ENCEPHALITOGENIC ACTIVITY IN RABBITS OF THE C-TERMINAL REGION OF BOVINE BASIC MYELIN PROTEIN: LOCALIZATION TO TWO DIFFERENT REGIONS

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1. Introduction

The basic encephalitogenic protein, which is a major component of the central nervous system myelin and is often referred to as the A1 protein [1], has recently attracted considerable attention. When minute amounts are injected into animals, it has the property of inducing a disease, experimental allergic encephalomyelitis (EAE), which has often been taken as an animal model for human demyelinating diseases, notably multiple sclerosis. The experimental disease is usually considered to be due to cell-mediated immunity against the basic protein [2]. The sequence of the protein, or fragments of it, has recently been determined in three different laboratories [1, 3–7]. A number of small regions of the protein have proved capable of inducing the disease. Considerable species variability in the response to these regions seems to exist. Guinea-pigs respond almost exclusively to a region composed of nine amino acids (residues 114–122 in the bovine protein) and containing the single tryptophan residue of the protein [4, 7–10]. Borderline activity has been ascribed to three more regions; one within residues 1–20 [4, 9, 11], one within residues 44–116 [12] (or even within residues 44–89 [13]), and one site in region 117–170 [12]. On the other hand, rabbits respond much more vigorously to these regions. Fragment 44–89 is a major encephalitogenic region when tested in rabbits [6, 7, 11, 13], and region 117–170 has been found to have considerable activity [14]. When monkeys are used as experimental animals, region 44–89 seems to be fully active [11], whereas the tryptophan containing determinant is not [11, 12]. However, inability to create disease in monkeys with region 44–89 has been reported [12]. In this communication, we extend previous studies concerning the encephalitogenic activity of the C-terminal region of the bovine protein when tested in rabbits. We present evidence that there are at least two different disease-inducing sites in this part of the protein localized within the sequences 134–150 and 154–170, respectively.

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2. Experimental methods and results

The preparation and isolation of region 117–170, using oxidation of the native protein or the fragment 44–170 with the bromine adduct of 2-(2-nitrophenylsulphenyl)-3-methylindole and CM-Sephadex chromatography and gel filtration of the reaction products, has previously been described [14, 15]. To 125 mg of this peptide, dissolved in 15 ml acetic acid pH 3, 1 mg of pepsin (Sigma P 6875) was added, and the reaction was allowed to proceed at 37° for 3 hr. The reaction products were lyophilized and dissolved in 1 ml 0.25 M ammonium acetate buffer pH 5 (core material was centrifuged away). They were first fractionated on Sephadex G-50 superfine (1.5 × 89 cm). The column was calibrated as previously described [16] with specially chosen fragments of the encephalitogenic to provide standard peptides with similar conformational and charge density characteristics. The fraction

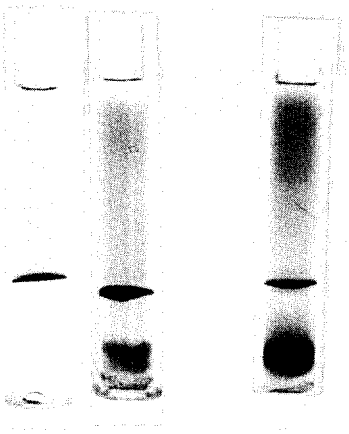

Fig. 1. Polyacrylamide gel electrophoresis pattern of the purified fractions. The first two photos show the patterns obtained with peptide 154–170 when stained with amido black 10B (left photo) and Coomassie blue and the third photo shows peptide 134–150 stained with Coomassie blue. The last peptide did not stain with amino black 10B. 10 μg was applied to each gel and migration is from the top to the bottom of the figure. The dark broad band in the cathodic end of the gels stained with Coomassie blue was obtained with gels without any sample applied and thus is not an impurity of the peptide.

Table 1
Amino acid composition of the purified fractions used in the present investigation.

	Amino acid residues in each peptide molecule		
	134–150	154–170	154–167
Lys	2.8 (3)	1.1 (1)	1.2 (1)
His	1.9 (2)		
Arg		4.2 (4)	2.1 (2)
Asp	1.1 (1)	1.2 (1)	1.0 (1)
Thr	0.9 (1)	trace	
Ser	0.9 (1)	2.5 (3)	3.2 (3)
Glu	1.0 (1)	trace	
Pro		0.7 (1)	1.6– (1)
Gly	2.6 (3)	3.1 (3)	3.4 (3)
Ala	1.9 (2)	1.2 (1)	
Met		0.7 (1)	
Leu	1.6 (2)	0.9 (1)	1.1 (1)
Phe		0.8 (1)	0.8 (1)

The analyses were performed in a Beckman 120 C analyser after hydrolyses in 6 N HCl at 110° for 22 hr. No corrections were made for hydrolytic losses. Also included in the table is the number of residues of regions 134–150, 154–170, and 154–167, respectively, according to the primary sequence. These figures are given in brackets. The composition of another preparation of the peptides has been given previously [16].

corresponding to molecules composed of 15 to 20 amino acids (the second of three major peptide peaks) was collected and further separated by chromatography on CM-Sephadex C-50 (1.0 × 10 cm) with the aid of a linearly increasing gradient of ammonium acetate buffer at pH 5 ranging from 0.02 M to 0.5 M. In the later part of the chromatogram, two major peaks emerged well separated from each other. Rechromatography of these fractions under identical conditions provided the final preparations. They showed a very little contamination when examined in a sensitive gel electrophoretic system. This gel electrophoretic method is a slight modification of the one described by Ahlroth and Mutt [17]. The basic proteins/peptides migrate towards the cathode in 15% gels, and the staining solution, Amido black 10 B or Coomassie blue migrate in the opposite direction.

