SYSTEMIC ENZYMATIC CHANGES IN GUINEA PIGS SUFFERING FROM EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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Experimental allergic encephalomyelitis (EAE) is one of the experimental models of human demyelinating diseases and recently is regarded also as a useful model for studying cell-mediated autoimmune diseases. Because of the possibility of induction of systemic changes in this model, we investigated enzymatic changes in serum and main organs of the diseased animals, including brain, spinal cord, limb muscle, heart muscle, spleen, liver and kidney. The enzymes measured consisted of 7 aminopeptidases, 5 endopeptidases, 3 glycosidases, creatine kinase, phosphatase and esterase. Significant changes of many enzymatic activities occurred in all the organs tested in 1 to 2 weeks after the administration of EAE antigen, myelin basic protein (MBP). Interesting correlations of the pattern of enzymatic changes were seen among most of the organs tested. Those patterns changed in the course of the 2 weeks and there remained marked changes characteristic for each organ. This model may represent some type of systemic autoimmune diseases.

Keywords — experimental allergic encephalomyelitis; hydrolytic enzyme, central nerve; muscle, spleen; kidney

INTRODUCTION

EAE has been regarded as one of the disease models for human demyelinating diseases such as multiple sclerosis, post-vaccinal encephalomyelitis, acute hemorrhagic leucoencephalitis, etc.^{1–3)} It can be induced in guinea pigs by sensitization with central nervous tissues, particularly MBP mixed with FCA. Recent studies have shown that EAE is mainly caused by T cell-mediated immune reactions and thereby pro-

vides a useful model for the studies of cell-mediated autoallergic immune diseases and delayed type hypersensitivity.^{4–6)} It has been known that anti-MBP antibody is not essential for inducing EAE. However, there still remains a possibility that the anti-MBP antibody, which is found in serum and in cerebrospinal fluid of the diseased animals, play at least some modulatory roles in the development of EAE. Since it is conceivable that the formed antibody or antigen-an-

Abbreviations used in this paper: Glu·NA, L-glutamic acid β -naphthylamide hydrochloride; Arg·NA, L-arginine β -naphthylamide hydrochloride; Pro·NA, L-proline β -naphthylamide hydrochloride; Leu·NA, L-leucine β -naphthylamide hydrochloride; fMet·NA, formyl L-methionine β -naphthylamide; Phe·NA, L-phenylalanine β -naphthylamide; Ser-Tyr·NA, L-seryl-L-tyrosine β -naphthylamide; Gly-Pro-Leu·NA, glycyl-L-prolyl-L-leucine β -naphthylamide hydrobromide; BAEE, *N* ^{α} -benzoyl-L-arginine ethyl ester hydrochloride; ATEE, *N*-acetyl-L-tyrosine ethyl ester; Ac(Ala)₃·ME, acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; NP-Glu, *p*-nitrophenyl- α -D-glucopyranoside; NP-Man, *p*-nitrophenyl- α -D-mannopyranoside; NP-GluNAc, *p*-nitrophenyl-*N*-acetyl β -D-glucosaminide; CK, creatine kinase; NPP, *p*-nitrophenyl phosphate; NPA, *p*-nitrophenyl acetate; Chy-try, chymotrypsin; GluNAc-ase, *N*-acetyl β -D-glucosaminidase; EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; FCA, Freund's complete adjuvant.

tibody complex or the sensitized lymphocytes induce systemic changes in the body and since no particular studies have so far been done in this direction, we directed our concerns not only to the nervous systems but also to other main visceral organs. Also, since several previous studies have suggested that the increased activities of various hydrolytic enzymes are related to pathogenetic mechanisms of various neuromuscular diseases,⁷⁻¹²⁾ we investigated the changes in such enzyme activities in this experimental model.

The results suggested that not only the absolute values of the hydrolytic enzymes but also the pattern of enzymatic changes have some meaning in the pathological processes of this disease.

MATERIALS AND METHODS

Animals — Outbred male Hartley guinea pigs, weighing 300 to 350 g, were used throughout this study. They were obtained from Shizuoka

Experimental Animal Farm (Hamamatsu, Shizuoka, Japan). MBP was prepared from the white matter of bovine brain by the modification of Eylar's method described previously.¹³⁾ The preparation caused 100% incidence of EAE in guinea pigs at a dose of 10 μ g in FCA. In this study 30 μ g of MBP were used for immunization of each guinea pig. Briefly, volumes of 0.25 ml of aqueous solutions of MBP and of mycobacteria were emulsified in a syringe with 0.1 ml of Arlacel A and 0.4 ml of Bayol F, and 0.1 ml portions were injected into the foot pads of both hind legs of guinea pigs. Heat-killed *Mycobacterium tuberculosis*, virulent Aoyama B strain suspended in Arlacel A was used for preparation of FCA and each 0.1 ml of MBP-FCA emulsion contained 100 μ g of mycobacteria.

