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Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide*

Three random basic copolymers of amino acids were tested for their effect on experimental allergic encephalomyelitis (EAE). One of these copolymers denoted as Cop 1, composed of alanine, glutamic acid, lysine and tyrosine, with a molecular weight of 23 000, showed a marked suppressive effect on the disease. The intravenous administration of Cop 1 in physiological saline, as late as 5 days following the challenge with the disease-inducing dose of the basic encephalitogenic protein, reduced the clinical incidence of EAE from 64 % in the control group to 22 %; the histological lesions were also decreased both in prevalence and in severity. The suppressive effect on the disease attained by the synthetic copolymer is of the same order of magnitude as that previously reported for the basic encephalitogen.

The effect of the copolymers appears to be specific, since neither an acidic amino acid copolymer, nor unrelated basic proteins, had any protective action. On the other hand, a second batch of Cop 1 showed activity identical to that of the first batch. The potential applicability of this non-encephalitogenic and non-immunosuppressive material is discussed.

1. Introduction

Experimental allergic encephalomyelitis (EAE) is an acute neurological autoimmune disease induced in laboratory animals by a single injection of brain or spinal cord tissue in complete Freund's adjuvant [1, 2]. The disease is characterized clinically by paralysis of the hind legs and histologically by perivascular infiltration in the brain tissue [2].

- * Supported in part by the Freudenberg Foundation.
+ Recipient of a long-term EMBO fellowship.

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Abbreviations: EAE: Experimental allergic encephalomyelitis
BE: Basic encephalitogen Cop 1: Copolymer 1 Cop 2: Copolymer 2
Cop 3: Copolymer 3 AGI: An acidic copolymer of alanine, glutamic acid and tyrosine LDH: Lactic dehydrogenase ICFA: Incomplete Freund's adjuvant CFA: Complete Freund's adjuvant.

The active encephalitogenic component in the nervous tissue is a basic low molecular weight protein which is a constituent of myelin, and has been purified by various procedures [3-7]. The basic encephalitogen (BE) is active in extremely low doses. Thus, 1-10 µg injected per guinea pig will induce the clinical symptoms, and a dose as low as 0.1 µg per guinea pig will bring about histological changes [6]. In recent years a number of reports appeared, demonstrating that high doses of the same protein injected by a different route can either suppress or prevent the induction of EAE [8-10]. Recently it was found that not only the basic encephalitogen was effective in such treatment, but other non-encephalitogenic basic neural proteins had a similar protective effect when injected in incomplete Freund's adjuvant [10]. In contrast, basic proteins of non-neural origin, such as histones, did not provide any protection.

In view of these results we have synthesized three random copolymers, of amino acid compositions approaching, to a

TEITELBAUM D., MESHORER A., HIRSHFELD T., ARNON R. & SELA M.
Suppression of experimental allergic encephalomyelitis by a synthetic
polypeptide.

Eur. J. Immunol. 1971, 1, 242-248

certain extent, that of the natural encephalitogen, and possessing a positive net electrical charge. The copolymers were tested for possible encephalitogenic activity, as well as for their capacity to suppress the disease. None of the copolymers had any encephalitogenic activity. On the other hand, these synthetic models were effective in suppressing EAE. The present report describes the effectiveness and specificity of this protective action.

2. Materials

2.1. Animals

D. H. Albino guinea pigs weighing 300–400 g were used in all the experiments, unless otherwise stated.

2.2. Proteins

The basic encephalitogenic protein (BE) was prepared from bovine spinal cord, as described previously [7], by column chromatography on SE-Sephadex (Pharmacia, Uppsala). The amino acid composition of this protein [7] is: Lys₅₁₂His₈Arg₁₆Asp₁₀Thr₄Ser₁₄Glu₁₀Pro₁₁Gly₂₁Ala₁₂Val₂Met₁Leu₃Leu₉Tyr₃Phe₇Trp₁, with a calculated molecular weight of approximately 16 000. Hen egg-white lysozyme (2 x crystallized) was obtained from Worthington Biochemicals, N. J. Bovine pancreatic ribonuclease (RNase type I–A, 5 x crystallized), and cytochrome c (from horse heart, type II) were from Sigma Chemical Co.

2.3. Copolymers

Four different random copolymers of amino acids were used in this study. Three were rich in basic amino acids, whereas the fourth one was an acidic copolymer. They were prepared from the N-carboxyanhydrides of the respective amino acids according to Kaichalski and Sela [11].

