EXPERIMENTAL ALLERGIC ENCEPHALITIS

Dissociation of Cellular Immunity to Brain Protein and Disease Production*

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Experimental allergic encephalitis (EAE)[1] is a disease produced in animals by the injection, in complete Freund's adjuvant (CFA), of encephalitogenic protein (EP) derived from brain. The evidence is strong that the disease results from an immune response to the injected antigen and, until recently, it was believed that delayed sensitivity to the EP was directly related to the production of disease. It is possible to chemically modify the EP by treating it with 2-hydroxy-5-nitro-benzyl bromide (HNB) which blocks the single tryptophan residue and renders the molecule inactive in producing disease (1). The HNB-treated molecule is therefore termed nonencephalitogenic protein (NEP).

The entire amino acid sequence of the EP derived from brain has recently been reported (2), and the major encephalitogenic determinant has been characterized and synthesized (3). It is a nonapeptide having the amino acid sequence Phe-Ser-Trp-Ala-Glu-Gly-Gln-Lys. Additional studies showed that three amino acids were essential for the encephalitogenic property of the peptides: Trp, Gln, and Lys, although the Lys could be replaced by another amino acid, Arg. The other amino acids in the sequence could be altered without destroying the encephalitogenic activity of the peptide (4).

We have used these well-characterized antigens to further study cellular immunity in relationship to EAE. The present communication reports the results of experiments to determine cellular immune response to the EP, the

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[1] *Abbreviations used in this paper*: BGG, bovine gamma globulin; CFA, complete Freund's adjuvant; EAE, experimental allergic encephalitis; EP, encephalitogenic protein; HNB, 2-hydroxy-5-nitro-benzyl bromide; KLH, keyhole limpet hemocyanin; MIF, migration inhibitory factor; NEP, nonencephalitogenic protein; PHA, phytohemagglutinin; PPD, purified protein derivative of tubercule bacillus.

NEP, and the synthetic peptides in animals which have been immunized with the various encephalitogenic and nonencephalitogenic peptides, in animals immunized with the EP and the NEP, and in animals protected from disease by immunization with the NEP. The results suggest a dissociation between cellular immunity to the EP and the production of EAE.

Materials and Methods

Encephalitogenic Basic Protein (EP).—This was purified from bovine spinal cord myelin as described (5). The preparation included lipid and acid extractions, precipitation of contaminants at pH 7, dialysis in acetylated tubing, diethylaminoethyl (DEAE)-cellulose chromatography and, finally, gel filtration. The homogeneity of the preparation was demonstrated by polyacrylamide gel electrophoresis, immunoelectrophoresis, and immuno-double diffusion.

Nonencephalitogenic Modified Protein (NEP).—This was prepared by treating the EP with 2-hydroxy-5-nitro-benzyl bromide (HNB) (6) as described (1). Briefly, the EP was dissolved

TABLE I

Amino Acid Sequence of Peptides Used in These Experiments

Peptide	Encephalitogenic	Amino acid sequence*
Tryptophan	Yes	Phe-Ser- Trp- Gly-Ala-Glu-Gly-Gln-Lys
1	Yes	Ser-Arg-Phe-**ALA**-Trp- Gly-Ala-Glu-Gly-Gln-Lys
2	No	Ser-Arg-Phe-Ser- Trp- Gly-Ala-Glu-Gly-Gln
3	Yes	Ser-Arg-Phe-Ser- Trp- Gly-Ala-**ILU**-Gly-Gln-Lys
4	Yes	Ser-Arg-Phe-Ser- Trp- Gly-Ala-Glu-Gly-Gln-**ARG**
5	No	Ser-Arg-Phe-Ser- **VAL**- Gly-Ala-Glu-Gly-Gln-Lys
6	No	Ser-Arg-Phe-Ser- **PHE**-Gly-Ala-Glu-Gly-Gln-Lys

* The amino acids which differ from those of the encephalitogenic determinant of the encephalitogenic protein are indicated by bold-face type.

in 8 M urea, treated with dry acetone containing HNB, and purified on a Sephadex G-25 column (Pharmacia Fine Chemicals Inc., Piscataway, N.J.).

Encephalitogenic and Nonencephalitogenic Peptides.—The peptides were synthesized according to the solid-phase procedure described by Merrifield (7) and purified by Sephadex gel filtration. (Some of the peptides were synthesized by Dr. Fred Westall of Salk Institute, San Diego, Calif.) The amino acid sequence of the peptides utilized in these studies is shown in Table I, and the amino acid content, as determined by the Beckman 121 Automatic Amino Acid Analyzer (Beckman Instruments Inc., Fullerton, Calif.) is shown in Table II. This analysis shows a close correlation between the expected and observed amino acid content.

Immunization and Testing.—Random-bred guinea pigs each weighing approximately 500–600 g were utilized. They were immunized and tested according to the following schedules. The skin tests and the in vitro studies were performed on different groups of animals to avoid any possible effect of the injection of the skin test antigen on the response in vitro. Immunized animals which had been used for skin testing were kept alive and observed for signs of disease.