Observations — Challenged animals were inspected daily for 4 weeks. The animals were weighed and examined for signs of EAE such as body weight loss, loss of the righting reflex, and paralysis of the hind legs, sometimes accompa-

TABLE I. *List of the Enzymes Measured and Their Substrates*

Enzyme	Abbreviation	Substrate	Reference for assay method
Aspartate aminopeptidase (EC 3.4.11.7)	AP-A	Glu·NA	11, 14-17
Arginine aminopeptidase (EC 3.4.11.6)	AP-B	Arg·NA	11, 14, 15, 18
Proline iminopeptidase	Pro-AP	Pro·NA	11, 14, 16
Leucine aminopeptidase (EC 3.4.11.1)	Leu-AP	Leu·NA	11, 14, 16, 18
Formylmethionine aminopeptidase	fMet-AP	fMet·NA	11, 19
Phenylalanine aminopeptidase	Phe-AP	Phe·NA	11, 14, 16
Tripeptide aminopeptidase (EC 3.4.11.4)	Gly-Pro-Leu-AP	Gly-Pro-Leu·NA	11, 14, 16, 18, 20
Serine proteinase (EC 3.4.21)	Trypsin-like	BAEE	11, 14, 21
Serine proteinase (EC 3.4.21)	Chy-try-like	ATEE	11, 14, 22
Serine proteinase (EC 3.4.21)	Elastase-like	Ac(Ala) ₃ ·ME	11, 14, 23
Dipeptidylaminopeptidase I (EC 3.4.14.1)	Cathepsin C	Ser-Tyr·NA	11, 14, 16
Carboxyl proteinase (EC 3.4.23.5)	Cathepsin D	Hemoglobin	11, 24
α -D-Glucosidase (EC 3.2.1.20)	Glucosidase	NP-Glu	11, 14, 16
α -D-Mannosidase (EC 3.2.1.24)	Mannosidase	NP-Man	11, 14, 16
β -N-Acetyl-D-glucosaminidase (EC 3.2.1.30)	GluNAc-ase	NP-GluNAc	11, 14, 16
Creatine kinase (EC 2.7.3.2)	CK	1- ¹⁴ C-creatine	25, 26
Alkaline phosphatase (EC 3.1.3.1)	Phosphatase	NPP	11, 14, 16, 27
Arylesterase (EC 3.1.1.2)	Esterase	NPA	11, 14, 16

nied by diarrhea and incontinence. The animals given MBP together with FCA started to develop clinical signs of EAE about 9 d after the injection and died within 14 to 16 d, whereas those given FCA alone did not. The animals were killed by cervical dislocation, and organ homogenates were prepared in phosphate buffered saline (PBS) using a tissue homogeniser, Ultra-turrax, at the maximum speed for 1 min. The homogenate was centrifuged ($3000 \times g$, 20 min) and the supernatant fluid was withdrawn to measure enzymatic activity.

Substrates for Enzyme Assay — The sources of substrates were as follows: Glu·NA, Arg·NA, Pro·NA and Met·NA from Mann Research, N.Y., U.S.A.; Leu·NA, Phe·NA, Ser-Tyr·NA and Ac(Ala)₃·ME from Bachem Feinchemikalien AG, Budendorf, Switzerland; Gly-Pro-Leu·NA from Peptide Institute, Osaka, Japan; BAEE from Sigma Chemical Co., Saint Louis, U.S.A.; ATEE and NPA from Tokyo Kasei Kogyo Co., Tokyo, Japan; NP-Glu, NP-Man and hemoglobin from Calbiochem-Behring Co., San Diego, U.S.A.; NP-GluNAc and NPP from BDH Chemicals Ltd., Poole, England; 1-¹⁴C-creatine from Daiichi Chemical Co., Tokyo, Japan. fMet·NA was synthesized in our laboratory.

Buffers — Buffers employed were as follows: 0.02M phosphate buffered saline (PBS, pH 7.2) for all enzyme systems; 0.2M sodium acetate buffer (pH 3.2) for cathepsin D; 0.5M sodium glycine buffer (pH 9.0) for creatine kinase.

Determination of Enzyme Activities — The supernatant fluids of homogenates were dispensed into test tubes (1.5 × 10 cm) with PBS containing the respective substrates. The assays were done at pH 7.2 except for cathepsin D and CK, which assayed at pH 3.2 and pH 9.0, respectively. The test tubes were incubated for 1 h at 37°C. All the enzyme assays were done in triplicate, and their errors were within 10% of the average values. The values presented are the averages of triplicate assays. The references for the assay methods and the substrates used are listed in Table I. For most assays the units of the enzyme activities are ex-

