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Experimental Autoimmune Encephalomyelitis*

DIGESTION OF BASIC PROTEIN OF HUMAN MYELIN WITH CYANOGEN BROMIDE AND TRYPSIN

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Summary. From a cyanogen bromide digest of the encephalitogenic basic protein of human brain a polypeptide—derived from the N-terminal end of the protein—was isolated and shown to induce experimental autoimmune encephalomyelitis. At low doses this peptide induced typical histological lesions unaccompanied by clinical symptoms and humoral antibody. Digestion of the protein with trypsin did not destroy its encephalitogenic activity. The basic protein of human myelin contains more than one encephalitogenic determinant.

INTRODUCTION

Experimental autoimmune (allergic) encephalomyelitis (EAE) is a disease of the central nervous system produced by injection of white matter from brain or spinal cord in Freund's complete adjuvant (FCA) into animals. The antigen from the bovine spinal cord which induces the disease is a well characterized basic protein of approximately 16,000 molecular weight (Carnegie, Bencina and Lamoureux, 1967; Eylar and Thompson, 1969; Palmer and Dawson, 1969; Kibler, Shapira, McKneally, Jenkins, Selden and Chou, 1969). Human brain contains a similar protein (Einstein, Csejtey, Davis and Rauch, 1965; Caspary and Field, 1965) which comprises 30 per cent of the total protein of myelin (Eng, Chao, Gerstl, Pratt and Tavaststjerna, 1968). Lumsden, Robertson and Blight (1966) and Carnegie and Lumsden (1967) found that diffusible peptides extracted from acetone dried powders of bovine spinal cord were encephalitogenic. These peptides were shown to be produced by the action of an acid proteinase on the myelin basic protein during extraction of acetone dried powders with acid (Nakao, Davis and Einstein, 1966; Carnegie *et al.*, 1967; Chao and Einstein, 1968). The structure of one of these peptides was recently determined by Kibler *et al.* (1969).

This paper described the isolation and pathogenicity of peptides obtained by digestion of the human basic protein with cyanogen bromide and trypsin, the correlation between humoral antibody to the basic protein, and the incidence of EAE produced by these peptides.

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METHODS

Human encephalitogenic protein (HEProt)

Since the acid proteinase of brain is denatured with chloroform–methanol (2 : 1) this solvent was used to defat human brain which was obtained 12 hours after death. The basic protein was extracted as described by Kies (1965) and purified by treatment with carboxymethyl–Sephadex and gel filtration on Sephadex G-100, G-75 and G-50 (Carnegie, Bencina and Lamoureux, 1967). HEProt is a highly basic protein composed of approximately 170 amino acids.

Digestion with cyanogen bromide

HEProt (200 mg) was treated with cyanogen bromide (800 mg) in 70 per cent v/v formic acid at room temperature for 16 hours. Two polypeptides were separated on Sephadex G-50 using 0·1 per cent acetic acid as solvent and a column of 144×5·2 cm. The polypeptide of high molecular weight (CNBr-H) was eluted close to the exclusion volume while the other (CNBr-L) emerged at a volume 1·9 times that of the exclusion volume. CNBr-L was further purified by paper electrophoresis in pyridine acetate at pH 6·5 (Offord, 1966) and its amino acid sequence determined (Carnegie, 1969a). Gel electrophoresis (Carnegie *et al.*, 1967) was used to examine the fractions.

In addition a small peptide was isolated by electrophoresis of the whole digest on Whatman 3MM paper at pH 6·5. This peptide had a mobility of 0·93 relative to lysine. Its amino acid sequence was determined as described previously (Carnegie, 1969a).

