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Allergic Encephalomyelitis: Evidence for Lack of Significant Encephalitogenic Activity of Purified Peptide L in the Monkey†

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ABSTRACT: A highly purified preparation of peptide L of basic protein from bovine spinal cord was obtained by Cellex-P ion-exchange chromatography and repeated Sephadex G-50 filtration; the peptide L preparation was shown to be free from peptide T contamination. When tested for activity, it induced experimental allergic encephalomyelitis (EAE) in the Rhesus monkey after 5–15 weeks at the concentration above 5 mg, suggesting the presence of a weak encephalitogenic site. When peptide L was further cleaved by cyanogen bromide, two peptides, peptide J (residues 1–20) and peptide D (residues 21–115), were obtained. Peptide J was inactive in inducing EAE, while peptide D was as weakly active as peptide L. Conformational studies and identical rate of tryptic hydrolysis of native and denatured peptide L show that it exists in a highly unfolded conformation. Two regions, CD1 (residues 1–43) and peptide R (residues 44–89) present in peptide L, have been previously reported to be encephalitogenic in the monkey; on a molar basis these peptides were much more active than our peptide L preparation. The high activity observed in these peptides is probably due to contamination with the basic protein. It was concluded from our previous studies that the major disease-inducing site in the monkey is a 14-residue peptide (peptide M) localized near the COOH-terminal end of the molecule (Karkhanis, Y. D., Carlo, D. J., Brostoff, S. W., & Eylar, E. H. (1975) *J. Biol. Chem. 250,* 1718).

Experimental allergic encephalomyelitis is an autoimmune disease induced by the basic protein of CNS[1] myelin (Laatsch et al., 1962; Einstein et al., 1962; Eylar et al., 1969) which comprises 30% of the total myelin protein. The protein is believed to have a highly ordered and folded structure and is best described as a prolate ellipsoid with an axial ratio 10:1 (Epand et al., 1974). The ease with which the basic protein can be isolated from myelin suggests that its location in the membrane is that of a so-called *peripheral* protein. The determination of the amino acid sequence of this protein has facilitated the definition of different encephalitogenic sites in this protein which are: a nonapeptide (residues 113–121) active in the guinea pig, peptide R (residues 44–89) active in the rabbit and two peptides, peptides CD1 and M (residues 1–43 and 154–167) active in the monkey (Eylar et al., 1970; Kibler et al., 1972; Jackson et al., 1972; Karkhanis et al., 1975). The rabbit peptide (residues 44–89) has also been shown to be active in the monkey (Kibler et al., 1972). Since the basic protein contains a single tryptophan residue, it is susceptible to treatment with BNPS-skatole (Omenn et al., 1970), the split product being peptides T and L. Peptide T is a COOH-terminal 54-residue peptide which contains one of the monkey sites, whereas peptide L contains two encephalitogenic regions, peptides CD1 and R. On a molar basis peptide L should be more active than peptides T, Y, and M alone. The data obtained so far on peptide L have not supported this interpretation (Eylar et al., 1972) and could be due to the difficulty in obtaining peptide L free from basic protein, since on ion-exchange and gel filtration columns it is eluted close to the basic protein. In the present communication we report the isolation and characterization of peptide L, containing 1% or less of basic protein, which shows weak encephalitogenic activity in the monkey. Since peptide L is comprised of peptides CD1 and R regions, the high activity on a molar basis observed by other workers (Brostoff et al., 1974; Kibler et al., 1972) is perhaps

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[1] Abbreviations used: CNS, central nervous system; peptide L, residues 1–115 of basic protein; peptide T, residues 116–170; peptide Y, residues 154–170; peptide M, residues 154–167; peptide CD1, residues 1–43; peptide R, residues 44–89; peptide J, residues 1–20; peptide D, residues 21–115; fluorescamine, 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-diane; CD, circular dichroism; UV, ultraviolet; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; BNPS-skatole, a bromide adduct of 2-(2-nitrophenylsulfenyl)-3-methylindole; EAE, experimental allergic encephalomyelitis.

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due to contamination of these peptides with the basic protein. Evidence is also presented to show that the weak encephalitogenic activity of peptide L resides in the residues 21–115.