pressed as nmols of reaction products generated in one minute of incubation per mg protein (nmol/min/mg protein). The absorbance values actually measured were translated into these units on the standard curves which had been prepared in advance. For the assays of aminopeptidases and cathepsin C, the reaction mixture was prepared by mixing 0.25 ml of 2 mM β -naphthylamide derivatives, 0.65 ml of PBS and 0.1 ml of diluted homogenates or serum in series of test tube. After the reaction, the mixture was processed as described previously^{11,14-20} and the absorbance at 525 nm of the final mixture was determined. For serine proteinase assays, 0.1 ml of 75 mM ester of peptides or *N*-acylamino acids were used as substrate. After the reaction, the mixture was processed as described previously^{11,14,21-23} and the absorbance at 525 nm was determined. For cathepsin D assay, 1.0 ml of 0.5% hemoglobin solution (pH 3.2) was used as the substrate. Two ml of 1.7M perchloric acid was added after the reaction, and the mixture was kept for 1 h at room-temperature. The supernatant fluid was withdrawn by centrifugation ($2000 \times g$ for 5 min) and the absorbance at 280 nm was determined.^{11,24} For glycosidase assays, 0.1 ml of 25 mM *p*-nitrophenyl derivative were used as the substrate. After the reaction, the mixture was processed as described previously^{11,14,16} and the absorbance at 400 nm was determined. For CK assay, 0.1 ml of 1 mM creatine containing 1-¹⁴C-creatine (50000 dpm) was used as the substrate. After the reaction, the radioactivity of the product in the unadsorbed fraction of Dowex 50 (H⁺) was determined.^{25,26} For the assays of phosphatase and esterase, 0.1 ml of 15 mM *p*-nitrophenyl derivatives were used as the substrate. After the reaction, the mixture was processed as described previously^{11,14,16,27,28} and the absorbance at 400 nm of the final mixture was determined.

Protein Determination — The method of Lowry *et al.*²⁹ was used, with bovine serum albumin as the standard.

Statistical Method — The observed values of enzyme levels of normal and diseased animals

were handled by the usual method of regression analysis.³⁰⁾

RESULTS

Before the determination of the 18 enzymatic activities in organ tissues, we confirmed the linearity of each activity relative to incubation time and enzyme concentration.¹¹⁾ Also we confirmed that no significant amount of enzymatic activities can further be extracted by repeating homogenization of the tissue residues using the same buffer containing 0.1% Triton X-100. Thus, it seems certain that the most part of the enzymatic changes obtained, at our hands, should be ascribed to the changes in the tissue levels.

The clinical courses of the diseased animals were almost the same as the ones reported previ-

ously.³¹⁾ Briefly, after a latent period of 5 to 7 d, body weight loss was evident on 8th and 9th day, which was soon followed by appearance of neurological signs, such as hyperirritability, ataxia and paralysis and very frequently also by diarrhea and incontinence. Most of the animals died until 15 to 16th days. Table II presents the enzymatic changes in hindlimb muscle of the guinea pigs suffering from EAE as compared with the control guinea pigs of the same age. At the time of 1 week after the MBP administration, a few enzymes showed various changes in their activities. Namely, the activities of fMet-AP and CK increased, whereas those of Gly-Pro-Leu-AP decreased significantly. At the time of 2 weeks 11 out of the 18 enzymatic activities showed statistically significant increases. Only the activity of glucosidase significantly de-

TABLE II. *Changes in Enzymatic Activity in Hindlimb Muscle of EAE*

Enzyme	Specific activity $\pm$ SD (nmol /min/mg protein)			
	1W (N=5)		2W (N=4)	
	Control	Disease	Control	Disease
AP-A	1.28 $\pm$ 0.09	1.24 $\pm$ 0.11	0.92 $\pm$ 0.05	1.69 $\pm$ 0.28 ^{c)}
AP-B	9.85 $\pm$ 0.54	9.99 $\pm$ 0.81	8.89 $\pm$ 0.37	10.88 $\pm$ 0.20 ^{d)}
Pro-AP	4.79 $\pm$ 1.04	4.22 $\pm$ 0.55	3.42 $\pm$ 0.50	4.61 $\pm$ 0.80 ^{b)}
Leu-AP	10.25 $\pm$ 0.29	9.47 $\pm$ 1.11	9.05 $\pm$ 0.69	10.32 $\pm$ 0.90 ^{a)}
fMet-AP	0.96 $\pm$ 0.04	1.10 $\pm$ 0.06 ^{c)}	0.98 $\pm$ 0.06	1.29 $\pm$ 0.25 ^{a)}
Phe-AP	13.66 $\pm$ 0.72	12.13 $\pm$ 1.38 ^{a)}	10.78 $\pm$ 0.95	14.09 $\pm$ 1.60 ^{b)}
Gly-Pro-Leu-AP	2.32 $\pm$ 0.14	1.56 $\pm$ 0.14 ^{d)}	2.03 $\pm$ 0.22	2.93 $\pm$ 0.22 ^{c)}
Trypsin-like	9.48 $\pm$ 1.06	10.94 $\pm$ 2.15	8.73 $\pm$ 1.36	16.06 $\pm$ 3.59 ^{c)}
Chy-try-like	12.25 $\pm$ 1.76	13.44 $\pm$ 1.50	11.08 $\pm$ 1.20	18.90 $\pm$ 3.47 ^{c)}
Elastase-like	17.11 $\pm$ 1.56	17.61 $\pm$ 3.61	15.58 $\pm$ 0.92	17.22 $\pm$ 3.37
Cathepsin C	1.46 $\pm$ 0.05	1.32 $\pm$ 0.16 ^{a)}	1.18 $\pm$ 0.07	2.12 $\pm$ 0.47 ^{c)}
Cathepsin D	0.0011 $\pm$ 0.0001	0.0012 $\pm$ 0.0001	0.0011 $\pm$ 0.0001	0.0038 $\pm$ 0.0008 ^{d)}
Glucosidase	0.23 $\pm$ 0.02	0.24 $\pm$ 0.01	0.26 $\pm$ 0.02	0.20 $\pm$ 0.02 ^{c)}
Mannosidase	0.05 $\pm$ 0.01	0.07 $\pm$ 0.02 ^{a)}	0.06 $\pm$ 0.01	0.09 $\pm$ 0.03
GluNAc-ase	0.43 $\pm$ 0.02	0.40 $\pm$ 0.10	0.48 $\pm$ 0.03	0.99 $\pm$ 0.09 ^{d)}
CK	46.10 $\pm$ 4.64	55.26 $\pm$ 5.59 ^{b)}	45.38 $\pm$ 2.45	44.73 $\pm$ 4.71
Phosphatase	2.74 $\pm$ 0.22	2.49 $\pm$ 0.51	2.43 $\pm$ 0.25	2.96 $\pm$ 0.51
Esterase	45.31 $\pm$ 4.36	37.70 $\pm$ 6.50 ^{a)}	37.32 $\pm$ 3.31	57.90 $\pm$ 6.94 ^{c)}

