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Limiting-dilution analysis of the frequency of myelin basic protein-reactive T cells in Lewis, PVG/c and BN rats. Implication for susceptibility to autoimmune encephalomyelitis

Y. MATSUMOTO, K. KAWAI, Y. TOMITA & M. FUJIWARA *Department of Immunology, Niigata University School of Medicine, Niigata, Japan*

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SUMMARY

Susceptibility to experimental autoimmune encephalomyelitis (EAE), which is an autoimmune disease inducible by immunization with a brain-specific antigen in complete Freund's adjuvant (CFA), is different among strains. In an attempt to resolve the immune mechanisms by which the difference in susceptibility to EAE is regulated, we re-estimated susceptibility of several strains of rats, and the frequency of antigen-reactive T cells in each strain was determined by limiting-dilution analysis. EAE was induced in Lewis (LEW), PVG/c and BN rats using four different methods: (i) active immunization with guinea-pig myelin basic protein (GPBP) in CFA; (ii) immunization with GPBP in CFA that had been further supplemented with *Mycobacterium tuberculosis* H37Ra (supplemented CFA); (iii) adoptive transfer of GPBP-activated spleen cells into syngeneic rats; and (iv) transfer of a GPBP-specific T-cell line. The LEW strain was susceptible to all four methods. The PVG/c strain was resistant to immunization with GPBP in conventional CFA (GPBP/conv. CFA), but was susceptible to immunization with GPBP in supplemented CFA (GPBP/suppl. CFA) and to transfer of activated spleen cells. The BN strain was resistant to all methods. Limiting-dilution analysis using T cells from LEW, PVG/c or BN rats has revealed that each strain of rat displays a different pattern of frequencies of GPBP-reactive or the 68–88 sequence (GP68–88)-reactive T cells. LEW rats showed relatively high frequencies of GPBP-reactive and GP68–88-reactive T cells after immunization with either GPBP/conv. CFA or GPBP/suppl. CFA, symptomatic rats showing higher values than asymptomatic rats. In asymptomatic PVG/c rats, the frequency of GP68–88-reactive T cells was lower than that of GPBP-reactive T cells. In PVG/c rats with clinical EAE, however, GP68–88-reactive T cells increased in frequency and were almost the same as GPBP-reactive T cells. BN rats, on the other hand, responded very poorly not only to the GP68–88 sequence but also to the whole GPBP molecule, even after immunization with GPBP/suppl. CFA. These findings, obtained by limiting-dilution analysis, strongly suggest that the development of EAE in LEW, PVG/c and BN rats is closely related to the frequency of GPBP-reactive T cells. Furthermore, it is shown that resistance to EAE found in PVG/c and BN rats may be generated by different immune mechanisms.

INTRODUCTION

It is well known that various strains of rats differ in their susceptibility to the induction of experimental autoimmune encephalomyelitis (EAE) (Levine & Wenk, 1961). The Lewis (LEW) strain is susceptible to EAE whereas the Brown Norway (BN) strain has been described as 'resistant'. By using both strains, and their F_1 and backcross progeny, it has been reported that susceptibility to EAE in the rat is controlled by an autosomal dominant gene, designated as Ir-EAE, which is linked to major histocompatibility complex (MHC) (Gasser, Palm & Gonatas, 1975; Gasser *et al.*, 1973; Williams & More, 1973). However, studies of the Lewis-resistant strain (Le-R) (Gasser, Hickey & Gonatas, 1983; Waxman *et al.*, 1981) and careful observations of EAE elicited in (LEW × BN) F_1 and backcross rats (Günther, Odenthal & Wechsler, 1978; Levine & Sowinski, 1975; Moore, Singer & Williams, 1980) have indicated a possibility that, in addition to Ir-EAE, another MHC-unrelated gene might be involved in determining susceptibility or resistance to EAE.

Recent studies using myelin basic protein (MBP)-specific T-cell lines and clones have also contributed to the elucidation of the immune mechanisms involved in the development of EAE. Vandenbark *et al.* (1985a) and Beraud *et al.* (1986) reported that a LEW T-cell line responded to the encephalitogenic 68–88 peptide of guinea-pig MBP (GP68–88), whereas a

Correspondence: Dr Y. Matsumoto, Dept. of Immunology Niigata University School of Medicine, Asahimachi-1, Niigata 951, Japan.