Immunization with Encephalitogenic and Nonencephalitogenic Peptides.—44 guinea pigs were immunized with 20 μg of the various synthetic peptides in saline emulsified with an equal volume of CFA (H37Ra, Difco Laboratories, Detroit, Mich.). Injection schedule for each animal was 0.1 ml administered intradermally into each foot pad and 0.2 ml given subcuta-

neously. Skin tests were performed on day 10 after immunization since previous study had shown that the intensity of the reaction diminished from days 13–17 as the disease progressed (5). Macrophage migration and lymphocyte stimulation studies were also performed on the 10th day after immunization. The number of skin tests and in vitro studies which could be done using the peptides as the test antigens was necessarily limited by the high dose of peptide required for these studies (200 μg per test or 1200 μg for complete testing of each animal) and the necessity that the peptides be synthesized. Four additional guinea pigs (two for skin testing and two for in vitro studies) in each subgroup were immunized with 100 μg of each of the peptides to determine whether a larger dose of the immunizing antigen might produce different results.

Immunization with EP.—To test the response to the peptides in guinea pigs immunized with the whole protein, guinea pigs were inoculated with 250 μg of the EP in saline emulsified with CFA in a manner similar to that described for immunization with the peptides. Skin tests and studies in vitro were performed 10 days after immunization.

TABLE II

*Amino Acid Composition of Synthetic Peptides**

Peptide	Molar ratio‡								
	Lys	Arg	Ser§	Glu	Gly	Ala	Val	Ilu	Phe
Tryptophan	1.19	—	0.86	1.86	1.78	0.86	—	—	1.44
1	1.17	0.98	.63	2.32	2.07	1.89	—	—	0.93
2	—	1.17	1.74	2.27	2.23	0.84	—	—	0.77
3	1.22	1.00	1.00	1.11	2.43	1.15	—	1.01	0.96
4	Not tested								
5	1.00	1.01	1.17	2.25	2.40	1.16	1.08	—	0.91
6	1.18	1.05	0.99	2.08	2.39	1.27	.04	—	1.98

* Tryptophan was destroyed during acid hydrolysis.

‡ Number of residues in each peptide was based on number of cycles performed in the synthesis.

§ The molar ratio of serine is minimal because no correction has been made for possible losses during acid hydrolysis.

Immunization with NEP.—To test the immunologic responses of the guinea pigs immunized with NEP, which does not produce disease, guinea pigs were inoculated with 250 μg of NEP in saline emulsified with CFA. Skin tests and studies in vitro were performed 10 days after immunization.

Protection from Disease with NEP.—Guinea pigs were injected with 1 mg of NEP emulsified in CFA. 1 and 2 wk later they were again injected with 1 mg of NEP in Freund's incomplete adjuvant. This procedure has been shown to protect the animals from EAE after subsequent challenge with EP (8). Skin tests, lymphocyte stimulation studies, and macrophage migration tests were performed 1 month after the last injection. A second group of guinea pigs was similarly injected with a protective dose of NEP, and was challenged with the injection of 250 μg of EP in CFA 1 month after the last injection of NEP. Skin tests and in vitro studies were performed 10 days after challenge.

Controls.—These animals were immunized and tested exactly as described for the peptides, except that the immunizing antigen was 250 μg of bovine gamma globulin (BGG) or keyhole limpet hemocyanin (KLH).

Skin Tests.—Skin tests were performed on a shaved area of the flank by intradermal injections of 0.1 ml of saline containing the specified antigen. The doses of antigens used for each

skin test were EP, 50 μg; NEP, 50 μg; purified protein derivation of tubercule bacillus (PPD), 10 μg; BGG, 10 μg; peptides, 200 μg. The diameter of the erythema and the induration was carefully observed and recorded at the end of 1, 2, 3, 5, and 6 hr (immediate reactions) and at the end of 24 and 48 hr (delayed reactions). Reactions were considered positive if they consisted of erythema and clear-cut induration of 5 mm or greater.

Lymphocyte Response.—Lymph node lymphocytes were cultured according to our modifications of the method of Dutton and Eady (9): 10^7 cells were cultured in 4 ml of Eagle's minimal essential medium for suspended cultures (Spinner's medium, Grand Island Biological Co., Grand Island, N. Y.) containing 100 units of penicillin and 100 μg of streptomycin/ml, 1% L-glutamine, and 5% calf serum. All cultures were prepared in triplicate. The following doses of antigen in 0.1 ml of saline were added to each 4 ml of culture: EP, 50 μg; peptides, 200 μg; PPD (Merck Sharp & Dohme, West Point, Pa.), 10 μg; and phytohemagglutinin (PHA) (Difco Laboratories). The antigens were added at the beginning of the cultures, and the mixture was incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 2 days, at the end of which period thymidine-2-^{14}C (specific activity, 59.2 mCi/mole) (New England Nuclear Corp., Boston, Mass.) was introduced into each culture tube. The cells were harvested in the cold 24 hr later by serially suspending them once in saline, twice in 5% trichloroacetic acid, and once in methanol. The precipitate was dissolved by incubation in 1 ml of Hyamine at 56°C for 1 hr, transferred to counting vials, and prepared for scintillation counting by adding 12 ml of Omnifluor (New England Nuclear Corp.) in toluene. The radioactivity was measured on a Packard liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.). In a few experiments, cell death was evidenced by uniformly low radioactivity (20–40 cpm) in all tubes and by lack of any stimulation by PHA; these results were eliminated from our calculations and the experiments were repeated. The cell death did not occur with cells from any particular group of animals. Results were analyzed by the Mann-Whitney U test. Statistical analysis indicated that for the EP, less than 5% of cultures from control animals would produce a ratio of 1.4 or greater when tubes with EP were compared with tubes without EP.