a) $p < 0.1$, b) $p < 0.05$, c) $p < 0.01$, d) $p < 0.001$.

Cathepsin D activity is expressed as absorbance (280 nm)/ min/ mg protein.

creased.

Table III shows the enzymatic changes in brain. The activities of AP-A, cathepsin D, glucosidase and GluNAc-ase, decreased, while those of Gly-Pro-Leu-AP increased significantly at 1 week. At 2 weeks, however, the pattern of enzymatic changes became entirely different. The increased activities were those of Chy-try-like, elastase-like, cathepsin D and GluNAc-ase, whereas the ones which decreased were those of glucosidase, mannosidase and esterase. Especially, it is to be noted that the movements of cathepsin D and GluNAc-ase reversed during the course between 1 and 2 weeks.

In Table IV are presented the enzymatic changes in spleen. At 1 week, there occurred significant decreases in various enzymatic activities including those of AP-A, Pro-AP, Leu-AP,

Phe-AP, Glu-Pro-Leu-AP, elastase-like, cathepsin C, phosphatase and esterase. At 2 weeks, the pattern of enzymatic changes became completely different, involving significant increases in the activities of most enzymes as follows: AP-A, Leu-AP, Phe-AP, Gly-Pro-Leu-AP, trypsin-like, Chy-try-like, elastase-like, cathepsin C, cathepsin D, GluNAc-ase and esterase.

Table V shows the enzymatic changes in kidney. At 1 week after the MBP injection the activities of AP-A, AP-B, Pro-AP, Leu-AP, fMet-AP, Phe-AP, Gly-Pro-Leu-AP, Chy-try-like, cathepsin C, cathepsin D, phosphatase and esterase decreased, whereas only that of mannosidase increased significantly. Comparing these enzymatic changes with those in spleen (Table IV), one may notice that there is a similarity of the pattern of the enzymatic changes. In

TABLE III. *Changes in Enzymatic Activity in Brain of EAE*

Enzyme	Specific activity $\pm$ SD (nmol/min/mg protein)			
	1W (N=5)		2W (N=4)	
	Control	Disease	Control	Disease
AP-A	1.52 $\pm$ 0.17	1.22 $\pm$ 0.08 ^{c)}	1.31 $\pm$ 0.21	1.33 $\pm$ 0.27
AP-B	17.89 $\pm$ 0.56	16.79 $\pm$ 2.86	17.72 $\pm$ 2.30	16.71 $\pm$ 1.72
Pro-AP	8.09 $\pm$ 1.00	7.87 $\pm$ 1.63	9.55 $\pm$ 2.09	7.41 $\pm$ 3.67
Leu-AP	8.91 $\pm$ 0.34	8.08 $\pm$ 1.02	8.02 $\pm$ 0.81	8.65 $\pm$ 2.20
fMet-AP	0.76 $\pm$ 0.13	0.71 $\pm$ 0.05	0.76 $\pm$ 0.07	0.80 $\pm$ 0.04
Phe-AP	15.21 $\pm$ 0.76	13.78 $\pm$ 2.19	13.83 $\pm$ 1.63	13.32 $\pm$ 4.80
Gly-Pro-Leu-AP	1.65 $\pm$ 0.08	1.83 $\pm$ 0.14 ^{b)}	1.64 $\pm$ 0.07	1.76 $\pm$ 0.20
Trypsin-like	14.21 $\pm$ 1.29	13.61 $\pm$ 2.37	12.79 $\pm$ 1.89	17.04 $\pm$ 3.06 ^{a)}
Chy-try-like	17.48 $\pm$ 1.15	16.04 $\pm$ 1.42	16.50 $\pm$ 2.02	25.81 $\pm$ 6.85 ^{b)}
Elastase-like	28.27 $\pm$ 5.35	25.16 $\pm$ 3.42	29.31 $\pm$ 2.49	34.93 $\pm$ 2.13 ^{b)}
Cathepsin C	1.63 $\pm$ 0.06	1.51 $\pm$ 0.19	2.06 $\pm$ 0.37	2.02 $\pm$ 0.46
Cathepsin D	0.0043 $\pm$ 0.0002	0.0038 $\pm$ 0.0002 ^{c)}	0.0039 $\pm$ 0.0001	0.0060 $\pm$ 0.0005 ^{d)}
Glucosidase	0.50 $\pm$ 0.02	0.45 $\pm$ 0.04 ^{b)}	0.95 $\pm$ 0.13	0.56 $\pm$ 0.07 ^{c)}
Mannosidase	0.06 $\pm$ 0.02	0.05 $\pm$ 0.01	0.49 $\pm$ 0.06	0.38 $\pm$ 0.04 ^{b)}
GluNAc-ase	2.01 $\pm$ 0.09	1.75 $\pm$ 0.15 ^{b)}	2.05 $\pm$ 0.09	2.83 $\pm$ 0.50 ^{b)}
CK	—	—	—	—
Phosphatase	4.60 $\pm$ 0.34	4.45 $\pm$ 0.86	6.42 $\pm$ 0.10	6.37 $\pm$ 0.83
Esterase	96.47 $\pm$ 5.36	97.51 $\pm$ 6.79	116.14 $\pm$ 3.46	81.69 $\pm$ 3.61 ^{d)}