Experimental Procedure

Materials. Basic protein from bovine spinal cord was a kind gift from Mr. J. J. Jackson of our laboratory; cyanogen bromide was obtained from Matheson, Coleman and Bell; Cellex-P, acrylamide, bis(acrylamide), and $NaDodSO_4$ were from Bio-Rad; BNPS-skatole and plastic coated silica gel thin-layer plates were from Eastman; BSA was from Gallard Schlesinger; ovalbumin, chymotrypsinogen A, ribonuclease, blue dextran 2000, Sephadex gels, and Sephacryl S-200 were from Pharmacia; myoglobin, cytochrome *c*, and DNP-alanine were from Calbiochem; thrice recrystallized lysozyme was from Sigma; Tos-PheCH_2Cl-trypsin was from Worthington Biochemical Corp.; succinic anhydride was from Pierce. Other reagents used in this study were of analytical grade. Water was glass distilled and deionized.

Isolation of Myelin Basic Protein. Basic protein was purified from bovine spinal cord according to the procedure of Oshiro & Eylar (1970). The purity of the protein was confirmed by acrylamide electrophoresis and ultracentrifugation (Eylar et al., 1969).

Isolation of Peptides L and T. Treatment of the basic protein with BNPS-skatole has been previously described (Burnett & Eylar, 1971). The peptide mixture containing peptide T, peptide L, and some unreacted basic protein was dissolved in 0.025 N phosphoric acid, pH 2.5, and chromatographed on a Cellex-P column equilibrated with 0.025 N phosphoric acid, pH 2.5 at 20 °C. Fifty milliliters of this buffer was initially used to wash the column. After the initial wash, peptides were then eluted with a KCl gradient formed in a three-chamber mixer with 500 mL of starting buffer in each chamber containing 0.17, 0.5, and 1.5 M KCl, respectively (Chin & Wold, 1972). The eluate was monitored at 245 nm. The fractions containing peptide L, peptide T, and basic protein were pooled and lyophilized.

Sephadex Chromatography of Peptides. All Sephadex gel filtrations of peptides reported in this study were carried out in 10% acetic acid at 20 °C. The lyophilized peptides obtained from Cellex-P chromatography were dissolved in 10% acetic acid and desalted on a G-50 column. The eluates were monitored at 245 nm and the elution positions of peptides L and T and basic protein were determined by using pure preparations of these components.

$NaDodSO_4$-Poly(acrylamide) Electrophoresis of Peptide L and Basic Protein. $NaDodSO_4$-poly(acrylamide) electrophoresis of peptide L was done according to the procedure of Weber & Osborn (1969); the peptide L was stained with Fast Green (Greenfield et al., 1971). For $NaDodSO_4$-poly(acrylamide) electrophoresis of fluorescamine-labeled peptide L and basic protein, a procedure developed in our laboratory was used. Peptides were incubated (1 mg/mL) in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 8.5, containing 1% $NaDodSO_4$, 4 M urea, 1% mercaptoethanol (v/v), and 10% glycerol at 45 °C for 1 h. Twenty microliters of this mixture was mixed vigorously on a Vortex Mixer with 5 μL of fluorescamine (3 mg/mL) in acetone (Udenfriend et al., 1972). The acrylamide–bis(acrylamide) solution was made as described by Weber & Osborn (1969) with a final gel concentration of 10%. Electrophoresis was done in the sodium phosphate buffer diluted 1:1 with water. The current applied was 8 mA per tube; electrophoresis took 4 h to complete. Standard proteins such as BSA, ovalbumin, chymotrypsinogen lysozyme, and RNase treated under the same conditions were also electrophoresed. Although a fast moving fluorescent band which moves with the buffer front served as an excellent marker, 3 μL of bromophenol blue was also added to these proteins before they were applied to the gel. Different concentrations of peptide L and basic protein were used; for the standard proteins, 2 μL was used. Peptides were located by a UV lamp.

Determination of Stokes Radius and Frictional Ratio. Stokes radius, *a*, was determined by molecular sieving on G-100 (2.5 × 60 cm) at 4 °C and the data were expressed in terms of K_{av}, a parameter defined by Laurent & Killiander (1964) as follows:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the elution volume, V_0 is the void volume of the column, and V_t is the total volume of the gel bed. The K_{av} values of standard proteins were plotted against Stokes radii according to the equation:

$$(-\log K_{av})^{1/2} = \alpha(\beta + a)$$

where α and β are constants. Frictional ratio f/f_0 was calculated according to the equation:

$$f/f_0 = \frac{a}{\left(\frac{3\bar{v}M}{4\pi N}\right)^{1/3}}$$

The partial specific volume $\bar{v}$ and the molecular weight M calculated from amino acid analysis were 0.71 and 12 608, respectively. N is the Avogadro's number. To determine the molecular weight of peptide L under denaturing condition, gel filtration was carried out at 20 °C on S-200 column (2.5 × 60 cm) in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0, containing 8 M urea and 0.1% (v/v) mercaptoethanol. Prior to chromatography, 10 mg of peptide L and standard proteins BSA, ovalbumin, chymotrypsinogen, myoglobin, and cytochrome *c* were incubated at 20 °C in 1.0 mL of the above denaturation buffer but containing 1% mercaptoethanol (v/v) under N_2 for 24 h. Blue dextran 2000 and DNP-alanine were used to determine V_0 and V_t, respectively. The molecular weight of peptide L was calculated from its elution position relative to that of the standard proteins.

