic alga *Cyanidium caldarium* is found (*9*). At temperatures below 40°C in these thermal effluents a wide variety of other eukaryotic algae were found, but blue-green algae were absent.

Observations were also made on natural *p*H gradients in thermal habitats. One *p*H gradient studied in some detail (*10*) is in Waimangu Cauldron, New