BN T-cell line did not respond to GP68–88 but did respond to guinea-pig MBP (GPBP) outside the 68–88 sequence. Furthermore, a (LEW × BN)F_1 T-cell line, which responded to GP68–88 as long as it was cultured with F_1 antigen-presenting cells (APC), lost its response to GP68–88 when cultured with BN APC. These findings suggest that encephalitogenicity of T cells is strongly influenced by the genotype (probably MHC) of APC.

Instead of using long-cultured T-cell lines, we wished to know what kind of immunological reactions take place in EAE-susceptible or EAE-resistant strains of rats shortly after active immunization. We first re-evaluated the susceptibility to EAE of LEW, PVG/c and BN rats by applying four different methods for the induction of EAE. Then, the frequency and antigen specificity of GPBP-reactive T cells were determined by limiting-dilution assay. It was revealed that LEW rats were susceptible to all four methods, whereas BN rats were resistant. PVG/c rats showed intermediate susceptibility. More importantly, limiting-dilution analysis revealed that susceptibility to EAE in each strain of rat correlated well with the frequency of GPBP-reactive, particularly GP68–88-reactive, T cells.

MATERIALS AND METHODS

Animals

LEW rats were obtained from Charles River Japan Inc. (Kanagawa). PVG/c and BN rats were purchased from Seiwa Sangyo (Fukuoka). (LEW × PVG/c)F_1 (LPVGF1), (LEW × BN)F_1 (LBNF_1) and (PVG/c × BN)F_1 (PVGBNF1) were obtained from Seiwa or bred in the animal facilities of our laboratory. All animals were used at 8–12 weeks of age.

Antigens

GPBP, purified by the method of Deibler, Matenson & Kies (1972), was a gift from Dr Amaya, Department of Neurology, Brain Research Institute, Niigata University. Porcine MBP (PBP) and keyhole limpet haemocyanin (KLH) were obtained from Calbiochem (La Jolla, CA). Ovalbumin (OVA) was obtained from Sigma (St Louis, MO). Purified protein derivative (PPD) was purchased from Mitsui Pharmaceutical Co. (Tokyo). Highly purified GP68–88 (GSLPQKSQRSQDENPVVHF) and GP43–67 (FGSDRAAPKRGSGKDSHHAARTTHY) synthetic peptides were custom-prepared by the Peptide Institute Inc. (Osaka) using a Peptide Synthesizer 430-A (Applied Biosystems Inc.) and purified to 99·8% homogeneity. Preliminary study had revealed that as little as 6 μg of GP68–88 synthetic peptide in conventional CFA elicited EAE in LEW rats.

Active immunization of LEW, PVG/c and BN rats

Rats were immunized with GPBP in two different complete adjuvants. Groups of rats were injected into both hind footpads with an emulsion of an appropriate concentration of GPBP and conventional complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI) containing 1 mg/ml *Mycobacterium tuberculosis* H37Ra. Each rat received 100 μg GPBP and 100 μg H37Ra. Other groups of rats were immunized with GPBP and CFA that had been further supplemented with 10 mg/ml of H37Ra (Difco) (final concentration, 11 mg/ml). In the latter case, each rat received 100 μg GPBP and 1100 μg H37Ra. For the purpose of simplicity, CFA further supplemented with H37Ra will be referred to as supplemented CFA in the present paper.

Adoptive transfer of GPBP-activated spleen cells or GPBP-specific line cells

Adoptive transfer of GPBP-activated spleen cells was achieved as described elsewhere (Matsumoto & Fujiwara, 1987). Spleen cells from the rats previously immunized with GPBP in conventional CFA were cultured with 2 μg/ml GPBP for 3 days. Then, $2–6 \times 10^7$ viable cells were injected i.v. into naive syngeneic animals.