Digestion with trypsin

In typical digestions of HEProt or CNBr-H, 100 mg of protein in 7 ml of water were treated in a pH stat at pH 8 with 100 μg of trypsin (Worthington batch 6111 treated with chloromethyl 2-phenyl-L-1-toluene-*p*-sulphonamidoethyl ketone) at 30° for 1½ hours. Tryptic digests were fractionated by gel filtration on Sephadex G-25 using phenol–acetic acid–water (1 : 1 : 1, w/v/v). This strongly dissociating solvent prevents adsorption of aromatic peptides to the Sephadex and makes it possible to correlate size and elution volume (Carnegie and Lumsden, 1967; Carnegie, 1965). The solvents were removed after additions of water by repeated evaporations at 30° on a rotary evaporator. A typical fractionation of a tryptic digest of CNBr-H is shown in Fig. 3. Any trace of undigested protein was removed in fractions prior to 75 ml. In some experiments digests of HEProt were first fractionated on a Sephadex G-25 column (59×2 cm) with 0·1 per cent acetic acid as solvent, and the zone merging between 112 and 140 ml was then fractionated on the phenol–acetic acid–water column. A peptide T10 (Fig. 2) which contained tryptophan, was isolated from zone A (Fig. 3) by paper electrophoresis and chromatography and its sequence determined (Carnegie, 1969b).

Encephalitogenic activity

The footpads of each hind foot of adult guinea-pigs were injected with 0·05 ml of an emulsion prepared from equal volumes of the test sample in water and Freund's complete adjuvant (FCA) (Difco, Detroit). The number of animals and doses are stated in Tables 1 and 3. Animals were examined daily for signs of EAE (Lamoureux, Carnegie, McPherson and Johnston, 1967). Guinea-pigs which showed between 11 and 20 days after injection, loss of righting reflex and incontinence were classed as having 'mild' clinical EAE

and those which also became paralysed as 'severe' EAE. Histological examination was as described by Robson, McPherson, Mackay and Carnegie (1970): in addition to typical lesions, margination of cerebral vessels with polymorphonuclear leucocytes was observed after low doses of HEProt in FCA.

Radioimmunoassay

Sera were taken from guinea-pigs when they were killed. This was 11–18 days after injection if they showed signs of EAE, or at 21 days if severe disease had not developed. Sera were assayed for antigen binding capacity by radioimmunoassay in which HEProt labelled with ^{125}I ([^{125}I]HEProt) was used as the test antigen and the antigen–antibody complex separated on a column of Sephadex G-75 (McPherson and Carnegie, 1968). An antigen binding curve was constructed for each assay with the percentage antigen bound plotted against serum dilution. For the purposes of calculating and comparing the amount of [^{125}I]HEProt bound (μg per ml undiluted test serum), the point corresponding to 40 per cent binding was interpolated from the linear part of each antigen binding curve.

CNBr-H and CNBr-L were iodinated by the procedure described for HEProt (McPherson and Carnegie, 1968). Preparations with a specific activity between 5 and 50 μCi/μg were used.

For competitive inhibition experiments serum from a rabbit injected in all four foot pads twice with FCA and HEProt (4 and 7 mg) was used.

RESULTS

CYANOGEN BROMIDE DIGESTION

Cyanogen bromide was used to cleave HEProt at its two methionine residues. Fractionation of the digest yielded a polypeptide CNBr-L composed of twenty-one amino acids, CNBr-H composed of approximately 145 amino acids and a small peptide Ala-Arg-Arg. The amino acid sequence of CNBr-L has been determined (Fig. 1) and it comprises the first 21 residues from the N-terminal end of HEProt (Carnegie, 1969a). CNBr-H accounted for the remainder of HEProt apart from the tripeptide which was from the C-terminal end of the protein.

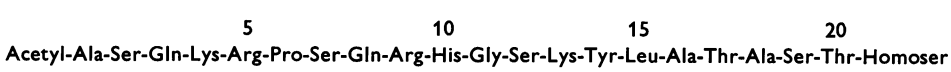

FIG. 1. Amino acid sequence of CNBr-L from the N-terminal region of encephalitogenic basic protein from human brain.

In both the gel filtration and paper electrophoresis procedures there was a clear separation of CNBr-L from HEProt. No trace of HEProt could be detected by electrophoresis of CNBr-L in polyacrylamide gel. However, examination of CNBr-H by gel electrophoresis showed traces of HEProt which could not be removed.

The occurrence of EAE in fourteen guinea-pigs injected with CNBr-L is shown in Table 1. Five n-moles induced typical histological lesions in two of four animals but there were no clinical signs of disease. Larger doses (33 n-moles) resulted in both typical clinical and histological EAE.

