IMMUNE RESPONSE OF LEWIS RATS TO PEPTIDE C1 (RESIDUES 68–88) OF GUINEA PIG AND RAT MYELIN BASIC PROTEINS*

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In the Lewis (Le)[1] rat, guinea pig (GP) myelin basic protein (BP) is by far the most encephalitogenic species of BP that has been tested (1, 2). Recently, Chou et al. (3) have shown that a 19-amino acid fragment of GP BP, peptide C1 (residues 68–88), is as active in the Le rat as the parent protein. The amino acid sequence of this peptide is as follows:

68 79 88
NH_2-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe-COOH

(4, 5).[2] Although the rat C1 peptide differs from the GP C1 peptide only by a threonine for serine substitution at position 79 (1, 6), rat BP is appreciably less active than GP BP in the Le rat. We have compared, therefore, the immune response of the Le rat to the two C1 peptides; including their encephalitogenic activities, capacity to stimulate incorporation of [^{3}H]thymidine by lymph node cells (LNC), and their capacity to induce the production of antibody.

Materials and Methods

Experimental Design. Female Le rats were challenged with each of the two C1 peptides, respectively, in complete Freund's adjuvant (CFA). Assessment of clinical signs was made daily and some animals were killed for histology 1–2 days after the peak of clinical signs. Other animals were sacrificed 8–9 days after challenge and the in vitro response of their LNC to the two C1 peptides was determined by the incorporation of [^{3}H]thymidine. Other groups of rats were bled from the tail at varying intervals after challenge through day 35; they were then boostered intraperitoneally (i.p.) with the respective C1 in incomplete Freund's adjuvant (IFA)

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[1] *Abbreviations used in this paper:* BMB, *Mycobacterium butyricum;* BP, myelin basic protein; Cl, residues 68–88 of BP; CFA, complete Freund's adjuvant; CM-52, carboxymethylcellulose; EAE, experimental allergic encephalomyelitis; GP, guinea pig; IFA, incomplete Freund's adjuvant; i.p., intraperitoneally; Le, Lewis; LNC, lymph node cells; P1, residues 43–88 of BP; SI, stimulation index.

[2] The numbering system for bovine BP (4) is used throughout since the complete sequence of GP BP is not known. It should be noted that in GP Cl residues 76–77 are deleted.

and exsanguinated 13 days later. Their sera were assayed for antibody by the sodium sulfate method of radioimmunoassay (7).

Induction of Experimental Allergic Encephalomyelitis (EAE). Le rats 3–6 mo of age were obtained from Microbiological Associates, Bethesda, Md. The appropriate amount of the respective C1 peptide was dissolved in Ringer's solution and homogenized with an equal volume of incomplete adjuvant (Difco Laboratories, Detroit, Mich.) to which crushed, killed *Mycobacterium butyricum* (BMB) had been added. Rats were lightly anesthetized with ether and injected intradermally in each of the hind footpads with 0.05 ml of the emulsion, i.e., a total of 0.1 ml/rat. The dose of BMB per rat was uniformly 250 μg.

Assessment of EAE. Clinical signs were graded on a scale of 1–6 and histological changes on a scale of 1–8, according to criteria previously described (1, 3).

Preparation of Fragment 43–88 (P1) and Peptide 68–88 (C1). GP and Wistar rat brains were from Pel-Freez Bio-Animals, Inc., Rogers, Ark. BP was prepared from these tissues by previously published methods (8). An acid extract of chloroform/methanol delipidated brain was chromatographed at 25°C on carboxymethylcellulose (CM-52, H. Reeve Angel & Co., Inc., Clifton, N. J.) using a linear NaCl gradient in 0.08 M glycine buffer, pH 10.4, 2 M urea. The last peak to elute from the column, component 1 of BP, is considered the native unmodified form of BP and was the material used in these studies. By this procedure, the smaller of the two rat basic proteins was obtained.

BP was subjected to limited pepsin (Worthington Biochemical Corp., Freehold, N. J.) digestion (9) at pH 3.0 for 1 h at 40°C, and fragment P1 isolated from the digest as previously described (8). The preparation of P1 was contaminated with ≅30% fragment 37–88.

Fragment P1 was subjected to limited chymotrypsin digestion (3) which results in selective cleavage at tyrosine 67 and phenylalanine 42. Peptide C1 was separated from peptides 37–42 and 43–67 by chromatography on CM-52 using a linear NaCl gradient in 0.02 M ammonium acetate, pH 4.0, followed by Sephadex G-50 gel filtration.