Succinylation of Peptide L. To a 2.0 mL (5 mg/mL) solution of peptide L in 0.1 M borate buffer, pH 9.0, 50 mg of succinic anhydride was added and the pH was maintained at 9.0 by adding a few drops of 1 N NaOH (Habeeb et al., 1958). After incubation at 20 °C for 4 h the mixture was dialyzed against 1 L of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0 at 4 °C for 24 h, and applied to a Sephadex G-100 column. A control was run using the same steps except for the addition of succinic anhydride. The extent of succinylation was determined by adding a 50-μL aliquot of the reaction mixture to 3 mL of 0.1 M borate buffer, pH 9.0, followed by a thorough mixing with 0.5 mL of fluorescamine solution (3 mg/10 mL) in acetone. The fluorescence of the reaction mixture was measured at 470 nm (excitation at 390 nm) on a Perkin-Elmer spectrofluorometer Model 204 and compared with the fluorescence of the control reaction mixture. The amount of succinylation was calculated from the formula

$$\frac{F_c - F_s}{F_c} \times 100$$

where F_c and F_s represent fluorescence of the control and succinylated peptide L.

Tryptic Hydrolysis of Native and Denatured Peptide L. Denatured peptide L was prepared by incubating a 10 mg/mL solution of peptide L for 18 h at 20 °C in 0.05 M Na_2HPO_4-

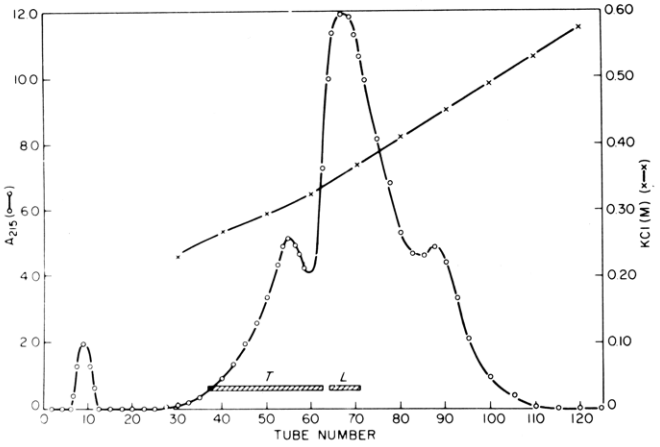

FIGURE 1: Chromatography on a Cellex-P column (20 × 1.4 cm) at 20 °C of basic protein (200 mg) treated with BNPS-skatole. The column was equilibrated with 0.12 M KCl. After the breakthrough peak it was eluted with a KCl gradient formed in three chambers containing 500 mL of 0.17 M KCl, 0.05 M KCl, and 1.15 M KCl, respectively. Fractions of 6 mL were collected in each tube. The flow rate was 30 mL per h. The contents of various tubes were pooled into fractions represented by striped bars. Each fraction was lyophilized and desalted on a Sephadex G-50 column.

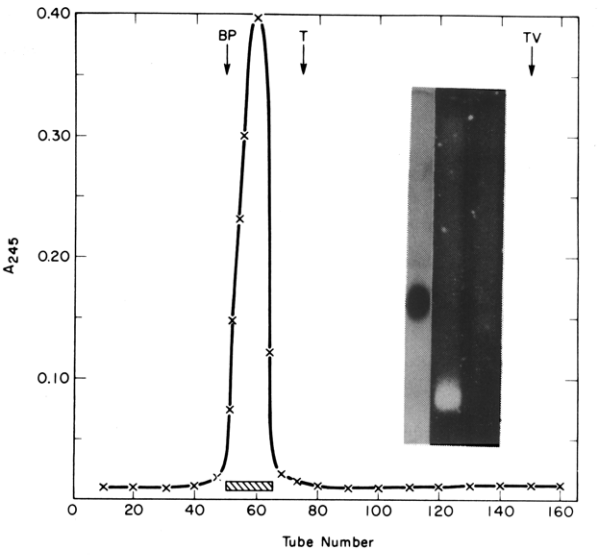

FIGURE 2: Desalting of peptide L on Sephadex G-50 column (51 × 4 cm) at 20 °C. The lyophilized fraction containing peptide L shown in Figure 1 was dissolved in 25 mL of 10% acetic acid and half of this amount was applied to the column equilibrated with 10% acetic acid. Each fraction was 4 mL in volume. The flow rate was 60 mL per h. Fractions represented by the striped bars were pooled and lyophilized for rechromatography on the same column. The arrows show the elution positions of basic protein (BP), peptide T (T), and the total volume of the column (TV). Inset shows, from left to right: $NaDodSO_4$–poly(acrylamide) electrophoresis pattern of peptide L, 300 μg; fluorescamine-labeled peptide L, 160 μg; basic protein, 1.6 μg.