a) $p < 0.1$, b) $p < 0.05$, c) $p < 0.01$, d) $p < 0.001$.

Cathepsin D activity is expressed as absorbance (280 nm)/min/mg protein.

both of these organs, there were highly significant decreases in the activities of Pro-AP, phosphatase and esterase, but the changes in trypsin-like, glucosidase and GluNAc-ase were not significant. What is more, the activity of mannosidase tended to increase in spleen ($p < 0.1$) and significantly increased in kidney ($p < 0.01$). In order to certify this similarity of the pattern of enzymatic changes, another statistical approach shall be tried below. At 2 weeks, the activities of AP-A, AP-B, Gly-Pro-Leu-AP, cathepsin C, glucosidase and mannosidase decreased while those of cathepsin D and phosphatase increased significantly. At this time, the pattern of enzymatic changes in kidney was much different from that in spleen, since in the latter organ most of the enzymatic changes were increased but none were decreased significantly. This observation,

also, shall be statistically ascertained afterwards.

The enzymatic changes in forelimb muscle, heart muscle, liver, spinal cord and serum are not presented in detail here. But they seem worth being commented briefly.

At 1 week after the MBP sensitization, the significantly increased activities were those of the following enzymes: AP-A, AP-B, Pro-AP, Leu-AP, fMet-AP, cathepsin C, cathepsin D, mannosidase, GluNAc-ase, CK in forelimb muscle; AP-B, fMet-AP, Chy-try-like, elastase-like, cathepsin C in heart; none in liver; phosphatase in spinal cord; AP-A and GluNAc-ase in serum. The decreased enzymatic activities were those of the following enzymes: none in forelimb muscle; Pro-AP, cathepsin D and esterase in heart muscle; AP-B, Pro-AP, Leu-AP, fMet-AP, Phe-AP, Gly-Pro-Leu-AP, Chy-

TABLE IV. *Changes in Enzymatic Activity in Spleen of EAE*

Enzyme	Specific activity $\pm$ SD (nmol /min/mg protein)			
	1W (N=5)		2W (N=4)	
	Control	Disease	Control	Disease
AP-A	0.86 $\pm$ 0.17	0.52 $\pm$ 0.05 ^{c)}	0.63 $\pm$ 0.23	1.29 $\pm$ 0.27 ^{c)}
AP-B	18.56 $\pm$ 1.97	16.30 $\pm$ 1.14 ^{a)}	15.50 $\pm$ 1.36	18.01 $\pm$ 3.49
Pro-AP	3.57 $\pm$ 0.56	2.15 $\pm$ 0.25 ^{d)}	2.18 $\pm$ 0.36	2.46 $\pm$ 0.70
Leu-AP	15.82 $\pm$ 1.86	12.89 $\pm$ 1.18 ^{b)}	13.43 $\pm$ 1.76	18.46 $\pm$ 1.14 ^{c)}
fMet-AP	1.85 $\pm$ 0.29	1.84 $\pm$ 0.16	1.16 $\pm$ 0.16	2.33 $\pm$ 0.53 ^{a)}
Phe-AP	14.80 $\pm$ 3.12	10.92 $\pm$ 0.63 ^{b)}	11.71 $\pm$ 2.71	20.72 $\pm$ 1.52 ^{c)}
Gly-Pro-Leu-AP	3.07 $\pm$ 0.42	2.08 $\pm$ 0.24 ^{c)}	2.20 $\pm$ 0.33	5.39 $\pm$ 0.63 ^{d)}
Trypsin-like	55.03 $\pm$ 10.74	51.52 $\pm$ 11.21	60.01 $\pm$ 6.16	104.93 $\pm$ 12.11 ^{d)}
Chy-try-like	75.85 $\pm$ 25.93	61.18 $\pm$ 7.42	89.65 $\pm$ 7.83	118.50 $\pm$ 19.77 ^{b)}
Elastase-like	36.55 $\pm$ 3.77	30.49 $\pm$ 1.79 ^{b)}	30.83 $\pm$ 3.83	39.49 $\pm$ 5.09 ^{b)}
Cathepsin C	29.82 $\pm$ 5.82	17.64 $\pm$ 6.05 ^{b)}	24.46 $\pm$ 3.53	67.15 $\pm$ 17.98 ^{c)}
Cathepsin D	0.0166 $\pm$ 0.0016	0.0148 $\pm$ 0.0020	0.0147 $\pm$ 0.0027	0.0378 $\pm$ 0.0061 ^{d)}
Glucosidase	0.71 $\pm$ 0.07	0.63 $\pm$ 0.08	0.65 $\pm$ 0.12	0.74 $\pm$ 0.10
Mannosidase	0.15 $\pm$ 0.05	0.24 $\pm$ 0.08 ^{a)}	0.16 $\pm$ 0.05	0.23 $\pm$ 0.03 ^{a)}
GluNAc-ase	4.72 $\pm$ 0.78	4.70 $\pm$ 0.98	3.91 $\pm$ 0.61	16.44 $\pm$ 4.83 ^{d)}
CK	—	—	—	—
Phosphatase	11.04 $\pm$ 1.01	4.10 $\pm$ 2.19 ^{d)}	5.45 $\pm$ 1.93	9.78 $\pm$ 4.87
Esterase	61.29 $\pm$ 4.66	44.43 $\pm$ 2.75 ^{d)}	48.52 $\pm$ 3.23	57.95 $\pm$ 1.07 ^{d)}