Lymphocyte Stimulation. The response of LNC was determined by modification of a previously published method (10). Rats were killed and their popliteal and inguinal lymph nodes removed immediately under sterile conditions and teased apart. LNC obtained in this manner were suspended in a medium containing 15% fresh Le rat serum at a concentration of 2.5×10^6 cells/ml and cultured in the presence of the appropriate concentration of antigen. Quadruplicate cultures were established for each variable. Incorporation of [Methyl-^{3}H]thymidine was determined after 24 and 48 hr incubation. The stimulation index (SI) was calculated by dividing the mean counts per minute (cpm) of stimulated cultures by the mean cpm of unstimulated cultures. In preliminary experiments, it was determined that the optimal day after challenge to obtain LNC was one day before the onset of clinical signs.

Radioimmunoassay (RIA). GP P1 and rat P1 (1.0 mg) were labeled with 1.0 mCi of carrier-free ^{125}I-Na, according to the lactoperoxidase method (11). The labeled fragment was separated from free iodide on a Sephadex G-10 column (30 × 0.7 cm inside diameter) in 0.01 N HCl. Fractions were read at 278 nm and the peak tube, which contained the majority of the labeled fragment, used in RIA. The concentration of P1 in the peak tube was estimated from a standard curve constructed with unlabeled P1. The efficiency of labeling was 85–90%.

Sera were assayed for antibody by the technique of Day and Pitts (7). 210 μl of normal rabbit serum (1:3 in 0.01 M phosphate-buffered saline) was mixed with 20 μl of rat antiserum and 20 μl of ^{125}I-P1 for a final concentration of 1×10^{-7} M. After 5 min at 25°C, 500 μl of 1.9 M Na_2SO_4 at 30°C was added. After 1 h the tubes were centrifuged for 30 min, the supernate removed and discarded, and the precipitates washed three times each with 500 μl 1.27 M Na_2SO_4. The final precipitates were dissolved in 0.01 M phosphate-buffered saline and transferred to counting tubes. Percent binding was based on total count tubes (50,000–100,000 cpm) since the percent ^{125}I-P1 left in the original tubes, after the precipitates had been transferred, was <1.0% of the total. Percent binding was converted to picomol antigen bound/milliliter of serum. All sera were tested in triplicate and agreement was within 5% of the mean. Normal and adjuvant control rat sera bound <10 pmol P1/ml.

Results

Encephalitogenic Activity. The encephalitogenic activity of the two C1 peptides is shown in Table I. Four quantitative criteria of encephalitogenic

TABLE I
Encephalitogenic Activity of C1 (68-88)

Challenge		EAE			
Dose (10^{-4} μmol)	Source	N*	Day of onset	Maximal clinical grade‡	Histology grades§
250‖	GP	4/4	9.4	5.5	3.5
250	Rat	4/4	10.8	4.8	3.0
25	GP	4/4	9.2	5.4	3.1
25	Rat	4/4	11.0	4.5	3.2
15	Rat	3/4	11.6	4.3	3.2
7.5	Rat	1/4	(12)	(4.5)	3.3
2.5	GP	4/4	10.3	5.8	3.3
2.5	Rat	0/4	–	–	
0.25	GP	4/4	12.2	5.0	2.5
0.15	GP	4/4	13.2	4.8	3.2
0.075	GP	2/4	14.0	5.0	2.0
0.025	GP	0/4	–	–	

* Ratio number rats with EAE to number challenged. Each rat received 250 μg BMB.
‡ Scale of 1-6; 1 – limp tail, 6 moribund.
§ Rats killed 1-2 days after peak of clinical disease. Scale of 0-8, 1 – minimal lesions, 8 extensive in all areas of central nervous system.
‖ 250 × 10^{-4} μmol ≅ 50 μg.

potency were used; number of animals which developed EAE out of the number challenged (N), day of onset, and the clinical and histological grades. The data shown in Table I are representative of a number of experiments. EAE in the Le rat is a highly reproducible phenomenon and the results among different experiments were almost identical. The minimal encephalitogenic dose of GP C1 was 0.075 × 10^{-4} μmol (15 ng); for rat C1 the minimal dose was 7.5 × 10^{-4} μmol (1,500 ng). Neither peptide produced disease in all animals at these dose levels, but GP C1 at 0.15 (30 ng) and rat C1 at 15 (3,000 ng) × 10^{-4} μmol had good activity as judged by the various quantitative parameters.