Macrophage Migration.—Macrophage migration was performed as described by David et al. (10). Peritoneal exudates were induced by the intraperitoneal injection of 30 ml of light mineral oil (Marcol) (Humble Oil & Refining Co., Houston, Tex.) into the guinea pigs 7 days after immunization. 3 days later the cells were harvested by washing the peritoneal cavity with 200 ml of Hanks' solution. The packed cells were suspended in Spinner's medium, 7.5% by volume, and capillary tubes were filled with the cell suspension. The tubes were sealed and spun in the cold for 5 min at 900 rpm. They were cut at the cell-fluid interface and placed in Mackaness-type culture chambers, two tubes per chamber. All chambers were prepared in duplicate. The chambers were filled with Spinner's medium containing 100 units of penicillin and 100 μg of streptomycin/ml, 1% L-glutamine, 15% normal guinea pig serum, and 0.1 ml of saline containing test antigens in the same concentrations as for lymphocyte culture, except that PHA was not used. (The PPD utilized in the macrophage migration studies was kindly supplied by the Ministry of Agriculture, Fisheries, and Food, Weybridge, England.) The chambers were sealed and incubated for 24 hr at 37°C. The cell image was projected and traced, and the area of migration measured using a planimeter. The areas of migration in duplicate test chambers (four tubes) were averaged, and the results were expressed as percentage of migration of peritoneal cells in experimental chambers with antigen compared with that in the control chambers containing no antigen. Results of the in vitro studies were analyzed by the Mann-Whitney U test. Statistical analysis indicated that less than 5% of the cultures from control animals would result in migration below 71% for EP or 62% for NEP when migration in chambers with antigen was compared with migration in chambers without antigen.

RESULTS

The results of the study of cellular immunity in guinea pigs immunized with the tryptophan peptide, which is encephalitogenic and has the same amino acid

sequence as that appearing in the EP, are shown in Table III. The animals showed both immediate and delayed skin test reactivity to the EP, and the migration of their peritoneal exudate cells was inhibited by the EP; however, there was no increase in DNA synthesis of their lymph node lymphocytes in response to EP. Only one of six animals showed delayed skin reactivity to NEP, and there was no significant lymphocyte stimulation or inhibition of macrophage migration by this antigen. As expected, the animals showed cellular im-

TABLE III

Results of the Study of Cellular Immunity in Guinea Pigs Immunized with the Tryptophan Peptide

Test antigen	Skin test				In vitro	
	Immediate		Delayed		Lymphocyte response	Macrophage migration
	Induration	No. of positive	Induration	No. of positive		
	mm		*mm*		*E/C**	%
EP	5.8 ± 1.2‡	5 (6)§	4.5 ± 0.7	5 (6)	1.4 ± 0.2 (6)	57.7 ± 3.4‖ (6)
NEP	3.2 ± 1.4	2 (6)	1.3 ± 0.8	1 (6)	1.0 ± 0.1 (6)	90.3 ± 5.3 (6)
BCG	0.8 ± 0.8	1 (6)	0.0 ± 0.0	1 (6)	NT	100.8 ± 5.6 (6)
KLH	NT	NT	NT	NT	1.0 ± 0.0 (6)	NT
PPD	6.2 ± 1.2	5 (6)	13.6 ± 0.6	6 (6)	2.8 ± 0.5 (6)	56.7 ± 6.9 (6)
PHA	NT	NT	NT	NT	27.2 ± 6.8 (6)	NT

NT, not tested.

* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.

‡ All results reported as mean ± SEM.

§ Numbers in parentheses are numbers of animals tested.

‖ Significantly different from controls ($P < 0.05$).

munity to PPD, since they had been immunized by antigen in CFA, which contains tubercle bacilli. The stimulation by PHA of the lymphocytes appeared less than that observed in the control animals, but the difference is not statistically significant.

The results of immunization with peptide 1 (Tables I and II) are shown in Table IV and illustrated in Fig. 1. This peptide has an amino acid sequence different from that of the EP, but it produces disease since the three amino acids essential for this activity are present. Four guinea pigs were immunized with single doses of 20 μg and two with single doses of 100 μg. The results were the

same in both groups and so have been pooled. Only one of six animals showed delayed skin reactivity to the EP. Lymphocytes derived from these animals did not show increased DNA synthesis in response to this antigen, nor was there inhibition of the migration of their peritoneal exudate cells. The observation of 80% migration with EP or NEP added to the chamber was the same as that which occurred when these antigens were added to cells from control animals immunized with BGG. The animals did show cellular immunity to the im-

TABLE IV

Results of the Study of Cellular Immunity in Guinea Pigs Immunized with Peptide 1