Fig. 1 gives the electrophoresis patterns obtained. Amino acid analyses, performed as previously described [15], of the two fractions are given in table 1. An excellent correspondence can be seen between the figures obtained for the first eluting CM-Sephadex fraction and the figures expected from the amino acid sequence of region 154–170 [1, 3]. The same is valid for the second fraction and region 135–150. The first fraction has an N-terminal phenylalanine, which is in accordance with its identification as peptide 154–170, but we were unable to identify the N-terminal residue of the second fraction with the use of the DNS-method as previously described [15].

Tyrosine is situated in position 134 in the intact protein [1, 3] but only traces of tyrosine could be detected in the amino acid analyses of the present peptide. The cleaving reaction used to prepare fragment 117–170 is known to oxidize almost completely the two tyrosine residues in it [15] and in the amino acid analyses of another preparation of this peptide, a substantial amount of an unknown substance was observed. Moreover, the $A^{1\%}_{1\,cm}$ value at 280 nm was determined to 10, which shows that a UV-light absorbing moiety is part of the peptide. It is thus concluded that this peptide constitutes region 134–150 in which the tyrosine residue has been oxidized. We do not know the nature of the oxidation product.

Table 2
Encephalitogenic activity in rabbits of the three purified peptides.

Peptide injected	Number of animals showing disease/ number of animals challenged
134–150 20 μg	4/9 (3/9)
154–170 20 μg	4/10 (3/10)
154–170 100 μg	2/5 (2/5)
154–167 100 μg	1/9 (0/9)

The peptides were injected into the animals as described in the text. The figures given are number of animals showing clinical disease/number of injected animals. In brackets are given the corresponding ratios of animals showing histological signs of disease to total numbers of animals.

Cyanogen bromide cleavage was performed with 7 mg of peptide 154–170 in 1 ml of 70% formic acid to which 100 mg of CNBr was added. The reaction was allowed to take place for 24 hr at room temp. The products were lyophilized and separated on CM-Sephadex (1.9 X 10 cm) with the same gradient of ammonium acetate buffer as described above. Peptide 168–170 eluted well ahead of peptide 154–167. The recovery of peptide 154–167 was almost of the theoretical yield. Table 1 gives the amino acid analyses of peptide 154–167.

Random bred rabbits were injected with the peptide preparations in the toe pads of three legs. The peptide solutions were emulsified with equal parts of Freund's complete adjuvant (Difco). Table 2 lists the total amount of antigen (in 3 X 0.05 ml) given to each animal. The animals were observed daily and killed for histological examinations when showing signs of clinical disease or 4 weeks after challenge.

Table 2 shows that both peptide 134–150 and 154–170 are encephalitogenic when injected into rabbits. Both peptides were tested with material from two different preparations and no obvious difference in activity between the preparations was observed. Peptide 154–167 induced disease in only one animal out of nine when tested in 5-fold amounts compared with peptide 154–170. In most animals, paresis tended to develop late, seldom before the 20th day after challenge.

3. Discussion

The finding in the present investigation that the encephalitogenic activity of the C-terminal region of bovine myelin basic protein when tested in rabbits can be traced to two different regions, 134–150 and 154–170, respectively, merits some comments. Up to the present, five different regions in the entire protein have been ascribed encephalitogenic activity in rabbits. The activity of three of these – the tyrosine containing region 1–20, the site around the tyrosine residue in position 68, and the tryptophan containing region – may, according to Shapira et al. [11], be dependent on the common presence of an aromatic residue localized in the neighbourhood of a Gln–Lys (or Gln–Arg) sequence. In the two most active sites, the Gln–Lys residues are situated 5–6 residues C-terminal to the aromatic residue. These two sites are extremely similar in shape [11].

In the present case, it is clear from the amino acid sequence of the peptide that the activity cannot be ascribed to such properties. As fig. 2 shows, there are no apparent similarities between the peptides. The activity of region 154–170 does not seem to be completely destroyed when it is cleaved at the methionine residue. This would indicate that an intact structure of the C-terminal part of the peptide is probably not absolutely essential for activity. The preparation of

Tyr–Lys–Ser–Ala–His–Lys–Gly–Leu–Lys–Gly–His–Asp–Ala–Gln–Gly–Thr–Leu
134 140 150

Phe–Lys–Leu–Gly–Gly–Arg–Asp–Ser–Arg–Ser–Gly–Ser–Pro–Met–Ala–Arg–Arg
154 160 170

Fig. 2. Amino acid sequences of peptides 134–150 and 154–170 (bottom) as determined by Eylar et al. [1, 3].

substantial amounts of the peptides is rather tedious, which means that a closer examination of the active sites in these regions will probably await synthesis of these and analogous peptides. This is further borne out by the fact that the peptides seem unsuitable for specific chemical modification.

Recently, both of the peptides described in the present investigation have been shown to contain antigenic determinants capable of reacting in the macrophage migration inhibition assay in guinea-pig, a test which is usually considered as an *in vitro* correlate of delayed hypersensitivity. It is thus probable that one or both of these regions are also responsible for the very slight encephalitogenic activity in guinea-pigs of the C-terminal third of the protein that has been observed [12].

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