Lymphocyte Stimulation. LNC were harvested from rats (six per group) 8 days after challenge with GP C1 and 9 days after challenge with rat C1. LNC from both groups of rats were incubated in the presence of 25 × 10^{-4} μmol/ml of GP and rat C1. As shown in Table II, LNC from rats challenged with 25 × 10^{-4} μmol of GP C1 gave a mean SI of 4.7 (range of 4.0–5.4) in the presence of GP C1 and a mean SI of 2.1 (range of 2.0–2.2) in the presence of rat C1. LNC from rats challenged with 25 × 10^{-4} μmol of rat C1 gave SI which were of borderline significance (not shown in Table II). LNC from rats receiving 250 × 10^{-4} μmol of rat C1 (Table II) gave a mean SI of 2.6 (range of 2.4–3.0) in the presence of GP C1 and a mean SI of 2.0 (range 1.9–2.1) in the presence of rat C1. These experiments have been repeated with similar results.

Fig. 1 shows typical in vitro dose-response curves of LNC from rats challenged with the two peptides. Fig. 1A gives the response of LNC pooled from two rats

TABLE II
*In Vitro LNC Responses**

Stimulant (10^{-4} μmol/ml)		SI‡ of LNC from rats challenged with:	
		GP Cl (25×10^{-4} μmol)	Rat Cl (250×10^{-4} μmol)
GP Cl	25	4.7 (4.0–5.4)	2.6 (2.4–3.0)
Rat Cl	25	2.1 (2.0–2.2)	2.0 (1.9–2.1)

* [^{3}H]Thymidine incorporation of LNC from groups of six rats each challenged with the respective Cl. Cells were harvested 8 (GP) and 9 (rat) days after challenge.

‡ SI-mean cpm of stimulated cultures/mean cpm of unstimulated cultures (quadruplicates). An SI > 1.5 is significant.

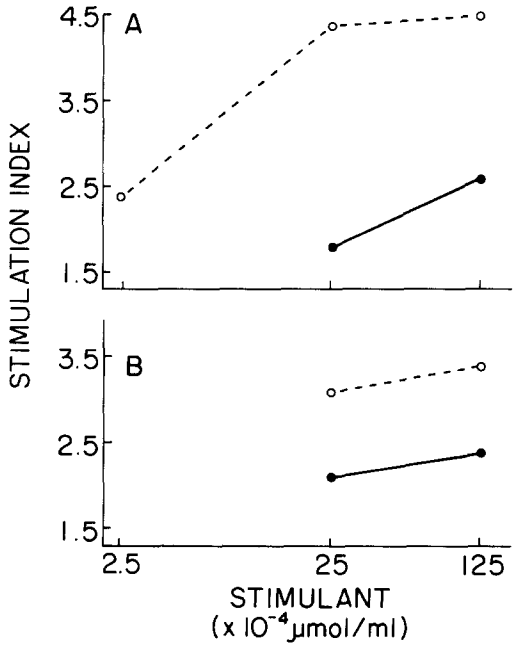

FIG. 1. In vitro stimulation of LNC from rats 8 days after challenge with 25×10^{-4} μmol GP Cl (A) and 9 days after challenge with 250×10^{-4} μmol rat Cl (B). Cells were incubated with $2.5–125 \times 10^{-4}$ μmol of GP BP (○) and rat BP (●), respectively, per ml of culture medium. Stimulation indices not shown at 2.5×10^{-4} μmol/ml were below the level of significance; i.e., 1.5.

8 days after challenge with 25×10^{-4} μmol of GP Cl and exposed in vitro to concentrations of GP BP and rat BP from 2.5 to 125×10^{-4} μmol/ml. The parent proteins were used for in vitro stimulation in these particular experiments, rather than the respective Cl peptide, since the results are comparable. Fig. 1B shows data from rats challenged with 250×10^{-4} μmol of rat Cl. SI below 1.5 have been omitted from Fig. 1 since such values are below the level of significance. Comparison of the two curves in Fig. 1A indicates that 2.5 ×

10^{-4} μmol/ml of GP BP and 125 × 10^{-4} μmol/ml of rat BP gave roughly comparable degrees of stimulation. In general, the in vitro response to the two BP was lower in Fig. 1B, but again the response to GP BP was greater.

Antibody Response. Groups of four rats each were challenged with GP and rat C1 at both 25 and 250 × 10^{-4} μmol in CFA in the same manner as that described for induction of EAE. A control group of four rats received CFA only. Sera were obtained from tail bleeds 9, 14, 23, and 35 days after challenge. All four experimental groups were boostered i.p. on day 38 with the respective C1 peptide in IFA at the same dose as that used in challenge and the rats were exsanguinated 13 days later. All sera were tested for their capacity to bind ^{125}I-P1. P1, which contains tyrosine at residue 67, was used instead of C1, which contains histidine at position 87, since it was found that iodination of the latter residue interfered with the binding of certain anti-C1 sera.