Test antigen	Skin test				In vitro	
	Immediate		Delayed		Lymphocyte response	Macrophage migration
	Induration	No. of positive	Induration	No. of positive		
	mm		*mm*		*E/C**	%
EP	0.8 ± 0.8‡	1 (6)§	0.8 ± 0.8	1 (6)	1.2 ± 0.1 (6)	81 ± 4.5 (6)
NEP	4.2 ± 1.2	4 (6)	1.5 ± 0.6	0 (6)	1.1 ± 0.1 (6)	82 ± 3.1 (6)
BGG	0.7 ± 0.6	0 (6)	0 ± 0	0 (6)	1.0 ± 0.1 (5)	80 ± 2.7 (5)
PPD	5.7 ± 1.8	4 (6)	16.7 ± 2.0	6 (6)	1.8 ± 0.3 (6)	44 ± 6.9 (6)
Peptide 1	6.0	2 (2)	5.5	2 (2)	3.3 (2)	68 ± 7.7 (4)
PHA	NT	NT	NT	NT	69 ± 22.0 (6)	NT

Tryptophan peptide, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys

Peptide 1, Ser-Arg-Phe-**ALA**-Trp-Gly-Ala-Glu-Gly-Gln-Lys

NT, not tested.

* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.

‡ All results reported as mean ± SEM.

§ Numbers in parentheses are numbers of animals tested.

munizing antigen, peptide 1, by all three measures. There was no reactivity with NEP, and they did show immunity to PPD, as expected.

The results of immunization with peptide 2 are shown in Table V. This peptide has the same amino acid sequence as that in the EP, but does not produce disease because it lacks the terminal lysine. Five of six animals showed delayed skin reactivity to EP, but cellular immunity to this antigen was not demonstrated by studies in vitro. Similarly, delayed skin reactivity to NEP was observed without demonstrable cellular immunity by the in vitro studies. The results with the control antigens, BGG, PPD, and PHA, were as expected.

Cellular immunity to the immunizing antigen, peptide 2, could not be tested in vivo or in vitro as this antigen produced nonspecific reactions in control animals.

The results of immunization with peptide 3 are shown in Tables VI and VII. This peptide is encephalitogenic but has an amino acid sequence different from that of EP. No cellular immunity to the EP could be demonstrated in animals which had been immunized with 20 μg of peptide 3, even though animals immunized with this dose of antigen regularly develop EAE. Peptide 3 produced inhibition of migration of peritoneal exudate cells derived from these animals,

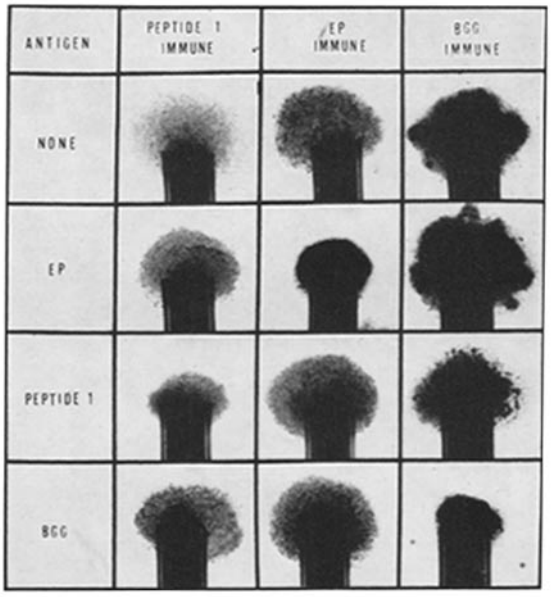

FIG. 1. Results of macrophage migration studies in guinea pigs immunized with the EP peptide 1, and BGG.

but did not cause increased DNA synthesis in their lymphocytes. When the animals were immunized with a larger dose (100 μg) of peptide 3, cellular immunity to EP could be demonstrated by all three criteria. (The lymphocyte response was borderline.)

Guinea pigs which were injected with each of the encephalitogenic peptides, and not further manipulated, developed clinical EAE and died. Detailed histologic evaluation of these animals will be reported elsewhere.[2] Some of the

[2] Hoffman, P., and L. E. Spitler. EAE: A comparison of histologic changes produced by immunization with the encephalitogenic peptides, encephalitogenic protein, and whole spinal cord. Manuscript in preparation.

TABLE V

Results of the Study of Cellular Immunity in Guinea Pigs Immunized with Peptide 2

Test antigen	Skin test				In vitro	
	Immediate		Delayed		Lymphocyte response	Macrophage migration
	Induration	No. of positive	Induration	No. of positive		
	mm		*mm*		*E/C**	%
EP	3.7 ± 1.6‡	3 (6)§	5.2 ± 0.4	5 (6)	1.0 ± 0.1 (4)	84 ± 4.2 (6)
NEP	4.5 ± 1.4	4 (6)	4.5 ± 1.0	4 (6)	1.0 ± 0.0 (4)	86 ± 4.8 (6)
PPD	7.3 ± 1.6	4 (6)	16.5 ± 0.9	6 (6)	4 ± 1.0 (4)	61 ± 6.4 (6)
BGG	0	0	1.5 ± 0.5	0 (6)	0.6 ± 0.1 (4)	91 ± 7.0 (6)
PHA	NT	NT	NT	NT	124 ± 30.5	NT

Tryptophan peptide, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys
Peptide 2, Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln
NT, not tested.
* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.
‡ All results reported as mean ± SEM.
§ Numbers in parentheses are numbers of animals tested.