TABLE 1

ENCEPHALITOGENIC ACTIVITY OF FRAGMENTS FROM DIGESTION OF ENCEPHALITOGENIC BASIC PROTEIN OF HUMAN BRAIN WITH CYANOGEN BROMIDE (SEE TEXT FOR ABBREVIATIONS)

	Dose		No. of guinea-pigs	Disease			
				Clinical		Histological	
				Mild	Severe	Polymorph margination only	Typical
HEProt	2·5	n-moles (50 μg)	4	0	4	0	4
CNBr-L	5	n-moles	4	0	0	1	2
	8	n-moles	3	0	0	1	2
	20	n-moles	3	1	0	1	2
	33	n-moles	4	0	3	0	4
CNBr-H	8	n-moles	4	2	1	0	2

'Polymorph margination only', polymorphonuclear leucocyte margination of vessels within CNS as sole abnormality.

'Typical', lesions showed vascular endothelial proliferation, accumulation of polymorphs and mononuclear cells within vessels and cellular migration into perivascular areas.

CNBr-H induced both clinical and histological EAE in three of the eight animals injected. However traces of undigested HEProt present in the CNBr-H could have been responsible for induction of EAE.

When HEProt was injected with Freund's complete adjuvant into guinea-pigs humoral antibody was usually produced but the amount present at the time of signs of disease was variable (Table 2) and there was no correlation between the amount of antibody and the incidence or severity of EAE. CNBr-H also induced humoral antibody against [^{125}I]-HEProt and [^{125}I]CNBr-H. Although CNBr-L induced typical clinical and histological disease, in contrast to HEProt and CNBr-H the disease was not accompanied by humoral antibody to either [^{125}I]HEProt nor [^{125}I]CNBr-L.

TABLE 2

ANTIGEN BINDING CAPACITY OF SERA FROM ANIMALS INJECTED WITH PEPTIDES FROM ENCEPHALITOGENIC BASIC PROTEIN (HEProt) (SEE TEXT FOR ABBREVIATIONS AND TABLES 1 AND 3 FOR AMOUNT INJECTED)

Peptide	No. of sera examined	No. positive	[^{125}I]HEProt bound (μg/ml undiluted serum)
HEProt	4	4	38, 15, 4, 80
CNBr-L	14	0	0
CNBr-H	2	2	27, 7
Tryptic digest HEProt	6	2	0·2, 0·2
Zone C	4	2	4·8, 1·6
Zone D	6	3	4·8, 1·9, 1·6
Zone E	6	1	5·6
Zones F and G	5	0	0

In competitive inhibition experiments in which [^{125}I]HEProt (0·1 μg) was used as antigen and a serum from a rabbit hyperimmunized with HEProt as the source of antibody, CNBr-L in amounts up to 500 μg caused no inhibition of binding, whereas 0·6 μg of HEProt caused 50 per cent inhibition. These competitive inhibition experiments provide additional evidence on the purity of CNBr-L. It is clear that there must be less than 0·12 per cent contamination of CNBr-L by HEProt. Even with a contamination of 0·1 per cent the amount of HEProt (0·01 μg) present in 5 n-moles of CNBr-L would be insufficient to

TABLE 3

ENCEPHALITOGENIC ACTIVITY OF FRACTIONS FROM A TYPTIC DIGEST OF ENCEPHALITOGENIC BASIC PROTEIN (HEProt) AND CNBr-H

Fraction	Approximate molecular weight*	Dose (μg) of amino acids	No. of guinea-pigs injected	Animals with disease			
				Clinical		Histological	
				Mild	Severe	Polymorph margination only	Typical
Tryptic digest of HEProt		50	6	1	2	1	4
Zone A	2000	30	7	3	0	3	0
Zone C	1400–1800	10	6	1	4	2	4
Zone D	1000–1400	30	6	0	1	1	2
Zone E	600–1000	30	10	2	7	4	5
Zone F plus Zone G	600	20	5	0	0	0	0
Tryptic digest of CNBr-H							
Zone D	1000–1400	85	14	3	7	–	–
Zone E	600–1000	50	5	1	4	–	–

* Approximate molecular weight of peptides contained in these fractions was calculated from calibration of the column with peptides of known molecular weight (Carnegie, 1965).

induce the marked histological lesions as the minimum dose for a 50 per cent incidence of such lesions is between 0·5 and 1 μg (Robson *et al.*, 1970).