2.3.1. Copolymer 1

Cop 1 was prepared from the N-carboxyanhydrides of tyrosine [12], alanine [13], γ -benzyl glutamate [14], and ϵ , N-trifluoroacetyllysine [15] (Table 1). The polymerization reaction was carried out at room temperature in anhydrous dioxane with diethylamine as initiator. The deblocking of the γ -carboxyl groups of the glutamic acid was carried out with hydrogen bromide in glacial acetic acid [16], and was followed by the removal of the trifluoroacetyl groups from the lysine residues by 1 M piperidine [17]. A second batch of this polymer was prepared in an identical manner. The molecular weight and amino acid composition of these polymers are listed in Table 1.

2.3.2. Copolymer 2

Cop 2 was prepared from the N-carboxyanhydrides of the following amino acids (Table 2): ϵ , N-trifluoroacetyllysine, N-benzylhistidine [18], O-benzylserine [19], γ -benzyl glutamate, proline [12], glycine [20] and tyrosine, by polymerization in dioxane (dimethylamine as initiator). In the first deblocking stage the benzyl groups were removed by hydrogen bromide in glacial acetic acid [16] to liberate the carboxyl groups of glutamic acid and the hydroxyl groups of serine. In the second stage the benzyl groups were removed from the histidine residues by hydrogenation with palladium as catalyst. The trifluoroacetyl groups were removed from the lysine residues by 1 M piperidine.

Table 1. Composition of copolymer 1

Amino acid	N-Carboxyanhydride derivative used for reaction	Amount used in the reaction		Molar ratio of amino acid in copolymer	
		g	mM	Batch I	Batch II
Alanine	Alanine	8.6	75	6.0	6.7
Glutamic acid	γ -Benzyl glutamate	6	23	1.9	2.1
Lysine	ϵ , N-Trifluoroacetyllysine	14	52	4.7	4.2
Tyrosine	Tyrosine	3	14	1.0	1.0
Molecular weight				23 100	22 800

Table 2. Composition of copolymer 2

Amino acid	N-Carboxyanhydride derivative used for reaction	Amount used in the reaction		Molar ratio of amino acid in copolymer
		g	mM	
Glutamic acid	γ -Benzyl glutamate	7.9	30	2.0
Glycine	Glycine	4.5	22	1.4
Histidine	N-Benzylhistidine	6.7	22	0.4
Lysine	ϵ , N-Trifluoroacetyllysine	10.1	37	2.8
Proline	Proline	2.1	15	0.4
Serine	O-Benzylserine	4.9	22	1.1
Tyrosine	Tyrosine	3.0	14	1.0
Molecular weight				4000

Table 3. Composition of copolymer 3

Amino acid	N-Carboxyanhydride derivative used for reaction	Amount used in the reaction		Molar ratio of amino acid in copolymer
		g	mM	
Alanine	Alanine	3.5	30	4.0
Arginine	δ , N-Trifluoroacetyl ornithine	7.5	29	1.0
Aspartic acid	β -Benzyl aspartate	5	20	2.7
Glutamic acid	γ -Benzyl glutamate	5.2	20	3.2
Glycine	Glycine	6	59	6.5
Histidine	N-Benzylhistidine	9.2	30	1.7
Leucine	Leucine	1.6	10	1.0
Lysine	Carbobenzyloxyllysine	6	20	3.8
Phenylalanine	Phenylalanine	1.9	9	1.2
Proline	Proline	2.8	20	1.8
Serine	O-Benzylserine	6.6	30	3.9
Tyrosine	Tyrosine	2.8	14	1.0
Molecular weight				5000

2.3.3. Copolymer 3

Cop 3 was prepared by the random polymerization in dioxane of the *N*-carboxyanhydrides of the following amino acids: alanine, *N*-trifluoroacetylornithine [21], β -benzyl aspartate [22], γ -benzyl glutamate, glycine, *N*-benzylhistidine, leucine [23], ϵ -carbobenzoxylysine [11], phenylalanine [11], proline, *O*-benzylserine and tyrosine (Table 3). The first deblocking stage was treatment with 1 M piperidine for the removal of the trifluoroacetyl groups from the δ -amino groups of the ornithine residues. They were subsequently guanidinated with 1-guanyl-3,5-dimethylpyrazol nitrate [21] to form arginine residues. The second deblocking stage was treatment with hydrogen bromide in glacial acetic acid. This liberated the carboxyl groups of glutamic and aspartic acids, the hydroxyl groups of serine and the amino groups of lysine. The benzyl groups of the histidine were then removed by hydrogenation.

The amino acid composition and molecular weights of copolymers 2 and 3 are listed in Tables 2 and 3, respectively.