TABLE VI

Results of the Study of Cellular Immunity in Guinea Pigs Immunized with 20 μg of Peptide 3

Test antigen	Skin test				In vitro	
	Immediate		Delayed		Lymphocyte response	Macrophage migration
	Induration	No. of positive	Induration	No. of positive		
	mm		*mm*		*E/C**	%
EP	0	0 (2)‡	0	0 (2)	1.0 (2)	103 (2)
NEP	0	0 (2)	0	0 (2)	0.8 (2)	96 (2)
PPD	14.0	2 (2)	23.0	2 (2)	2.7 (2)	59 (2)
BGG	0	0 (2)	0	0 (2)	0.8 (2)	94 (2)
Peptide 3	NT	NT	NT	NT	0.9 (2)	56 (2)
PHA	NT	NT	NT	NT	55	NT

Tryptophan peptide, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys
Peptide 3, Ser-Arg-Phe-Ser-Trp-Gly-Ala-ILU-Gly-Gln-Lys
NT, not tested.
* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.
‡ Numbers in parentheses are numbers of animals tested.

TABLE VII

Results of the Study of Cellular Immunity in Guinea Pigs Immunized with 100 μg of Peptide 3

Test antigen	Skin test				In vitro	
	Immediate		Delayed		Lymphocyte response	Macrophage migration
	Induration	No. of positive	Induration	No. of positive		
	mm		*mm*		*E/C**	%
EP	5.5	2 (2)‡	5.0	2 (2)	1.4 (2)	63 (2)
NEP	5.5	2 (2)	4.5	1 (2)	1.4 (2)	65 (2)
PPD	8.5	2 (2)	14.0	2 (2)	1.9 (2)	55 (2)
BGG	1.0	0 (2)	0	0 (2)	0.8 (2)	89 (2)
PHA	NT	NT	NT	NT	92 (2)	NT

NT, not tested.

* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.

‡ Numbers in parentheses are numbers of animals tested.

TABLE VIII

Results of the Study of Cellular Immunity in Guinea Pigs Immunized with Encephalitogenic Protein

Test antigen	Skin test				In vitro	
	Immediate		Delayed		Lymphocyte response	Macrophage migration
	Induration	No. of positive	Induration	No. of positive		
	mm		*mm*		*E/C**	%
Peptide 1	1.7 ± 0.6‡	1 (8)§	1.1 ± 0.5	0 (8)	NT	102 ± 12.1 (6)
Peptide 4	1.5 ± 1.3	3 (4)	0	0 (4)	NT	118 ± 19.3 (3)
EP	4.0 ± 1.4	3 (6)	9.0 ± 1.0	6 (6)	2.7 ± 0.6‖ (6)	68 ± 3.2‖ (10)
NEP	3.6 ± 1.4	4 (8)	8.0 ± 0.7	7 (8)	2.3 ± 0.5‖ (6)	73 ± 6.1 (10)
PPD	7.5 ± 1.6	5 (6)	13.0 ± 1.3	6 (6)	2.8 ± 0.6 (6)	47 ± 3.9 (10)
BGG	0	0 (8)	NT	0 (8)	0.9 ± 0.1 (5)	93 ± 9.7 (8)
PHA	NT	NT	NT	NT	162 ± 38.2 (6)	NT

Tryptophan peptide, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys

Peptide 1, Ser-Arg-Phe-**ALA**-Trp-Gly-Ala-Glu-Gly-Gln-Lys

Peptide 4, Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-**ARG**

NT, not tested.

* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.

‡ All results reported as mean ± SEM.

§ Numbers in parentheses are numbers of animals tested.

‖ Significantly different from controls ($P < 0.05$).

guinea pigs immunized with encephalitogenic peptides and subsequently used for skin testing did not develop clinical disease. This could be due to suppression of disease by the amount of antigen necessary for skin testing; alternatively, the guinea pigs could have developed mild disease which was not detected clinically and they may have subsequently recovered.

The results of the studies of cellular immunity in animals immunized with EP are listed in Table VIII. It is evident that animals immunized with EP did not show cellular immunity, as measured by skin reactivity or macrophage

TABLE IX

Results of the Study of Cellular Immunity in Guinea Pigs Immunized with Nonencephalitogenic Protein (NEP)

Test antigen	Skin test			In vitro	
	Immediate	Delayed		Lymphocyte culture	Macrophage migration
	No. of positive	Induration	No. of positive		
		mm		*E/C**	%
EP	5 (6)‡	6.1 ± 0.7§	5 (6)	2.8 ± 0.8‖ (6)	60 ± 5.3‖ (6)
NEP	6 (6)	1.3 ± 0.4	6 (6)	8.4 ± 2.7‖ (6)	35 ± 6.0‖ (6)
PPD	4 (6)	11.6 ± 1.3	6 (6)	1.4 ± 0.3 (6)	45 ± 6.0 (6)
BGG	0 (6)	0	0 (6)	0.9 ± 0.1 (6)	89 ± 2.8 (6)
KLH	NT	NT	NT	85.0 ± 25.1 (6)	
PHA	NT	NT	NT		

NT, not tested.

* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.

‡ Numbers in parentheses are numbers of animals tested.