GPBP-specific T-cell lines from LEW and BN rats were established by the method described by Vandenbark *et al.* (1985a) with a few modifications (Matsumoto, Kawai & Fujiwara, 1989). Rats were immunized with 100 μg GPBP in conventional CFA. Ten days later, popliteal lymph nodes were removed and a single-cell suspension was prepared by passing the tissue through a steel mesh screen. Viable cells at a concentration of 10^7 cells/ml in the stimulation medium were cultured in a 24-well plastic plate with 100 μg of GPBP for 3 days. The stimulation medium consisted of RPMI-1640, 1% sodium pyruvate, 1% non-essential amino acids, 5×10^{-5} M 2-mercaptoethanol, 100 μg/ml kanamycin and 1% fresh rat serum. Then, lymphocytes were cultured for 7 days in the propagation medium in the presence of 10% rat concanavalin A (Con A) supernatant, which had been prepared as described previously (Watanabe *et al.*, 1985). The propagation medium contained 10% fetal calf serum instead of 1% rat serum. Cells were restimulated with GPBP and propagated with Con A supernatant. After several cycles of stimulation and propagation, the optimal concentration of GPBP was determined by proliferation assay. For transfer experiments, propagated line cells were always stimulated with 5 μg/ml GPBP for 3 days in the presence of irradiated normal thymus cells, and then $0·1–2 \times 10^7$ cells were transferred into syngeneic animals.

Proliferation assay

Activation of line cells *in vitro* was assayed in microtitre wells by uptake of [^{3}H]thymidine after cultivation of $2–10 \times 10^4$ T lymphocytes and 10^6 irradiated syngeneic thymus cells in the presence of various concentrations of GPBP (1–100 μg/ml) or PPD (12·5 μg/ml). The microwell cultures were incubated for 3 days, the last 24 hr in the presence of 0·5 μCi [^{3}H]thymidine (Amersham International, Amersham, Bucks, U.K.). The cells were harvested on glass fiber filters, and the label uptake was determined using standard liquid scintillation techniques.

Limiting-dilution assay

Limiting-dilution assay was performed on the same time schedule as that of the proliferation assay. LEW, PVG/c or BN rats were immunized with 100 μg of GPBP, KLH or OVA in conventional or supplemented CFA. Ten or 15 days later, the spleen and bilateral popliteal lymph nodes were removed, and a single-cell suspension was prepared. After lysing red blood cells, a T-cell-enriched fraction was obtained by passing cell suspension through a nylon-wool column twice.

For the assay, various concentrations of T cells were cultured in 24 wells of 96-well flat-bottomed culture plates with GPBP (15 μg/ml) or with GP68–88 (4·5 μg/ml) in the stimulation medium containing 1% fresh syngeneic serum or in 12 wells without the antigen for controls. KLH and OVA were used at a concentration of 20 μg/ml. Irradiated normal thymus cells, at a dose of 10^6, were added to each well. They were then cultured, pulsed with [^{3}H]thymidine and harvested as exactly the same

as the proliferation assay. Cultures containing antigens were scored as positive if the c.p.m. exceeded the mean c.p.m. of the control plus 2 SD. Statistical analysis was performed according to Lefkovits & Waldmann (1979) and the frequencies of GPBP-reactive or GP68–88-reactive T cells were calculated. When the frequency could not be calculated precisely because too many or too few antigen-reactive T cells existed in the culture wells, the concentration point of leading the highest or lowest frequency was used to calculate and represented as $1/>X$ or $1/<Y$. In the previous reports, the frequency of MBP-reactive T cells was assayed in the presence of Con A supernatant (Cohen *et al.*, 1987) or after 5-day culture (Fallis *et al.*, 1987). We did not employ these methods, because we thought that the exact frequency could not be obtained after such augmentation.

Assessment of clinical EAE

After immunization with GPBP or transfer of GPBP-activated cells, rats were observed daily for clinical signs of EAE, which were graded into the following categories: flaccid tail, grade 1; mild paraparesis, grade 2; severe paraparesis, grade 3; and tetraparesis or moribund condition, grade 4.

Histological examination

Rats with or without clinical EAE were examined histologically. Symptomatic rats were killed under ether anaesthesia when they showed full-blown EAE, or 1–3 days after this stage. Asymptomatic rats were examined between 15 and 21 days after challenge. The spinal cord was removed and segments from multiple levels were embedded in paraffin or snap-frozen. Paraffin-embedded tissues were sectioned and stained with haematoxylin and eosin (H&E). Frozen tissues were sectioned in a cryostat and stained with monoclonal antibodies specific for various T-cell markers (Matsumoto & Fujiwara, 1986; Matsumoto *et al.*, 1986).