However, this antiserum to HEProt did bind [^{125}I]CNBr-L. However, binding of [^{125}I]HEProt by the rabbit serum was 80 μg/ml whereas that for [^{125}I]CNBr-L was only 0·4 μg/ml. Thus it appears that CNBr-L contains sufficient determinants to bind to some antibodies directed against HEProt but insufficient to inhibit the stronger binding of the main determinants in the basic protein.

TRYPTIC DIGESTION

HEProt was readily digested with trypsin. A peptide map of the digest is shown in Fig. 2. The whole tryptic digest of HEProt induced EAE (Table 3) and antibody directed against HEProt. However the level of antibody was much lower than that induced by a comparable amount of undigested HEProt. After complete removal of any traces of undigested HEProt by gel filtration in phenol–acetic acid–water, zones C, D and E (Fig. 3) which were composed of peptides from about 600 to 1800 molecular weight still readily induced severe clinical and histological EAE in 50 per cent of the animals 11–20 days after injection. These zones could be consistently isolated from tryptic digests of both HEProt and from CNBr-H. Zones C, D and E contained peptides T1, T2, T4, T6, T13, T15 and T17 which are shown on the peptide map (Fig. 2). Zones F and G, which were composed of smaller peptides, did not induce EAE.

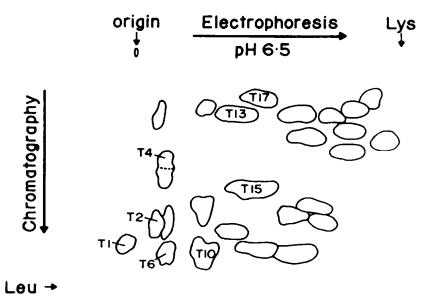

FIG. 2. Peptide map of tryptic digest of human encephalitogenic protein. Electrophoresis was in pyridine acetate pH 6·5 on 3MM paper at 3 kV for 70 minutes. Chromatography was in n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 by volume).

Zones C, D and E induced low levels of antibody to [^{125}I]HEProt in some animals (Table 2) but there was no correlation between the level of antibody and the severity of disease.

In preliminary tests for encephalitogenicity on peptides T1, T2, T4, T13, T15 and T17 from zones D and E, at a dose per guinea-pig of approximately 4 n-moles mixed with FCA, no clinical or histological EAE was induced. Peptide T6, which has the sequence of Phe-Phe-Gly-Gly-Asp-Arg did induce a mild disease (loss of righting reflex and incontinence) in two of four animals injected with 4 n-moles. Histological examination showed only polymorph margination. Further work will be necessary to ascertain which peptide is responsible for the activity of zones D and E.

The largest peptide (T10) in the tryptic digest has been isolated from zone A. It contains seventeen amino acids, including tryptophan, and the sequence of amino acids has been

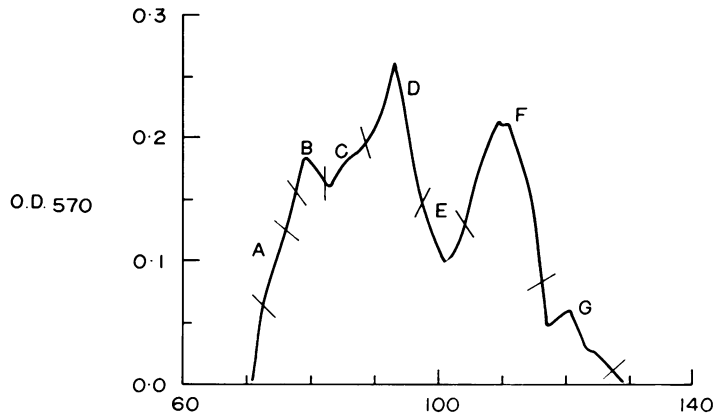

FIG. 3. Gel filtration of a tryptic digest of CNBr-H on a column of Sephadex G-25 (70 × 2·5 cm) in phenol–acetic acid–water (1 : 1 : 1; w/v/v). From each fraction (2·2 ml) 0·1 ml was removed and treated with ninhydrin. Fractions were pooled to give zones A to G. Tryptic digest of HEProt gave a similar pattern.

determined (Carnegie, 1969b, see below). This peptide, in doses of 10 n-moles with FCA, did not induce clinical nor histological EAE in any of six guinea-pigs neither did it induce antibody to [^{125}I]HEProt.