§ All results reported as mean ± SEM.

‖ Significantly different from control ($P < 0.05$).

migration, to either of the two encephalitogenic peptides tested. Peptides 2, 3, and 5 could not be utilized for test purposes because they gave nonspecific reactions in animals immunized with BGG. Some guinea pigs immunized with EP were tested with the peptides alone without simultaneous testing with other antigens (EP or PPD) because of the possibility that testing with other antigens at the same time might diminish the response to the peptide; however, these animals similarly did not show reactions to the test peptides. As expected, the animals immunized with EP did show delayed sensitivity to EP by all three measures.

The results of the study of cellular immunity in the guinea pigs immunized with NEP, which does not produce disease, are shown in Table IX. Five of six

animals showed delayed reactivity to EP. As expected, they also showed delayed reactivity to the immunizing antigen, NEP; this was stronger than the reaction to the EP, suggesting that cellular reactivity to the HNB group is also involved in the reaction. Lymphocytes from the guinea pigs immunized with NEP responded to EP by increased incorporation of radioactive thymidine into DNA in vitro. They also responded to NEP and to PHA, but not to the control antigen, BGG. Migration of peritoneal exudate cells derived from these animals

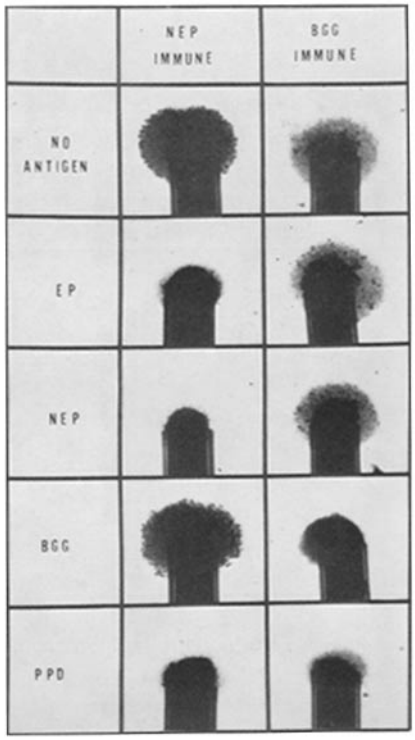

FIG. 2. Effect of test antigens on migration of peritoneal exudate cells derived from guinea pigs immunized with NEP or with BGG.

was inhibited by EP, NEP, and PPD, but not by BGG. These results are illustrated in Fig. 2. The animals inoculated with NEP and skin tested with EP were kept alive and observed for 2 months after immunization. None showed clinical signs of EAE.

The results of the study of cellular immunity in the control guinea pigs immunized with BGG or KLH are shown in Table X. Some inhibition of macrophage migration was produced by the EP and NEP, and this was taken into account in the statistical analysis of the data.

The results of protection from disease by immunization with NEP are sum-

marized in Table XI. Guinea pigs which had received protective immunization with the NEP did not show delayed skin reactivity to the EP, but they did show both immediate and delayed skin reactivity to the EP after protective immunization with the NEP and challenge with the EP in CFA. All of the guinea pigs showed immediate and delayed skin reactivity to the NEP. The results of the studies in vitro were similar in animals which had received pro-

TABLE X

Results of Study of Cellular Immunity in Control Guinea Pigs Immunized with BGG or KLH

Test antigen	Skin test				In vitro	
	Immediate		Delayed		Lymphocyte response	Macrophage migration
	Induration	No. of positive	Induration	No. of positive		
	mm		*mm*		*E/C**	%
EP	0.4 ± 0.3‡	0 (10)§	0.5 ± 0.3	0 (10)	0.9 ± 0.04 (6)	80 ± 5.1 (11)
NEP	0.8 ± 0.5	1 (10)	0.5	0 (10)	0.9 ± 0.2 (6)	71 ± 4.8 (11)
BGG	5.7 ± 1.5	5 (10)	12.4 ± 1.8	9 (10)	NT	63 ± 8.4 (11)
KLH	NT	NT	NT	NT	24.2 ± 4.8 (6)	NT
PPD	10 ± 1.2	9 (10)	18 ± 1.7	9 (10)	0.9 ± 0.4 (6)	78 ± 9.9 (10)
PHA	NT	NT	NT	NT	204 ± 56.3 (6)	NT
Peptide 1	1.7 ± 1.0	2 (6)	0.7 ± 0.6	0 (6)	1.0 ± 0.0 (4)	83 ± 0.9 (4)
Peptide 4	0	0 (2)	0	0 (2)	NT	NT

NT, not tested.

* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.

‡ All results reported as mean ± SEM.

§ Numbers in parentheses are numbers of animals tested.

tective immunization with NEP alone, or protective immunization followed by challenge with the EP in CFA. The EP and the NEP produced both inhibition of macrophage migration and lymphocyte stimulation of cells derived from the animals. None of these guinea pigs developed signs of EAE or died during an observation period of 2 months after the last immunization.

DISCUSSION

The results reported here show that the cellular immune responses to the EP, the NEP, and the synthetic peptides in general are those which would be ex-

pected according to the principles of cellular immunity, and are independent of whether the antigens utilized are encephalitogenic or nonencephalitogenic. A complete dissociation between delayed sensitivity to the brain protein and the production of EAE has not before been reported; this is the first demonstration that it is possible to produce EAE in animals without cellular immunity to the brain protein and, conversely, that it is possible to produce cellular immunity to the brain protein in animals which do not develop and, indeed, are protected from, the disease.