The acidic copolymer, poly(Ala³³Glu³⁷Tyr³⁰) [24], abbreviated as AGT, was a gift from Dr. Sara Fuchs. It had an average molecular weight of 17 000.

3. Methods

3.1. Induction of EAE

Guinea pigs were injected with 10 μ g of the purified BE in complete Freund's adjuvant into the footpads of the two hind legs. They were then observed daily for loss in weight, and for the appearance of clinical symptoms of the disease, as reflected by paralysis of the hind legs.

3.2. Delayed hypersensitivity reactions [25]

The skin test reactions were carried out on the 10th or the 11th day after the challenge injection. The basic encephalogen (20 μ g in 0.1 ml saline) was injected intradermally into each of the guinea pigs. The appearance of erythema at the spot of the injection was observed 24 h later, and the extent of the delayed hypersensitivity was quantitated by measuring the diameter of the skin reaction. Only reactions of above 5 mm diameter were considered positive.

3.3. Determination of lactic dehydrogenase (LDH) activity

The level of LDH in the sera of the guinea pigs was assayed spectrophotometrically by the rate of oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) after adding sodium pyruvate to the serum, according to Schwartz and Bodansky [26]. The determinations were carried out on sera from bleedings at days 4, 11 and 14 after the induction of EAE. A rise in value of LDH was observed only in samples from the 14th day.

3.4. Histological tests

The animals were sacrificed after 4 weeks by means of nembutal. The brain was taken out in its entirety after the skull had been sawn. The *medulla oblongata* was also removed till the atlas. After fixation of the brain in formalin for a period of 24 h a median section was taken 3 mm from the center line (along the *sulcus lateralis*). A whole median section of

following areas: a cross-section along the line of the *vermis*, the *corpus mamillare*, the *tuberculum olfactorium* and of the *medulla oblongata*. The sections were stained with Luxol-fast-blue [27] with counter stain "kern echt rot".

All histological examinations were carried out under code. The histopathological changes which we observed were: in mild cases (+) a small percentage of blood vessels showed a slight perivascular infiltration with mononuclear cells (Consisting mainly of small lymphocytes and very few granulocytes (Fig. 1 b). The perivascularitis could be observed in different parts of the brain without any predominating area. Figure 1 a shows, for comparison, a normal blood vessel in the control brain tissue.

In more advanced cases (++) the histological changes were much more obvious in comparison to the former group, namely, a higher percentage of the blood vessels were affected and the infiltrated area around them was thicker.

In severe cases (+++) the perivascularitis was very massive (Fig. 1 c) together with periventriculitis (Fig. 1 d). The ventricles were surrounded by inflammatory cells, mainly with small lymphocytes and a few macrophages. In many places in the severe cases, groups of lymphocytes were seen in the brain parenchyma, not being a part of the immediate perivascular or periventricular zone (Fig. 1 e).

As mentioned, we used the Luxol-fast-blue dye to stain myelin, but no stage of demyelination appeared.

3.5. Prevention of EAE

Each animal received 8 repeated intradermal injections of 100 μ g of the respective polymer in incomplete Freund's adjuvant (ICFA). The material was injected over the back and sternum of the guinea pigs twice a week for four weeks [10]. Three days after the last injection the animals were challenged with 10 μ g of BE in CFA into the two hind footpads.

3.6. Suppression of EAE

After the initial challenge with 10 μ g of BE, two methods of suppression were tried: (1) each guinea pig received 6 intradermal injections of 100 μ g each of the respective polymer in incomplete Freund's adjuvant. The injections were given over the back and sternum of the guinea pig, twice a week for three weeks starting two days after the initial challenge [10]. (2) Each guinea pig received three intravenous injections of 1 mg each of the respective polymer or protein in saline. The injections were given according to schedules mentioned in the text, which differed in the various experiments.

3.7. Molecular weight determinants

The average molecular weights of the polymers were determined, in a Spinco model E ultracentrifuge, from sedimentation and diffusion measurements, as described earlier [24], and by the approach to equilibrium technique of Yphantis [29].

3.8. Amino acid analyses

Were carried out in a Beckman-Spinco automatic amino acid analyzer Model 120 B.