Fig. 2 shows the mean response of each group. In rats challenged with 25 × 10^{-4} μmol of antigen, low levels of anti-GP C1 were first detected on day 23, followed by a modest increase on day 35. No appreciable levels of anti-rat C1 were detected during this time. 13 days after an i.p. boost with the respective C1 peptide, substantial levels of anti-GP C1 were achieved but there was no response to the boost with rat C1. At 250 × 10^{-4} μmol there was a substantial and similar antibody response to the two peptides. The day-51 sera were tested for cross-reactivity and the results are shown in Table III. Anti-GP C1 and anti-rat C1 reacted equally with the two labeled P1 fragments, suggesting that the two sets of sera recognized an antigenic determinant common to both C1 peptides. These studies were carried out with 1:20 dilutions of the antisera where binding was in the range of 50%.

Discussion

In the Le rat a heterologous peptide, GP C1, is substantially more encephalitogenic than the homologous peptide. This presents an interesting paradox since T cells, which are the mediators of EAE (12), must presumably react with rat C1 within the myelin sheaths of the central nervous system in order to initiate the lesions of EAE, regardless of which peptide is used in challenge.

The difference in encephalitogenecity of GP and rat C1 is best explained by assuming that both peptides stimulate a population of T cells which have a greater affinity for serine than for threonine at position 79; so that at comparable, submaximal doses GP C1 stimulates a greater number of cells than does rat C1. The results of the in vitro lymphocyte stimulation assay presented in Table II and Fig. 1, are consistent with this proposal. These data show that LNC from rats challenged with either peptide were stimulated in vitro by both peptides, but in each instance the magnitude of the response was greater to GP C1. An alternative explanation; i.e. that blocking antibody accounts for the reduced encephalitogenecity of rat C1, is not supported by the data shown in Fig. 2, since rat C1 was a less, rather than a more, potent inducer of antibody than was GP C1. Other possibilities which seem less likely are that threonine 79 in rat C1, in addition to being a part of the EAE determinant, is part of a suppressor site or, alternatively, that serine 79 in GP C1 is also part of a helper determinant for the induction of EAE.

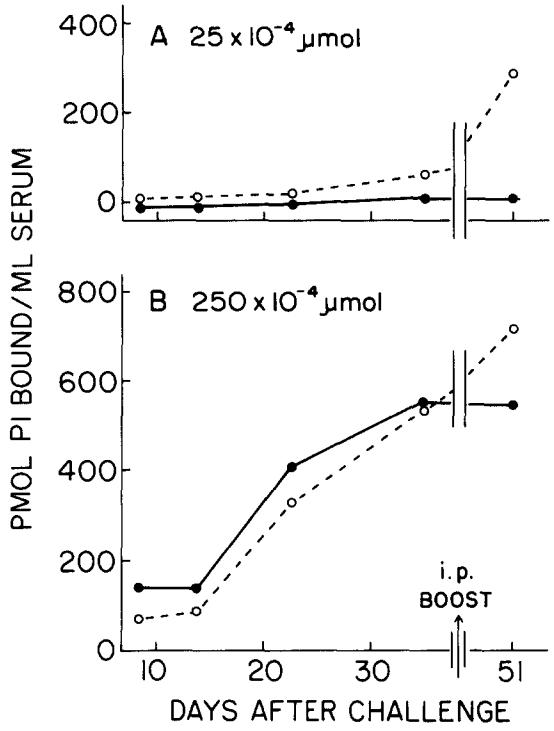

FIG. 2. Mean antibody response to GP (○) and rat (●) Cl of rats (four per group) challenged with 25 (A) and 250 (B) × 10^{-4} µmol of each peptide in CFA (250 µg BMB/rat). The animals were bled by tail at the intervals shown and boostered i.p. on day 38 with the respective Cl in IFA at the same dose as that used in challenge. They were exsanguinated on day 51. Antisera (diluted 1-10) were tested against the respective ^{125}I-P1 (1 × 10^{-7} M) by the sodium sulfate method of Day and Pitts (7).