Tryptic digests of the cyanogen bromide fragment CNBr-H were also active in inducing clinical and histological EAE. Fractionation of the digests by gel filtration in phenol–acetic acid–water (Fig. 3) consistently yielded fractions similar to those obtained from HEProt.

DISCUSSION

The encephalitogenic basic protein from human brain (HEProt) was digested with cyanogen bromide and trypsin to yield fragments which induced paralysis in guinea-pigs and histological lesions in the brains typical of EAE.

The basic protein from bovine spinal cord also has been digested with cyanogen bromide by Hashim and Eylar (1969a) and Palmer and Dawson (1969). Both groups found that the polypeptide of high molecular weight induced EAE, but neither group observed any clinical signs of EAE with the equivalent of our CNBr-L. However, histological examinations were not reported by Hashim and Eylar and not carried out by Palmer (personal communication). It was surprising that CNBr-L from the human protein induced severe histological lesions unaccompanied by clinical disease. The dose of CNBr-L (33 n-moles) required to induce severe EAE was approximately 60–100 times greater than that of HEProt necessary to cause similar clinical and histological EAE (Robson *et al.*, 1970).

The absence of antibody to HEProt in some animals with EAE produced by injection of CNBr-L (or tryptic peptides, see below) is consistent with the general view that the lesions of EAE are the result of an attack by sensitized lymphocytes on nervous tissue. Lisak, Heinze, Kies and Alvord (1969) using simply a qualitative assay for antibody to basic protein also found that there was no correlation between presence of antibody and disease.

Tryptic digests of the bovine basic protein have been found to retain encephalitogenicity (Carnegie *et al.*, 1967). Hashim and Eylar (1969b) claimed that tryptic peptides from the bovine protein showed negligible encephalitogenicity when compared to the parent protein and to a peptic digest. However as many as 20 per cent of their guinea-pigs, when injected with peptides, which were chromatographically and electrophoretically equivalent to those in our zones D and E, developed clinical EAE. Histological studies were not reported. The comparatively lower incidence reported by Hashim and Eylar may be due to differences in our injection procedure or different strains of guinea-pigs; e.g. there is an approximately 10 fold increase in the potency of a dose of HEProt if two foot pads rather than one are injected (Robson *et al.*, 1970). Caspary and Field (1965) reported only a partial loss in encephalitogenicity after digestion of the human protein for 18 hours with trypsin.

A fragment of the bovine basic protein which retained encephalitogenicity in rabbits was isolated from an acid proteinase digest of the protein (Kibler *et al.*, 1969). This polypeptide, which is composed of forty-five amino acid residues, starts with the sequence Phe-Gly-Ser-Asp-Arg- which is similar to our peptide T6—Phe-Phe-Gly-Gly-Asp-Arg. Although a tryptic digest of Kibler's polypeptide was inactive in rabbits a partial acid digest did produce EAE.

Although the largest peptide (T10) in our tryptic digest did not induce EAE it is very similar in sequence to part of another encephalitogenic determinant in the bovine basic protein, since Eylar and Hashim (1968) showed that peptic peptides which contain the sequence Ser-Arg-Phe-Gly-Ser-Trp-Gly-Ala-Glu-Gly-Gln are highly encephalitogenic. Peptide T10 has the sequence Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-Pro-Gly-Phe-Gly-Tyr-Gly-Gly-Arg. Eylar and Hashim (1968) found that trypsin destroyed the encephalitogenicity of such peptides by hydrolysing the Arg-Phe-bond, which would explain the lack of encephalitogenic activity of T10.

Since fractions from digestion of the human protein with cyanogen bromide and trypsin retain encephalitogenicity there must also be more than one region of this protein which will induce EAE. The actual number of encephalitogenic determinants, their localization in the protein and their relative quantitative capacity to induce EAE are now claiming our attention.

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