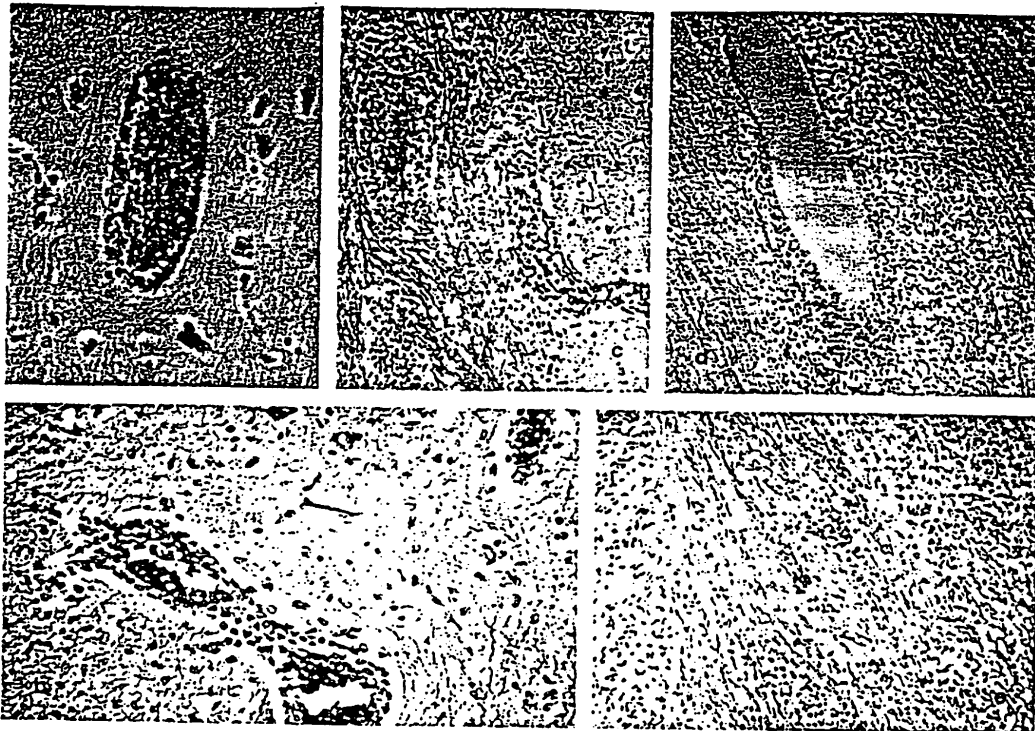


Figure 1. Typical examples of the histological changes in the brains of guinea pigs challenged with BE (the sections were stained with Luxol-fast-blue and counterstained with nuclear fast red).
 a) A normal control; a normal blood vessel. (The erythrocytes are stained deep blue). Note the thin vessel wall containing the endothelial and epithelial layers only (magnification: x 500).
 b) Perivasculitis; blood vessel from a mild positive case. Note the narrow mononuclear infiltration around the vessels (x 500).
 c) Perivasculitis; a strong positive case. Note the thick inflammatory area (x 200).
 d) Periventriculitis (x 200).
 e) Diffuse mononuclear infiltration of the brain parenchyma (x 200).

4. Results

4.1. Characterization of EAE

In the numerous reports which have appeared in the literature on EAE, several criteria were used for the diagnosis of the disease. The appearance of clinical symptoms, manifested mainly by the paralysis of the hind legs, was the criterion mostly used. A second parameter reported is the degree of histological changes in the brain. It has also been suggested that in many cases a correlation exists between delayed hypersensitivity reaction toward the encephalitogen and the appearance of clinical symptoms [30]. Recently, it has been shown that the level of serum LDH in rabbits increased as a result of EAE [31], and could thus serve as a useful index of the disease induction.

We have determined all four parameters mentioned above in ten individual guinea pigs, and the results are presented in Table 4. As shown, six out of the ten animals were paralyzed within 13 to 20 days after being challenged with BE. Histological lesions, of various degrees of severeness, were observed in the brains of all the guinea pigs tested. Delayed hypersensitivity reaction was observed on 8 of the guinea pigs. However, it is of interest that the animals that

in the delayed hypersensitivity test suffered paralysis, whereas all four animals that did not show the clinical symptoms reacted positively in the delayed hypersensitivity test. An increase was observed in the level of serum lactic dehydrogenase in several of the animals. This effect could not be correlated, however, with the other parameters listed in Table 4.

On the basis of these and similar data, we have chosen the incidence of clinical symptoms and of histological lesions in the brain as the two critical parameters for evaluation of our results. All animals suffering from paralysis were considered diseased, and the non-paralyzed animals were judged by the degree of their histological lesions. An added indication of the disease was the decrease in the weight of the animals. This, however, was not considered to be of major significance, as it is not specific for EAE.