TABLE III
*Cross-Reactivity of Anti-Cl Sera**

Serum	Rat	^{125}I-GP Pl	^{125}I-Rat Pl
		pmol Pl bound/ml serum‡	
Anti-GP Cl	1	1,200	1,020
	2	1,280	1,240
	3	840	840
	4	860	960
Avg.		1,045	1,015
Anti-Rat Cl	1	940	800
	2	1,080	940
	3	620	580
	4	540	580
Avg.		795	725

* Antisera are from rats challenged and boostered with 250 × 10^{-4} µmol of the respective Cl (Fig. 2, Day-51 sera).

‡ Sera diluted 1:20 (≅50% binding) and tested against 1 × 10^{-7} M Pl.

In this system the in vitro LNC stimulation assay appears to measure a T-cell response. Were B cells primarily involved, we would not have expected the marked difference in the response to the two peptides. The data in Table III indicate that antibodies raised against either peptide showed the same specificity for GP and rat C1, and it is generally assumed that receptors on B cells have the same specificity as the antibodies they produce. Thus it is more likely that the in vitro LNC response to the two peptides, which closely paralleled their respective encephalitogenic potencies, reflects activity of T cells, which also have a greater affinity for serine than threonine at position 79. Our prior studies (3) have established that residue 79 is part of the EAE determinant; but whether the determinant responsible for in vitro LNC stimulation is identical, or whether the same population of T cells are involved in the two responses, will require further study. The close relationship of the two activities demonstrated in this study are at variance with the report by Spitler et al. (13) which suggested that in guinea pigs, it was possible to dissociate production of EAE from cell-mediated immunity as measured by the in vitro lymphocyte stimulation test. In their studies, a synthetic peptide of the active tryptophan (residue 115) region of bovine BP was found to induce EAE in guinea pigs but failed to induce cell-mediated immunity.

GP C1 also had a significantly greater capacity to stimulate the cells involved in antibody production. This difference was most apparent after the i.p. boost in those groups of rats which received 25×10^{-4} μmol of the respective peptide (Fig. 2A). The antibody produced by either peptide, however, reacted equally well with both peptides in the method of RIA used in these studies (Table III). Fritz et al.[3] have recently shown that a high proportion of Le rat anti-GP C1 sera bind, or are competitively inhibited by peptide 79–88 to the same extent as peptide C1; but peptide 79–88 alone, at a dose of 250×10^{-4} μmol, does not produce antibody even after an i.p. boost. This suggests that peptide 79–88 contains an antigenic determinant which reacts with antibody but which requires a T-helper determinant for antibody production. Therefore, despite the similarity in specificity of antibody produced by the two peptides, GP C1 produces more antibody than rat C1 at the low dose because it stimulates a greater number of helper T cells. To decide whether or not the determinant for helper T-cell function is the same as the EAE determinant will require more precise delineation of the two sites.

Single amino acid substitutions, such as exist between GP and rat C1, are known to have profound effects on the immunological activity of proteins. Allotype differences among the immunoglobulins, due to single isopolar substitutions, show up readily when these proteins are compared by antibodies produced to them. Recently, Barcinski and Rosenthal (14) have shown that a serine (beef) interchange with glycine (sheep) in the alpha-loop region of the A chain of insulin caused a significant difference in the in vitro proliferative response of strain 2 GP. Other isopolar substitutions within this region were found to have an effect on T-helper function.

This study illustrates the manner in which well-defined antigenic prepara-

[3] Fritz et al. unpublished observations.

tions can be used to study immunological phenomena. A 19-amino acid peptide has been shown to induce EAE, produce circulating antibodies, stimulate LNC in vitro, and perhaps, stimulate helper T cells in the elaboration of antibodies. A more precise delineation of the determinants and of the LNC populations and/or subpopulations responsible for these various functions may answer some of the questions raised by this study.

Summary

Peptide C1 (residues 68–88) from GP and rat BP differ by a single amino acid interchange at residue 79. This residue is serine in GP C1 and threonine in rat C1. GP C1 was encephalitogenic in Le rats at doses as low as 15 ng. Rat C1 was encephalitogenic at doses of 1,500 ng or greater. LNC from rats challenged with 25×10^{-4} μmol of GP C1 and 250×10^{-4} μmol of rat C1 showed a proliferative response in vitro to both peptides, but in each instance the magnitude of the response was greater to the GP peptide. GP C1 also induced higher levels of circulating antibodies at 25×10^{-4} μmol, but the specificity of antibodies produced by the two peptides was the same. These results have been interpreted as indicating that the presence of serine at position 79 in GP C1 results in the stimulation of greater numbers of T cells involved in (*a*) the induction of EAE, (*b*) the in vitro proliferative response and (*c*) helper function in antibody production.

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