a) $p < 0.1$, b) $p < 0.05$, c) $p < 0.01$, d) $p < 0.001$.

Cathepsin D activity is expressed as absorbance (280 nm)/ min/ mg protein.

try-like, cathepsin C, glucosidase, phosphatase, and esterase in liver; AP-A, glucosidase and phosphatase in spinal cord; Gly-Pro-Leu-AP and elastase-like in serum.

At 2 weeks were significantly increased activities of the following enzymes: AP-A, AP-B, Leu-AP, Phe-AP, Gly-Pro-Leu-AP, trypsin-like, Chy-try-like, cathepsin C, cathepsin D, GluNAc-ase, CK in forelimb muscle; none in heart muscle; AP-B, Gly-Pro-Leu-AP, trypsin-like, cathepsin C, cathepsin D and GluNAc-ase in liver; cathepsin C, cathepsin D and GluNAc-ase in spinal cord; CK in serum. The decreased activities were those of the following enzymes: CK in forelimb muscle; fMet-AP, elastase-like, glucosidase and esterase in heart muscle; Leu-AP, glucosidase, mannosidase and esterase in liver; Pro-AP and elastase-like in spinal cord;

AP-B, Leu-AP, Phe-AP, Gly-Pro-Leu-AP, elastase-like and cathepsin C in serum.

As already mentioned, it was necessary to devise some statistical methods to compare the pattern of enzymatic changes among several organs. For this purpose, we utilized Student's *t*-values which had been already used in Tables II to V to test the statistical difference in enzymatic changes between the control and the diseased animals. The enzymes were ranked according to the magnitude of the *t*-values calculated. The ranking sequence thus made was compared among various organs using the Spearman's coefficients of rank correlation.³⁰⁾

As can be seen in Table VI, the above mentioned similarity of the pattern of enzymatic changes at 1 week after the MBP sensitization was endorsed by the highly significant correlation