4.2. Encephalitogenic activity of the synthetic copolymers

The copolymers 1, 2 and 3 were tested for encephalitogenic activity at dose levels of 10 μ g, 250 μ g, 1 mg and 5 mg. Each dose of each copolymer was injected into 5 guinea pigs in CFA in the two hind legs. No clinical symptoms were

Table 4. Characterization of EAE

Guinea pig no.	Delayed hyper-sensitivity reaction ^{a)}	Day of onset of paralysis	Histological lesions ^{b)}	Lactate dehydrogenase ^{c)} enzyme units	Weight change
1	negative	13	+	83	decrease
2	negative	14	+	124	decrease
3	6	20	+++	42	decrease
4	7	no paralysis	+	44	no decrease
5	8	no paralysis	++	80	decrease
6	8	17	++	86	decrease
7	12	15	+++	34	decrease
8	14	15	+++	136	decrease
9	15	no paralysis	+	33	no decrease
10	15	no paralysis	++	90	decrease

- a) The test was carried out on the 11th day after challenge. The numbers represent the diameter of the skin reactions in mm.
- b) The various degrees of the histological lesions are described and defined under methods.
- c) The LDH was assayed in serum samples taken on the 14th day after challenge. The level of LDH in the sera of untreated guinea pigs was between 25 and 50 enzyme units.

Table 5. Prevention and suppression of EAE by synthetic copolymers

Group	Treatment	Copolymer	Clinical incidence	Histological changes
Control	Injection with 10 µg BE in CFA into the footpads of the animal	none	15/20	20/20
Prevention	Eight intradermal injections of 100 µg of the polymer in ICFA, twice a week for four weeks, followed by challenge with 10 µg of BE in CFA	Cop 1	3/9	8/9
		Cop 2	5/10	9/10
		Cop 3	5/10	9/10
Suppression	Injection with 10 µg BE in CFA, followed by 6 intradermal injections of 100 µg copolymer in ICFA, two injections per week, starting two days after the injection of BE	Cop 1	2/9	5/9
		Cop 2	4/10	10/10
		Cop 3	3/10	10/10
		ICFA alone	2/5	5/5

Table 6. Suppression of EAE by intravenous injections of polymers^{a)}

Treatment	Clinical incidence	Histological changes	
		Incidence	Degree
Control: 10 µg of BE	4/5	5/5	++, +++
Cop 1	1/5	4/5	+, ++
Cop 2	6/8	7/8	++, +++

- a) The copolymers (1 mg) were injected intravenously at days 5, 10, 15 after challenge with BE.

4.3. Prevention and suppression of EAE with synthetic copolymers

In the first stage of this work experiments of prevention and suppression of EAE with the synthetic polymers were carried out under conditions identical with those described

Table 5. In the suppression experiment the copolymers were more effective. The best result was achieved with Cop. 1 which brought about reduction not only in the clinical incidence of the disease, but also in the prevalence of histological lesions.

The only doubt shed upon these data is the finding [32] that injection of incomplete Freund's adjuvant alone had a suppressive effect on EAE. Indeed, in the one experiment listed in Table 5, it was found to have a suppressive effect. For this reason an additional suppression experiment was carried out by applying intravenous injections of the copolymers in saline (Table 6). Only two copolymers (Cop 1 and Cop 2) were compared in this experiment, since Cop 3 was found toxic under the conditions employed. It is seen from Table 6 that under the experimental conditions used Cop 2 had no protecting effect against the disease. Cop 1, however, showed a drastic suppressive effect. It reduced the clinical incidence of the disease, as well as the degree of histological lesions.

4.4. Effect of schedule of injections

The effectiveness of Cop 1 was studied as a function of the schedule of the injections given for suppressing the EAE. The results given in Table 7 show that the injections of the suppressive agent may be started as late as 5 days after the challenge with the BE and still provide a protecting effect.

However, if the injections are started at 10 days after the challenge, the polymer is not effective any longer. The best conditions, as indicated by these experiments, are three injections of the material at days 5, 10 and 15 after the induction of EAE.

In later experiments it was found that when young guinea pigs (weighing less than 300 g) were used, the day of onset of paralysis occurred earlier (at days 12-15 as compared to days 15-21 in adult animals). In these cases the suppression injections have to be given at earlier dates, namely, days 1, 6 and 11 after the challenge.

4.5. Specificity of the suppressive effect

In an attempt to study the specificity of the suppressive effect, we first synthesized and tested another batch of Cop 1 prepared under the same conditions as the first one. The two batches of the polymer were shown to be similar in both amino acid composition and molecular weight (Table 1). In addition to the testing of the second preparation of Cop 1, we compared the efficiency of this suppression to that caused by the natural BE on the one hand, and by non-related materials on the other hand. The results listed in Table 8 show that the two batches of the polymer had similar activities. Both reduced considerably the clinical

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