RESULTS

Groups of rats were immunized with 100 μg GPBP emulsified in two different CFA. When immunized with GPBP in conventional CFA, LEW rats developed EAE 9–15 days after immunization. On the other hand, PVG/c and BN rats were resistant to this immunization protocol (Table 1). When rats were immunized with GPBP in another CFA in which H37Ra was further supplemented (supplemented CFA), LEW rats developed more severe EAE with earlier onset of clinical signs. In addition, the PVG/c resistance to active immunization with GPBP/conv. CFA was overcome by the immunization protocol using supplemented CFA (Table 1). BN rats did not develop EAE after immunization with GPBP/conv.CFA and with GPBP/suppl.CFA. F_1 hybrids (LPVGF1, LBNF1 and PVGBNF1) were all susceptible to EAE by active immunization using supplemented CFA. Since the BN strain is resistant to active immunization with supplemented CFA, PVGBNF1 susceptibility may have been attributable to PVG/c susceptibility to GPBP/suppl. CFA (Table 1).

Adoptive transfer of GPBP-activated spleen cells or GPBP-specific T-line cells was done using LEW, PVG/c and BN rats. GPBP-activated spleen cells at doses of $2–6 \times 10^7$ cells were transferred into syngeneic animals. As shown in Table 2, LEW and PVG/c rats developed EAE by transfer of 2×10^7 GPBP-activated syngeneic spleen cells. In contrast, BN rats did not develop clinical or histological EAE even by transfer of 6×10^7 activated spleen cells.

GPBP-specific T-cell lines from LEW and BN rats were established by repeated stimulation of sensitized lymph node cells (LNC) with GPBP. In the presence of irradiated syngeneic thymus cells, LEW line cells responded significantly to both the whole GPBP molecule and the GP68–88 sequence, as reported previously (Vandenbark *et al.*, 1985a, b). BN line cells, on the other hand, responded to GPBP and PBP, but not to GP68–88 and showed the only marginal response to GP43–67 (Fig. 1). We used PBP in the proliferation assay to know whether the line cells react with the 89–169 sequence of GPBP because the amino acid sequence of GPBP and PBP has a homology in the 89–169 sequence but not in the 43–67 and 68–88 sequences (Martenson,

Table 1. Active immunization with GPBP*

Strain	H37Ra in adjuvant (mg/ml)	Clinical EAE	Onset	Max. clinical score	Histological EAE
LEW	1	10/12	9–15	2·7	8/8
	11	5/5	10	3·1	5/5
PVG	1	0/4	—	—	0/4
	11	3/3	11	4·0	3/3
BN	1	0/3	—	—	0/3
	11	0/4	—	—	0/4
LPVGF1	11	13/13	9–10	3·1	9/9
LBNF1	11	6/6	9–10	ND†	6/6
PVGBNF1	11	6/6	9–10	3·0	6/6

*Each rat received 100 μg GPBP and 0·1 ml complete adjuvant divided into two hind footpads.

†Not determined because rats were killed for histology before expression of maximal signs.

Table 2. Adoptive transfer of GPBP-activated spleen cells* or line cells† into syngeneic rats

Strain	Cells transferred	($\times 10^{-7}$)	Clinical EAE	Onset	Max. clinical score	Histological EAE
LEW	Spleen	2	7/7	4·4	3·0	5/5
	Line	0·1–1·0	9/9	4·2	3·5	6/6
PVG/c	Spleen	2–6	5/5	6·5	ND	5/5
BN	Spleen	3–6	0/7	—	—	0/5
	Line	0·5–1·5	0/5	—	—	5/5

*Spleen cells from rats previously immunized with GPBP in conventional CFA were cultured with GPBP for 3 days and the indicated numbers of activated spleen cells were transferred i.v. into syngeneic rats.

†GPBP-specific T-cell line was established by repeated antigen stimulation and propagation of lymph node cells from GPBP-immunized rats with a optimal concentration of GPBP. The indicated numbers of T-line cels were transferred into syngeneic animals after 3-day stimulation with GPBP.

ND, not determined.

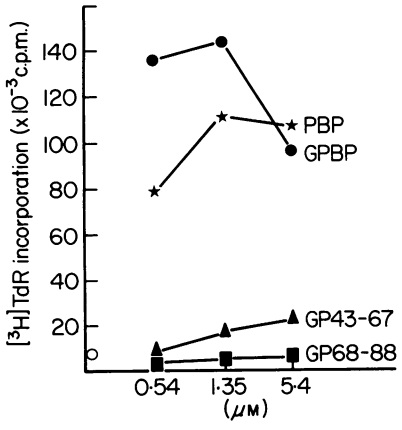

Figure 1. Proliferative responses of BN line cells to GPBP (●), PBP (★), GP43–67 (▲) and GP68–88 (■). Open circle indicates the response of line cells in the absence of antigen. Proliferation was measured after the third cycle of stimulation and propagation.