As expected, guinea pigs immunized with the tryptophan peptide, which has the same amino acid sequence as that which appears in the EP, show cellular

TABLE XI

Results of Study of Cellular Immunity in Guinea Pigs Protected from EAE by Immunization with NEP

Test antigen	Protected with NEP not challenged with EP				Protected with NEP challenged with EP			
	Skin test		Lymphocyte culture	Macrophage migration	Skin test		Lymphocyte culture	Macrophage migration
	Immediate	Delayed			Immediate	Delayed		
			*E/C**	%			*E/C*	%
EP	1‡ (2)§	0 (2)	4.0 (2)	46 (2)	2 (2)	2 (2)	2.9 (1)	48 (1)
NEP	2 (2)	2 (2)	2.7 (2)	30 (2)	2 (2)	2 (2)	8.2 (1)	58 (1)
PPD			8.5 (2)	39 (2)	2 (2)	2 (2)	10.0 (1)	61 (1)
BGG	0 (2)	0 (2)	2.0 (2)	88 (2)	0 (2)	0 (2)	0.82 (1)	75 (1)
PHA			65.0 (2)				184.0 (1)	

* Counts per minute in experimental tubes with antigen/counts per minute in control tubes without antigen.

‡ Number of animals showing reactivity.

§ Numbers in parentheses indicate number of animals tested.

immunity to the EP as determined by skin testing and macrophage migration inhibition. By contrast, guinea pigs immunized with peptide 1 or with 20 μg of peptide 3 do not show cellular immunity to the EP in vivo or in vitro, despite the fact that they do develop EAE. They do, however, show delayed sensitivity to the immunizing antigen. Both peptides 1 and 3 have amino acid sequences which are different from that in the EP. In view of the specificity of delayed sensitivity to the hapten and carrier (11–14), it is not too surprising that animals immunized with these peptides do not show delayed sensitivity to EP. Further, although guinea pigs immunized with peptide 2 do not develop EAE, they do show delayed sensitivity to the EP; peptide 2 has an amino acid sequence which is the same as that which appears in the native protein, so this response also would be anticipated based on the principle of the specificity of cellular immune responses.

One could argue that we failed to demonstrate sensitivity to the EP in the animals immunized with peptides 1 and 3 because of the steric configuration of the EP when used as a test antigen in the in vivo and in vitro systems such that the encephalitogenic determinant might be hidden. That this is not the case is demonstrated by the fact that sensitivity to the EP can be demonstrated by in vivo and in vitro criteria in animals immunized with the tryptophan peptide.

It is of interest that skin test reactivity and inhibition of macrophage migration by the EP could be demonstrated in guinea pigs immunized with the tryptophan peptide or with 100 μg of peptide 3, whereas the EP did not cause increased DNA synthesis in the lymph node lymphocytes derived from these animals. A similar dissociation between skin test reactivity and macrophage migration on the one hand and lymphocyte stimulation on the other has now been reported in several different systems. We first reported this dissociation in guinea pigs immunized with the tobacco mosaic virus protein and tested with the antigenic peptides of this protein (15). We noted a similar dissociation in patients with the Wiskott-Aldrich syndrome after treatment with transfer factor (16, 17). Using a tuberculin carbohydrate fraction as antigen, others have confirmed our findings (18). These observations led us to suggest that there may be two different kinds of thymus-derived lymphocytes or T cells: one which undergoes proliferation in response to antigen, and one which produces mediators of cellular immunity, such as migration inhibitory factor (MIF).

We believe the crucial question is the mechanism by which animals immunized with the encephalitogenic peptides develop EAE, although we were unable to demonstrate cellular immunity to the brain protein by the tests used. It is clear that sensitized cells, probably lymphocytes, are in some way involved in the pathogenesis of the disease, since EAE can be transferred by cells derived from animals immunized with brain protein (19–21) but not by serum derived from these animals (22, 23) even after direct intracerebral inoculation (24). It is now recognized that the lymphocyte is a multipotent cell and that there are numerous biologic functions of lymphocytes and of the products of the interaction of sensitized lymphocytes and antigen. These cells undergo increased RNA, DNA, and protein synthesis and produce substances with activities including inhibition of macrophage migration (25, 26), chemotaxis of mononuclear cells (27), increased DNA synthesis in nonsensitized lymphocytes (28–30), "activation" of nonsensitized lymphocytes (31), death of target cells (32), and the production of skin reactions (33). Interferon (34) and immunoglobulins (35) are also produced. It is now recognized that dissociation between these various lymphocyte activities occurs. Thus, it would seem possible that the sensitized lymphocytes in the animals immunized with the peptides might be reacting with the brain protein either directly or by producing a cell-free mediator, but that the reaction involved in producing disease is not one of the reactions which we have measured in this study, i.e., that skin reactivity, production of MIF, and lymphocyte stimulation are not necessarily concomitants of this reaction.