NaH_2PO_4, pH 7.0, containing 8 M urea. The mixture was chromatographed on a Sephadex G-25 column (2.4 × 12 cm) at 20 °C equilibrated with 10% acetic acid. Peptide L which eluted in the void volume was lyophilized and was used as the denatured peptide L preparation.

For tryptic hydrolysis, a 1 mg/mL solution of native and denatured peptide L in 0.1 M Na_2HPO_4–NaH_2PO_4, pH 7.4, containing 10% Tos-PheCH_2Cl-trypsin by weight was incubated at 37 °C. Every 20-min interval 50 μL was removed and added to 3 mL of 0.2 M borate buffer, pH 8.0. One hundred microliters of a fluorescamine solution was added and thoroughly mixed (see above). The percent hydrolysis was calculated from the formula $F_t/F_m \times 100$, where F_t and F_m are the fluorescence at hydrolysis time t and maximum fluorescence, respectively. The maximum fluorescence was considered as 100% hydrolysis of the peptide.

CD Spectra. The circular dichroism spectra were recorded on a JASCO J-41A spectropolarimeter at 20 °C with a 0.23 mg/mL solution of peptide L in 0.1 M Na_2HPO_4–NaH_2PO_4, pH 7.0. The protein concentration was calculated from amino acid analysis. The mean residue weight of 115 was calculated from the amino acid composition. The CD data were interpreted by method I of Greenfield & Fasman (1969). As a control, the spectrum of lysozyme was also recorded.

Cyanogen Bromide Cleavage of Peptide L and Basic Protein. Peptide L or basic protein (10 mg/mL) was dissolved in 70% formic acid and treated with cyanogen bromide (30 mg/mL) in a N_2 atmosphere in the dark for 24 h at room temperature. The mixture was diluted ten times with water and lyophilized. The lyophilized mixture was dissolved in 10% acetic acid and chromatographed on a Sephadex G-75 column.

Purity of Peptides. The purity of the peptides was confirmed by thin layer chromatography utilizing butanol–water–pyridine–acetic acid (15:12:10:3) as a solvent. Peptides were located by spraying with a 0.5% solution of ninhydrin in acetone. Fluorescamine-treated peptides were located by a UV lamp.

Other criteria of purity used were $NaDodSO_4$–poly(acrylamide) electrophoresis and amino acid analysis.

Amino Acid Analysis. For amino acid analysis, peptides were hydrolyzed in a sealed tube under N_2 containing 0.5 mL of 6 N HCl for 18 to 30 h. After hydrolysis, the samples were evaporated to dryness on a Buchler Evapomix and were dissolved in LPH-1 buffer for acid-neutral amino acids and LPH-4 buffer for basic amino acids. Amino acids were analyzed on a Perkin-Elmer KLA-5 amino acid analyzer using ligand exchange chromatography. This instrument was equipped with an interface and a PEP-1 computer for data retrieval.

Encephalitogenic Activity. The Rhesus monkeys (both sexes) used in this study were obtained from Primate Imports, Inc., Port Washington, N.Y. They ranged in age from 2 to 3 years and weighed 3500 to 5000 g. They were free of tuberculosis, diarrhea, and infection as shown by frequent periodic examinations. Their diet consisted of Purina Monkey Chow and fresh fruit.

To induce EAE, the antigen was dissolved in 0.15 M NaCl and was emulsified with an equal volume of complete Freund's adjuvant (Difco H37ra) containing 1 mg per mL of *M. tuberculosis.* Each animal received an intradermal injection into each footpad containing 0.1 mL of the emulsion for a total of 0.2 mL.

Animals were killed at appropriate times; brain and spinal cord were fixed in formalin and prepared for histological examination. Histological examination was carried out on sections from the frontal, temporal, parietal, and occipital lobes, including the periventricular white matter. Other sections were obtained from the cerebellum, brain stem, and spinal cord.