TABLE V. Changes in Enzymatic Activity in Kidney of EAE

Enzyme	Specific activity $\pm$ SD (nmol /min/mg protein)			
	IW (N=5)		2W (N=4)	
	Control	Disease	Control	Disease
AP-A	20.78 $\pm$ 5.68	9.68 $\pm$ 0.57 ^{c)}	12.04 $\pm$ 1.74	8.58 $\pm$ 1.48 ^{b)}
AP-B	48.95 $\pm$ 12.23	30.43 $\pm$ 3.73 ^{b)}	32.92 $\pm$ 2.51	29.32 $\pm$ 1.47 ^{b)}
Pro-AP	8.07 $\pm$ 1.42	4.33 $\pm$ 0.55 ^{d)}	4.47 $\pm$ 0.37	5.12 $\pm$ 0.63
Leu-AP	78.03 $\pm$ 9.40	46.02 $\pm$ 3.48 ^{d)}	55.27 $\pm$ 3.56	38.96 $\pm$ 13.70 ^{a)}
fMet-AP	3.04 $\pm$ 0.29	2.43 $\pm$ 0.44 ^{b)}	3.48 $\pm$ 0.53	3.16 $\pm$ 0.67
Phe-AP	135.33 $\pm$ 43.15	66.10 $\pm$ 7.15 ^{c)}	84.15 $\pm$ 7.79	65.92 $\pm$ 25.84
Gly-Pro-Leu-AP	28.34 $\pm$ 7.35	14.84 $\pm$ 1.25 ^{c)}	22.60 $\pm$ 2.78	14.74 $\pm$ 4.88 ^{b)}
Trypsin-like	30.83 $\pm$ 4.27	28.52 $\pm$ 2.14	28.61 $\pm$ 6.83	26.95 $\pm$ 10.28
Chy-try-like	85.16 $\pm$ 5.29	65.72 $\pm$ 10.45 ^{c)}	89.18 $\pm$ 14.75	87.85 $\pm$ 32.96
Elastase-like	31.30 $\pm$ 1.59	27.50 $\pm$ 3.80 ^{a)}	29.28 $\pm$ 0.94	30.55 $\pm$ 2.33
Cathepsin C	11.99 $\pm$ 3.44	6.79 $\pm$ 0.79 ^{b)}	8.80 $\pm$ 1.05	6.81 $\pm$ 0.23 ^{b)}
Cathepsin D	0.0082 $\pm$ 0.0006	0.0066 $\pm$ 0.0008 ^{c)}	0.0094 $\pm$ 0.0005	0.0162 $\pm$ 0.0023 ^{c)}
Glucosidase	0.85 $\pm$ 0.05	0.92 $\pm$ 0.32	0.86 $\pm$ 0.09	0.67 $\pm$ 0.06 ^{b)}
Mannosidase	0.16 $\pm$ 0.04	0.28 $\pm$ 0.04 ^{c)}	0.40 $\pm$ 0.06	0.15 $\pm$ 0.06 ^{c)}
GluNAc-ase	3.16 $\pm$ 0.71	2.86 $\pm$ 0.51	3.09 $\pm$ 0.40	3.49 $\pm$ 0.35
CK	—	—	—	—
Phosphatase	5.61 $\pm$ 0.45	3.58 $\pm$ 0.59 ^{d)}	4.15 $\pm$ 0.67	8.78 $\pm$ 1.28 ^{d)}
Esterase	491.08 $\pm$ 38.92	276.91 $\pm$ 45.01 ^{d)}	554.17 $\pm$ 51.65	470.45 $\pm$ 89.91

a) $p < 0.1$, b) $p < 0.05$, c) $p < 0.01$, d) $p < 0.001$.

Cathepsin D activity is expressed as absorbance (280 nm)/min/mg protein.

between spleen and kidney ($r_s=0.770$). Likewise, spleen showed significant correlations with hindlimb ($r_s=0.723$) and liver ($r_s=0.569$), but not so with forelimb muscle ($r_s=0.230$). In ad-

TABLE VI. *Correlation among Enzymatic Changes in Various Organs of Guinea Pig with EAE (1W)*

Organs	Muscle		Heart	Spleen	Liver	Kidney	Brain	Sp. cord	Serum
	Forelimb	Hindlimb							
Muscle									
Forelimb	—	0.145	-0.056	0.230	-0.012	-0.003	-0.238	-0.174	0.489 ^{b)}
Hindlimb	0.145	—	0.451 ^{a)}	0.723 ^{c)}	0.593 ^{b)}	0.578 ^{b)}	-0.297	-0.363	0.322
Heart	-0.056	0.451 ^{a)}	—	0.346	0.238	0.397	-0.022	0.196	0.061
Spleen	0.230	0.723 ^{c)}	0.346	—	0.569 ^{b)}	0.770 ^{d)}	-0.436 ^{a)}	-0.071	0.456 ^{a)}
Liver	-0.012	0.593 ^{b)}	0.238	0.569 ^{b)}	—	0.525 ^{b)}	-0.583 ^{b)}	-0.240	0.582 ^{b)}
Kidney	-0.003	0.578 ^{b)}	0.397	0.770 ^{d)}	0.525 ^{b)}	—	-0.260	-0.091	0.381
Brain	-0.238	-0.297	-0.022	-0.436 ^{a)}	-0.583 ^{b)}	-0.260	—	0.407	-0.510 ^{b)}
Sp. cord	-0.174	-0.363	0.196	-0.071	-0.240	0.091	0.407	—	-0.141
Serum	0.489 ^{b)}	0.322	0.061	0.456 ^{a)}	0.582 ^{b)}	0.381	-0.510 ^{b)}	-0.141	—

a) $p < 0.1$, b) $p < 0.05$, c) $p < 0.01$, d) $p < 0.001$. Sp. cord: spinal cord.

TABLE VII. *Correlation among Enzymatic Changes in Various Organs of Guinea Pig with EAE (2W)*

Organs	Muscle		Heart	Spleen	Liver	Kidney	Brain	Sp. cord	Serum
	Forelimb	Hindlimb							
Muscle									
Forelimb	—	0.647 ^{c)}	0.368	0.632 ^{c)}	0.706 ^{c)}	-0.020	0.635 ^{c)}	0.331	-0.071
Hindlimb	0.647 ^{c)}	—	0.324	0.480 ^{a)}	0.784 ^{d)}	0.054	0.277	0.248	0.169
Heart	0.368	0.324	—	0.422 ^{a)}	0.328	0.142	0.260	0.414	0.066
Spleen	0.632 ^{c)}	0.480 ^{a)}	0.422 ^{a)}	—	0.346	0.027	0.456 ^{a)}	0.378	-0.069
Liver	0.706 ^{c)}	0.784 ^{d)}	0.328	0.346	—	0.272	0.539 ^{b)}	0.235	0.202
Kidney	-0.020	0.054	0.142	0.027	0.272	—	0.517 ^{b)}	-0.081	0.322
Brain	0.635 ^{c)}	0.277	0.260	0.456 ^{a)}	0.539 ^{b)}	0.517 ^{b)}	—	0.348	0.014
Sp. cord	0.331	0.248	0.414	0.378	0.235	-0.081	0.348	—	0.237
Serum	-0.071	0.169	0.066	-0.069	0.202	0.322	0.014	0.237	—

a) $p < 0.1$, b) $p < 0.05$, c) $p < 0.01$, d) $p < 0.001$. Sp. cord: spinal cord.