1984) and because LNC from GP43–67- or GP68–88-immunized rats did not respond to PBP (our unpublished data). The response pattern of BN line cells indicates that the immunodominant epitope for BN rats lies within the 89–169 sequence of GPBP. After 3-day culture with GPBP, line cells were washed and transferred into syngeneic rats. As shown in Table 2, LEW rats developed full-blown EAE after transfer of as little as 1×10^6 line cells, while GPBP-specific BN line cells did not elicit clinical EAE even at a dose of $1{\cdot}5 \times 10^7$ cells.

Histological examination of the spinal cords from symptomatic and asymptomatic rats (the numbers of rats examined are shown in Tables 1 and 2) were carried out using H&E sections. All the symptomatic rats had moderate to severe inflammatory lesions in the spinal cords, whereas most of the asymptomatic rats had no lesions (Tables 1 and 2). One exception was the case of BN rats that had received GPBP-reactive T-line cells. Histological examination of the spinal cord from these rats revealed that there was mild inflammation in all cases (Table 2).

In order to know whether such a difference in susceptibility to EAE is related to the frequency of antigen-reactive T cells, limiting-dilution analysis was performed. In the first set of experiments, the frequencies of GPBP-reactive or GP68–88-reactive T cells in the spleens or regional lymph nodes were determined and compared. Five LEW rats were immunized with GPBP in conventional CFA (nos 1–3, Table 3) or in supplemented CFA (nos 4 and 5, Table 3), and examined on Days 10 and 15. In both GPBP/conv. CFA and GPBP/suppl. CFA groups, more GPBP-reactive or GP68–88-reactive T cells were generally found in the regional lymph nodes than in the spleens (Table 3). Since it was predicted that the frequency of the antigen-reactive T cells would be much lower in relatively resistant (PVG/c) or resistant (BN) rats, we decided to use LNC in further experiments. In the separate experiment, we confirmed that the immunization protocols employed in this study induced sufficient proliferative responses of LNC from immunized LEW, PVG/c or BN rats to PPD. Using LNC, the frequencies of GPBP-reactive or GP68–88-reactive T cells in LEW, PVG/c or BN rats were assayed on Days 10 and 15 after immunization. As shown in Table 4, each strain of rats showed a very characteristic pattern. In LEW rats, the frequencies of GPBP-reactive T cells of asymptomatic animals (nos 1 and 2, Table 4) were 1/105,000 and 1/51,000, whereas the rat with clinical EAE (no. 4) showed a higher frequency (1/12,000). Rats at the recovery stage (nos 3 and 5) showed intermediate values. The most characteristic finding in LEW rats was that the frequencies of GP68–88-reactive T cells were almost the same as those of GPBP-reactive T cells in four out of five cases (nos. 1, 2, 4 and 5), suggesting that the majority of GPBP-reactive T cells are reactive with the GP68–88 sequence.

PVG/c rats (nos 6–11, Table 4) showed a different pattern. As described earlier, PVG/c rats do not develop clinical EAE when immunized with GPBP/conv.CFA, but develop EAE after

Table 3. Frequency of GPBP- or GP68–88-reactive T cells in the spleen and regional lymph nodes of Lewis rats*

Animal no.	Immunization	Day examined	Clinical score	Antigen tested	Frequency (1×10^{-3}) Spleen	Lymph node
1	GPBP/conv. CFA	10	0	GPBP	1/1712	1/105
				GP68–88	1/606	1/181
2		15	0	GPBP	1/>115	1/51
				GP68–88	1/197	1/42
3		15	1IR†	GPBP	1/38	1/48
				GP68–88	1/73	1/196
4	GPBP/suppl. CFA	10	3	GPBP	1/<19	1/12
				GP68–88	1/<13	1/<8
5		15	1R	GPBP	1/164	1/68
				GP68–88	1/498	1/36

*T-cell-enriched fraction was prepared from the spleens or lymph nodes of GPBP-immunized LEW rats by passing through a nylon-wool column twice. Graded numbers of T cells were cultured with GPBP or GP68–88 in the presence of syngeneic thymus cells. They were pulsed with [^{3}H]thymidine on Day 2, and harvested on Day 3. Cultures containing antigens were scored positive if the c.p.m. exceeded the mean c.p.m. of the control plus 2 SD.