Results

Figure 1 shows the separation of peptides T and L and basic protein on Cellex-P chromatography. At a KCl concentration between 0.2 and 0.3 M, peptide T was eluted, followed by peptide L at the salt concentration above 0.3 M; the basic protein was eluted last, next to peptide L. The fractions containing peptides T or L were pooled and lyophilized for desalting.

Desalting of Peptides T and Peptides L. The lyophilized

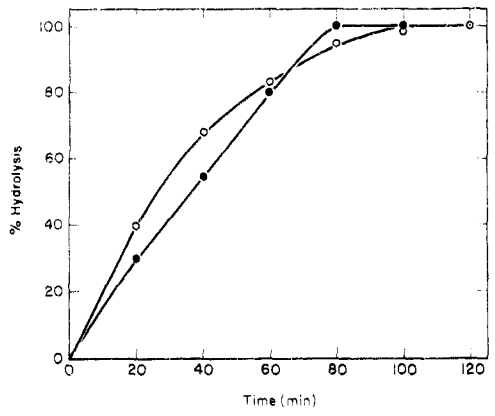

FIGURE 3: Tryptic hydrolysis of native and denatured peptide L. For conditions, see Experimental Procedure.

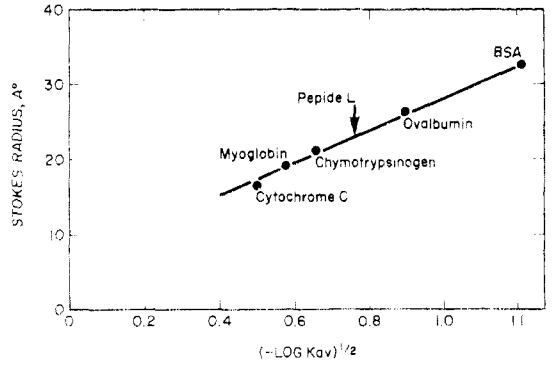

FIGURE 4: Stokes radius of peptide L as determined by gel filtration. Peptide L and standard proteins, 10 mg/mL in 0.1 M Na_2HPO_4-NaH_2PO_4, pH 7.0, were chromatographed at 4 °C on a Sephadex G-100 column (2.5 × 60 cm) equilibrated with the same buffer. The flow rate was 30 mL/h. Each tube contained a fraction of 3 mL in volume.

materials containing peptides T and L obtained from Cellex-P columns were dissolved in 10% acetic acid and desalted on G-50 columns equilibrated with the same solvent. Figure 2 shows the desalting of peptide L. The arrows show the elution positions of basic protein, peptide T and the total volume of the column. These positions were obtained using pure preparations of basic protein, peptide L and peptide T. It is interesting to note that peptide L was eluted near the basic protein which eluted at the void volume of the column. Although the peak containing peptide L is symmetrical, it contained a trace amount of basic protein when tested above 250 μg concentration on $NaDodSO_4$-poly(acrylamide) electrophoresis. Chromatography of the L peptide material had to be repeated several times to eliminate the trace contamination by basic protein.

$NaDodSO_4$-Poly(acrylamide) Electrophoresis of Peptide L. Figure 2 shows the $NaDodSO_4$-poly(acrylamide) electrophoresis of peptide L. No contaminating proteins or peptides were detected at a concentration of 300 μg. To detect the possible presence of any contaminants, peptide L and basic protein were labeled with fluorescamine and electrophoresed on $NaDodSO_4$-poly(acrylamide) (Figure 2). At the 160-μg concentration, the fluorescamine-labeled peptide L showed a single band without any impurity; the basic protein labeled with fluorescamine could be detected even at the concentration of 1.6 μg. This represents only 1% of the fluorescamine-labeled peptide L used in this experiment. Therefore, if any impurity was present in peptide L, it was less than 1%.

Tryptic Hydrolysis of Native and Denatured Peptide L. When both native and denatured peptide L were subjected to tryptic hydrolysis there was no significant difference in the rate of hydrolysis. The denatured peptide L was hydrolyzed 10 and 14% faster at times 20 and 40 min, respectively, although both were completely hydrolyzed at about the same time (Figure 3).

Molecular Weight and Stokes Radius by Gel Filtration. A Stokes radius of 23 Å was obtained for peptide L when chromatographed on Sephadex G-100 and compared with standard proteins (Figure 4). The frictional ratio of the peptide L cal-

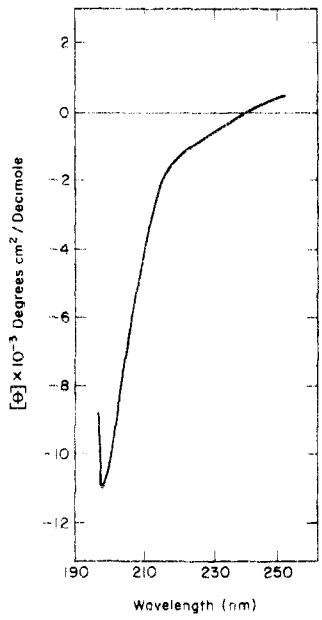

FIGURE 5: Circular dichroism spectra of 0.23 mg of peptide L in 0.1 M Na_2HPO_4-NaH_2PO_4, pH 7.0 at 20 °C.