TABLE VIII. *Enzymatic Correlations between 2 Stages, 1W and 2W of EAE*

Organs	Correlation	Organs	Correlation
	1W vs. 2W		1W vs. 2W
Forelimb muscle	0.005	Kidney	-0.238
Hindlimb muscle	-0.150	Brain	-0.341
Heart	-0.326	Sp. cord	0.005
Spleen	0.015	Serum	0.065
Liver	0.338		

dition to these, many more significant correlations were seen among the remaining organs and serum. There was no significant correlation between forelimb muscle and hindlimb muscle. The changes in brain was peculiar in that most of its correlations were negative implying the inverse pattern of enzymatic changes in this organ in comparison to other organs. It seems reasonable that its correlation with spinal cord exhibited a positive coefficient.

In Table VII are shown similar correlations at 2 weeks among the several organs and serum. Notably, the correlations of forelimb muscle with hindlimb muscle and with spleen became positive at this time. Because of the alteration in the pattern of enzymatic changes in spleen at 2 weeks, most of the significant correlations which were found at 1 week between this organ and others disappeared. Instead, several other correlations among the remaining organs appeared, reflecting the alteration of the pattern of enzymatic changes during this period. Especially, the changes in brain gained significant correlations with several organs such as forelimb muscle, liver and kidney.

In Table VIII are shown the correlations of the pattern of enzymatic changes between the two stages, 1 week and 2 weeks. No significant correlations were seen, indicating that the pattern of enzymatic changes completely altered during this interval between these two stages.

DISCUSSION

The administration of homogenate of central nervous tissue to susceptible animals is known to cause EAE which resembles human demyelinating diseases such as postvaccinal encephalomyelitis, multiple sclerosis and so on.¹⁻³⁾ In the present study using guinea pigs with MBP-induced EAE, however, extensive enzymatic changes occurred not only in brain and spinal cord but also in skeletal muscle, heart muscle, spleen, liver, kidney and serum. We have to admit that there remain some questions about the specificity of enzymatic changes measured. As long as we are not handling the purified

enzymes from the tissues, we should stay in the limitation that we are just measuring the activities of "ATEE-ase" instead of the activities of "chymotrypsin-like enzymes". Nevertheless, we still believe that this type of study is needed to understand the abnormalities of the enzyme networks in disease models. Of all the enzyme networks the focus was centered around the hydrolytic enzymes in the present study.

At 1 week after the MBP sensitization, many enzymatic activities tended to decrease in most organs except forelimb muscle. At 2 weeks, however, they tended rather to increase. As far as forelimb muscle is concerned, it is highly possible that the enzymatic activities had already decreased, once, at the very early stage and that they had begun to increase thereafter. At the time of 1 week after the MBP sensitization there were significant correlations of enzymatic changes especially between spleen and other organs such as hindlimb muscle, liver and kidney, implying that the pattern of enzymatic changes resembled among these organs. Judging from the insignificant correlations between the enzymatic changes at 1 week and those at 2 weeks, the pattern of enzymatic changes have completely changed during this period. In this connection, it is of particular interest to note that some immunological cellular responses already were observed around 5th to 6th day after the MBP sensitization.^{1,2)} At 2 weeks, most of the significant correlations between spleen and other organs disappeared. But significant correlations between brain and other organs appeared instead. According to another study of ours, the pattern of enzymatic changes in the diseased animals was generally different from that of enzymatic changes in the animals treated with FCA.³²⁾

Now one may offer two questions. First, what was the reason for the similarity of the patterns of the enzymatic changes among several organs either at 1 week or at 2 weeks? Second, what is meant by the complete alteration of the patterns of enzymatic changes during the course of 1 to 2 weeks after the MBP administration? An answer

to the first question may be that similar events simultaneously occurred in those organs which were mutually correlated. These events may be related to some autoimmune processes or to the changes in reticuloendothelial systems. As far as the second question is concerned, one may have to assume that there occurred some changes in the property of pathological processes during the period of 1 to 2 weeks after the sensitization with autoantigen. It is possible that same organ-specific reactions ensue after the systemic reactions induced by this autoimmune process. If so, the organ specific changes will get mixed with the systemic changes which had already occurred, and this may represent the pattern of enzymatic changes in each organ tested at 2 weeks.

Another important question is what is the mechanism of the pathological processes in many organs other than nervous systems. The mechanism may be somehow related to the accumulation of antigen-antibody complexes in most organs or to certain secondary events triggered by the essential autoallergic reactions. Whatever may be the mechanisms for the systemic events induced by MBP injection, they should be worth being studied furthermore. EAE may serve as one of the models for systemic autoimmune diseases.

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