†R, recovery.

Table 4. Frequency of GPBP- or GP68–88-reactive T cells in the regional lymph nodes of Lewis, PVG/c or BN rats

Strain	Animal no.*	Immunization	Day examined	Clinical score	Frequency (1×10^{-3}) GPBP	GP68–88
LEW	1	GPBP/conv. CFA	10	0	1/105	1/181
	2		15	0	1/51	1/42
	3		15	1R	1/48	1/196
	4	GPBP/suppl. CFA	10	3	1/12	1<8
	5		15	1R	1/68	1/36
PVG/c	6	GPBP/conv. CFA	10	0	1/39	1/228
	7		15	0	1/41	1/353
	8	GPBP/suppl. CFA	10	0	1/162	1/398
	9		10	0	1/61	1/167
	10		15	3	1/52	1/75
	11		15	3	1/40	1/61
BN	12	GPBP/conv.CFA	10	0	1/274	1/208
	13		15	0	1/612	1/1127
	14	GPBP/suppl.CFA	15	0	1/915	1/2939

*Nos 1–5 animals are the same as those in Table 3.

immunization with GPBP/suppl.CFA. The frequencies of GPBP-reactive T cells ranged from 1/39,000 to 1/162,000. There was no significant difference between GPBP/conv. CFA and GPBP/suppl. CFA groups (nos 6 and 7 versus 8–11, Table 4) or between symptomatic and asymptomatic rats (nos. 6–9 versus 10 and 11). GP68–88-reactive T cells, on the other hand, were less frequently seen than GPBP-reactive T cells in the asymptomatic rats after immunization with GPBP/conv. CFA, which did not induce EAE in the PVG/c strain (nos 6 and 7). In

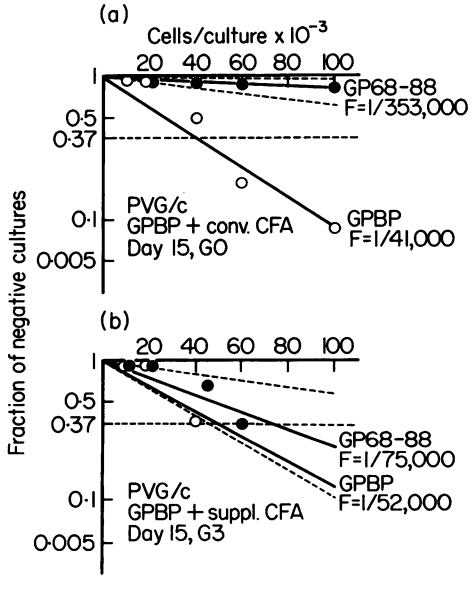

Figure 2. Limiting-dilution analysis of the frequencies of GPBP-reactive or GP68–88-reactive T cells from PVG/c rats 15 days after immunization with GPBP/conv. CFA (a) or with GPBP/suppl.CFA (b). Dashed lines indicate 95% confidence limits.

Table 5. Frequency of KLH- or OVA-reactive T cells in LEW or BN rats

Strain	Immunization	CFA	Day examined	Frequency (1×10^{-3}) KLH	OVA
LEW	KLH	Conv.	10	1/22	—
	KLH	Suppl.	10	1/29	—
BN	KLH	Conv.	10	1/20	—
	KLH	Suppl.	10	1/29	—
LEW	OVA	Conv.	15	—	1/85
	OVA	Suppl.	15	—	1/43
BN	OVA	Conv.	15	—	1/57
	OVA	Suppl.	15	—	1/63

contrast, the frequencies of GP68–88-reactive T cells in rats with clinical EAE (nos 10 and 11) were significantly higher than those in asymptomatic rats after immunization with GPBP/conv. CFA (Fig. 2). PVG/c rats that had been immunized with GPBP/suppl. CFA but were still asymptomatic showed low (no. 8) and intermediate (no. 9) values. Collectively, these findings suggest that PVG/c rats do not develop EAE unless GP68–88-reactive T cells increase in number to a certain level.