TABLE I: Amino Acid Composition of Peptides L, D, and J.[a]

amino acid	residues per mole		
	peptide L	peptide D	peptide J
Lys	6.8 (7)	4.8 (5)	2.0 (2)
His	8.0 (8)	8.0 (8)	
Arg[b]	13.2 (13)	11.1 (11)	1.9 (2)
Asp[c]	7.8 (8)	8.1 (8)	
Thr	5.7 (6)	5.1 (5)	0.81 (1)
Ser[d]	12.8 (13)	8.0 (8)	6.4 (6)
Glu	7.1 (7)	5.1 (5)	1.9 (2)
Pro	10.2 (10)	9.1 (9)	1.0 (1)
Gly	13.1 (13)	12.7 (13)	
Ala	8.7 (9)	4.9 (5)	4.1 (4)
Cys			
Val	2.9 (3)	2.7 (3)	
Met	1.1 (1)		
Ile	2.0 (2)	1.9 (2)	
Leu	6.8 (7)	5.9 (6)	1.0 (1)
Tyr	2.2 (2)	1.0 (1)	0.9 (1)
Phe	5.8 (6)	5.8 (6)	
total residues	115	95	20

[a] The expected number of residues of each amino acid is shown in parentheses. [b] No separate peak of methylated arginine was obtained. From the number of arginine residues, it appears that methylated arginine was eluted at the position of arginine. [c] Aspargine and glutamine were obtained as aspartic acid and glutamic acid residues, respectively. [d] Homoserine appeared at the position of serine, while homoserine lactone could not be eluted in ligand exchange chromatography. The complete disappearance of methionine was taken as the evidence for complete cleavage of the peptide due to cyanogen bromide.

TABLE II: Encephalitogenic Activity in Rhesus Monkey of Peptides Derived from CNS Myelin Basic Protein.[a]

antigen	dose (mg)	no. of animals tested	onset of disease (weeks)	no. of animals with disease	
				clinical	histological
CNS myelin basic protein	5	3	2	3	3
peptide L (residues 1-115)	5	2	15	1	1
			5	1	1
peptide L (residues 1-115)	10	2	4	1	1
			15	1	1
peptide T (residues 116-170)	2	3	2	3	3
	5	2	2-3	2	2
peptide Y (residues 154-170)	0.5	3	2	2	3
peptide M (residues 154-167)	0.4	3	2	3	3
peptide J (residues 1-20)	2	1	no	0	0
	4	1	disease	0	0
peptide D (residues 21-115)	2.7	1	7	1	1

[a] Two monkeys were injected with 5 mg of peptide L each. One did not show signs of clinical disease until 15 weeks at which time an additional 5 mg of peptide L was given. The monkey died 11 days later. The other monkey stopped eating after 5 weeks; he became lethargic and blind and died after 8 weeks. Two other monkeys were injected with 10 mg of peptide L each. One began to show clinical signs after 4 weeks and died at 5 weeks. The other monkey was blind after 4 weeks but lived 15 weeks; additional peptide L (5 mg) was given and the monkey died in 11 days.

culated was 1.55. The succinylated peptide L had a Stokes radius of 24 Å and had a frictional ratio of 1.62. Under denaturing condition, no change in the molecular weight of peptide L was obtained.

Circular Dichroism. The CD spectra of peptide L shown in Figure 5 are typical of a protein existing predominantly as a random coil. When the method I of Greenfield & Steiner (1969) was applied, 7% α helix and 13% β structure were calculated.

Cyanogen Bromide Cleavage. The chromatography of the cyanogen bromide digest of peptide L on Sephadex G-75 column (2 × 39 cm) gave three peaks. The first two peaks were peptides D and J, respectively; the third peak contained salt. From 14 mg of peptide L used in this experiment, 8 mg of pure peptide D and 2 mg of pure peptide J were obtained. This amount of peptide J was not sufficient for further studies; therefore this peptide was obtained from cyanogen bromide cleavage of basic protein. The digest (200 mg) was chromatographed on a Sephadex G-75 column (4 × 53 cm). Three peaks were obtained; the middle peak contained peptide J. It was further purified by its rechromatography on a Sephadex G-75 column (2.5 × 60 cm).