The studies in the animals immunized with the EP confirm and extend previous studies which have shown that animals immunized with brain antigens show delayed sensitivity in vivo and in vitro to the brain antigens (36–38). The lack of response of these animals to the encephalitogenic peptides in vivo and in vitro is in accord with expected results based on the principles of cellular immunity, since these peptides have amino acid sequences different from these in the immunizing protein.

The demonstration that animals immunized with NEP, which do not develop disease, show cellular immunity to EP has important implications for investigators engaged in the study of cellular immunity. Inhibition of migration of macrophages, or of leukocytes, has been reported in the presence of appropriate tissue antigens in diseases such as glomerulonephritis (39), Addison's disease (40), pernicious anemia (41), ulcerative colitis and Crohn's disease (42), multiple sclerosis (43, 44), and the Guillain-Barré syndrome (44). Most of these investigators have at least suggested that cellular immunity can, therefore, be considered to contribute to the pathogenesis of these diseases. It is probable that the cellular immunity that we have demonstrated in animals immunized with NEP is directed toward a part of the EP other than the tryptophan peptide and is, therefore, unrelated to the disease-producing site. It is likely that the same principle holds for human diseases in which cellular immunity to tissue antigens has been reported: cellular immunity may be directed towards parts of the antigen unrelated to production of the disease, a point which has not been clearly recognized by investigators involved in these studies.

EAE is the only "autoimmune" disease for which we have information regarding the determinant necessary for the production of disease; there is no similar information concerning other diseases. In the human diseases mentioned earlier, cellular immunity to tissue antigens, rather than causing the disease, may well result from the pathogenic process itself. A parallel can be drawn to autoantibodies, initially thought to be pathogenic, that have now been found to accompany, but not cause, certain diseases.

The animals protected from EAE by immunization with the NEP and subsequently challenged with the EP show cellular immunity to the EP in vivo and in vitro, although they do not develop EAE. This study does not indicate the mechanism of the protection produced by immunization with NEP, but it does show that the mechanism is not the prevention of cellular immunity to the EP as has been postulated by others. It has been postulated that the induction of antibodies may be the mechanism of protection, and it has been shown that EAE can be prevented by the passive transfer of immune serum (23). However, if this is the case, the antibody does not prevent the cellular immune reaction from occurring in vivo, since the protected animals show skin reactivity to the injection of the EP.

We have demonstrated a dissociation between cellular immunity and EAE in the three tests of these responses measured: skin reactivity, inhibition of

macrophage migration, and lymphocyte stimulation. In the past it was thought that antibodies played a role in the pathogenesis of many autoimmune diseases, but subsequent study showed that in many of these diseases the antibody was secondary to the disease rather than being responsible for its production; therefore, interest turned to delayed sensitivity in these diseases, because of the demonstrated cytopathic effects which can be produced in the cellular immune response. However, it is possible that cellular immunity, like antibody formation, may be a concomitant of the immunization process or a result of tissue damage and, hence, unrelated to the pathogenesis of disease.

The present study raises many questions about the mechanism of the production of EAE in animals immunized with encephalitogenic protein. The pathogenesis of disease may not even be the same in animals immunized with EP as compared with animals immunized with the encephalitogenic peptides. The sensitized lymphocyte may react with antigenic determinants in the central nervous system to produce EAE but this reaction may not be mediated by the type of responses measured here. In this regard, it would be of interest to study the local production of the demyelinating antibody (45) utilizing a system such as ours. It may be also that an appropriate combination of cellular immunity and antibody production is necessary for the production of disease.

The use of the well-characterized encephalitogenic and nonencephalitogenic peptides and highly purified preparations of brain protein and modified brain protein has made it possible to define some of the principles regarding the development of EAE. With the tools now available it should be possible to answer the questions raised in this paper regarding the pathogenesis of the disease.

SUMMARY

The encephalitogenic determinant of brain protein, a nonapeptide having the amino acid sequence Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys, has been characterized and synthesized. In a previous study, analogues of this encephalitogenic peptide were synthesized and some were shown to be encephalitogenic while others were not. Guinea pigs were immunized with encephalitogenic peptides having amino acid sequences different from that in the native protein. These guinea pigs did not show cellular immunity in vivo (skin reactivity) or in vitro (lymphocyte stimulation or macrophage migration inhibition) to the encephalitogenic brain protein (EP) although they did show cellular immunity to the immunizing antigenic peptide. Guinea pigs immunized with an encephalitogenic peptide having the same amino acid sequence as the brain protein, or with a nonencephalitogenic peptide having the same amino acid sequence as the native protein but lacking the terminal lysine, did develop cellular immunity to the EP. Animals immunized with EP showed cellular immunity to this protein, but not to the encephalitogenic peptides. Animals immunized with nonencephalitogenic protein (NEP), prepared by altering the tryptophan residue of EP, did not develop disease but did show cellular immunity in vitro and

in vivo to the EP. Animals protected from disease by immunization with NEP similarly showed cellular immunity to EP. Thus, the results suggest a dissociation between cellular immunity to EP and the production of experimental allergic encephalitis (EAE). Animals immunized with the encephalitogenic peptides develop EAE, but do not show cellular immunity to EP, and animals immunized with NEP show cellular immunity to EP but do not develop EAE. A fresh approach to the examination of the pathogenesis of EAE is now possible through the use of these well-characterized antigens.

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