Amino Acid Composition of Peptides. Table I shows amino acid composition of peptides L, D, and J, respectively. Absence of methionine in peptide D shows that it was free from peptide L and the cyanogen bromide cleavage of peptide L was complete.

Purity of the Peptides by Thin-Layer Chromatography. When peptides were tested for purity by thin-layer chromatography, peptide J (100 μg) and fluorescamine-labeled peptide J (10 μg) moved as single spots as detected by ninhydrin and UV lamp, respectively. Under the same conditions both labeled and unlabeled peptides L and D stayed at the origin. Fluorescamine-labeled peptide D was applied in sufficient amounts so that impurities could have been detected. Amino acid composition and the absence of methionine in peptide D confirmed its purity. It was interesting to note that the labeled peptide J (R_f = 0.41) moved faster than unlabeled peptide J (R_f = 0.19).

Yield of Peptide L. From 1 g of basic protein, 40 mg of pure peptide L was obtained.

Encephalitogenic Activity. The encephalitogenic activity of the peptides derived from bovine myelin basic protein in the Rhesus monkey is shown in Table II. Basic protein and peptides T, Y, and M were compared on a molar basis; these peptides have the same activity as the whole protein. Peptide L was given in two doses, 5 mg and 10 mg. At the 5-mg dose, it took between 5 to 15 weeks for clinical disease to be observed and at the 10 mg level, clinical signs were present after 4 to 5 weeks. Below 5 mg the disease could not be induced. Peptide D showed weak encephalitogenic activity since it took 7 weeks to induce disease, while peptide J was completely inactive in inducing clinical disease. It was interesting to note that the peptide L obtained from the first chromatography after desalting was active at a 3 mg concentration; however, this preparation showed a trace amount of basic protein when electrophoresed on $NaDodSO_4$-poly(acrylamide) above a concentration of 250 μg.

Discussion

Two peptides CDl and R, which comprise 77% of peptide L, have been shown to be encephalitogenic in the monkey (Brostoff et al., 1974; Kibler et al., 1972). However, Eylar et al. (1972) observed that peptide R is tenfold less active than the carboxyl terminal region of the basic protein. Such discrepancies arose in EAE studies because the peptides were not extensively purified and characterized. Eylar (1972) has pointed out that even a 5% contaminant can lead to misleading interpretations. If the peptides CDl and R are encephalitogenic, then peptide L should be very active in inducing the disease. To date peptide L has not been well characterized to answer this question (Burnett & Eylar, 1971). In the present studies we have obtained a highly purified preparation of peptide L, free from basic protein, using ion-exchange chromatography and repeated gel filtration. Our ion-exchange procedure differed from that of Burnett & Eylar (1971). The desalting step was very efficient in separating basic protein, peptide L, and peptide T from each other. Although this

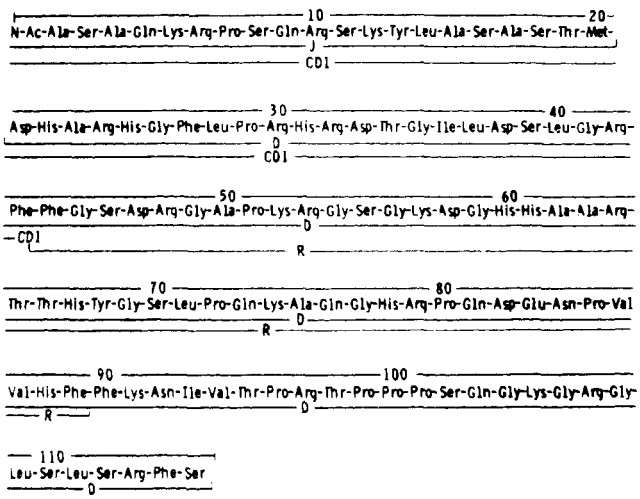

FIGURE 6: The amino acid sequence of peptide L, residues 1–115, of the bovine CNS myelin basic protein. The location of the region of this sequence represented by peptides J, D, CD1, and R is indicated.

chromatography separated peptide L from basic protein, this step had to be repeated several times to remove trace contamination of basic protein. To test the extent of basic protein present as an impurity, peptide L was labeled with fluorescamine (Udenfriend et al., 1972) and electrophoresed on $NaDodSO_4$–poly(acrylamide) gels. The amount applied was such that even 1% of the basic protein could have been detected. Absence of the band due to basic protein showed that our peptide L contained a maximum of less than 1% basic protein.

When this preparation of peptide L was injected into the Rhesus monkey, the induction of disease was delayed as compared with basic protein, peptide Y, and peptide M. In addition, the dose used was relatively high. With 5 mg, a weak disease was induced; and it took 5–15 weeks for clinical signs to appear. With 10 mg, disease could be induced in 4 weeks; however, on a molar basis this would suggest the presence of a weak disease-inducing site within peptide L. Below a 5 mg dose it was not possible to induce the disease. Two regions, found within the peptide L sequence, have been reported to be more active on a molar basis than peptide L (Figure 6). These peptides have been termed CD1 and peptide R (Brostoff et al., 1974; Eylar et al., 1972; Kibler et al., 1972). The CD1 peptide was obtained by Brostoff et al. (1974) by treatment of the basic protein with cathepsin D. The cathepsin D cleaved the Phe-Phe bond of the basic protein between residues 43 and 44 giving rise to two peptides, CD1 and CD2. The peptides were separated on Cellex-P chromatography which gave a profile showing poor resolution of CD1, CD2, and basic protein. Between CD1 and CD2 there was an unresolved peak comprised of 768 mL which presumably contained basic protein. The pure CD1 peptide reported by these workers was a fraction taken from the Cellex-P profile which was not further purified. All these facts reviewed together strongly suggest that CD1 was contaminated with basic protein. When 5.0 mg of this peptide was injected into Rhesus monkeys, it induced EAE within 16–18 days. Similar arguments can be raised about peptide R isolated by Eylar et al. (1971a) and Kibler et al. (1972). Kibler et al. (1972) obtained a 45-residue peptide from Rhesus monkey brain with the same sequence as peptide R reported by Eylar and his co-workers (1971a). These workers found this peptide to be extremely encephalitogenic in the monkey. It induced disease in the monkey in 3–4 weeks with a dose of 1.4 mg. The peptide was isolated from Sephadex G-50 and carboxymethylcellulose chromatography. The isolated fraction, an unresolved peak, was not further purified. The purity of the peptides was confirmed by a peptide map. Since the amount used in this analysis has not been mentioned, a 5–10% contamination can easily escape detection. The peptide was eluted last on a carboxymethylcellulose column, a typical characteristic of the basic protein on cation exchangers. The peptide R isolated by Eylar et al. (1971a) eluted as an unsymmetrical peak on a column composed of Sephadex G-50 and Bio-Rad P-10.

Since peptide L contained a single methionine residue, it was possible to split it further with cyanogen bromide. Two peptides, peptide J (residues 1–20) and peptide D (21–115), were obtained. Both were isolated in a purified state. When injected into the monkey, only peptide D induced disease similar to that induced by peptide L; peptide J was never shown to induce EAE over a wide dosage range. Therefore, the weak site of peptide L must be on the peptide D region.

We had shown previously that the region of the basic protein active in the monkey is a 14-residue peptide from the carboxyl end (Karkhanis et al., 1975). In order to delineate the region of this peptide responsible for disease induction, we cleaved this peptide with mild acid to give two peptides. None of these peptides were active suggesting that the region around aspartic acid is very critical, this region being the Gly-Arg-Asp-Ser. It is interesting to note that this sequence is reversed in peptide L as Ser-Asp-Arg-Gly, residues 45–48 (Figure 6). One could speculate that this is the region which may be contributing to the weak encephalitogenic activity of peptide L.

The possibility that both CD1 and R regions in peptide L are buried to express weak activity was also investigated. When subjected to trypsin treatment, very little change in the rate of hydrolysis between native and denatured peptide L was observed. The Stokes radius of peptide L was 23 Å with a frictional ratio of 1.55 suggesting a high degree of asymmetry. Chromatography in 8 M urea in mercaptoethanol at room temperature showed no change in these two parameters. The succinylated peptide L had a Stokes radius of 24 Å with a frictional ratio of 1.62. The small change in these two parameters could be due to the slightly higher molecular weight of the peptide since 97% of the peptide L was succinylated. The CD spectra of peptide L are typical of a random coil and therefore its weak encephalitogenic activity is not due to the unavailability of CD1 and R regions. The previous report on the activity of these peptides must be due to the presence of basic protein or its carboxyl-terminal peptide as a contaminant.

From the results obtained we conclude that there exists a weak disease-inducing site on peptide L from residues 21–115. We do not think that this site participates in inducing the disease when whole basic protein is injected into the monkey, since on the molar basis, this site is much less active than peptide M. This reinforces the concept that peptide L has a different role in disease induction, since several events are involved in the production of experimental allergic encephalomyelitis (Alvord, 1970). With highly purified preparation of peptide L, we now have an opportunity to elucidate this role.

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