BN rats (nos 12–14, Table 4) did not develop EAE even when immunized with GPBP/suppl.CFA. Compared with LEW or PVG/c rats, the frequencies of both GPBP-reactive and GP68–88-reactive T cells were very low. This result, however, raised the possibility that BN rats are general low responders, not only to GPBP and GP68–88 but also to other antigens. If so, analysis of BN rats would not be useful for the study on susceptibility to EAE. To address this question, we did a similar analysis using well-characterized antigens, i.e. KLH and OVA. As shown in Table 5, the frequencies of KLH-reactive T cells ranged from 1/20,000 to 1/29,000. There was no significant difference between LEW and BN rats. OVA-reactive T cells were less frequently seen than KLH-reactive T cells, but again no significant difference was noticed. These findings suggest that BN rats are not always low responders and that low responsiveness of BN rats to GPBP and GP68–88 results from specific immunological reactions.

DISCUSSION

Experimental allergic encephalomyelitis (EAE) is a T-cell-mediated autoimmune disease that is inducible in various animal species by immunization with brain-specific antigens such as MBP. Upon immunization with MBP, either rats or mice show strain-specific susceptibility to EAE. In the rat, the LEW strain is susceptible to EAE, whereas the BN and PVG/c strains are reported to be resistant (Ben-Nun, Eisenstein & Cohen, 1982; Hughes & Stredronska, 1972). The immune mechanisms by which susceptibility to EAE, especially its difference among strains, is regulated have been investigated using fragmented GPBP molecules (Chou *et al.*, 1977; Kibler *et al.*, 1977) or using GPBP-reactive T-cell lines and clones (Happ & Heber-Kats, 1988; Vandenbark *et al.*, 1985a, b). Most investigations believe that EAE-resistant rats do not respond to

the key peptide of the GPBP molecule that is encephalitogenic to the susceptible strain. In the present study, we intended not only to study the reactivity of various strains of rats to the encephalitogenic peptide and the whole GPBP molecule, but also to analyse this issue quantitatively.

In order to approach this, we first re-estimated susceptibility to EAE of LEW, PVG/c and BN rats using several induction protocols. Regarding LEW and BN rats, the results obtained were essentially the same as those reported previously (Hugh & Stredonska, 1972). The LEW strain was highly susceptible to all the induction protocols, whereas the BN strain was resistant. One exception for BN rats is that transfer of GPBP-reactive T-line cells elicited histological EAE in this study. PVG/c rats have revealed a different feature from that reported previously. It is already known that the PVG/c strain is resistant to active immunization with GPBP in conventional CFA and susceptible to transfer of GPBP-reactive T-line cells (Ben-Nun *et al.*, 1982). In addition, we have shown here that PVG/c rats developed EAE after immunization with GPBP in supplemented CFA and transfer of activated spleen cells. Since BN rats did not develop EAE by the latter two induction protocols, susceptibility of PVG/c rats to EAE is ranked between that of LEW and BN rats.

After confirming susceptibility of three different strains of rats to EAE, the frequencies of GPBP-reactive or GP68–88-reactive T cells were determined using these rats at various stages of EAE. In the experiments using LNC from immunized LEW rats, the frequency of GPBP-reactive T cells ranged from 1/12,000 to 1/105,000. The values obtained from symptomatic rats were higher than those from asymptomatic rats. The results obtained by our assay system do not contradict those reported previously using SJL mice (1/46,000–1/328,000) (Fallis *et al.*, 1987) and LEW rats (1/8,300 and 1/25,000 in LNC from GPBP-alone-immunized rats) (Cohen *et al.*, 1987). The latter authors reported slightly higher values, probably because they assayed in the presence of IL-2. Limiting-dilution analysis has revealed that each strain of rats showed a very characteristic pattern after immunization with GPBP. The first pattern was seen in LEW rats. The frequencies of GP68–88-reactive T cells were almost the same as those of GPBP-reactive T cells, and symptomatic rats showed higher values than asymptomatic rats. The second pattern was seen in PVG/c rats. The frequency of GP68–88-reactive T cells was lower than that of GPBP-reactive T cells in asymptomatic rats. By contrast, in symptomatic rats, both values became almost the same. These interesting findings suggest several possibilities. First, when immunized with GPBP/conv. CFA, most of the GPBP-reactive T cells may react with the GPBP molecule outside the 68–88 sequence. Second, such non-68–88-reactive T cells may not be encephalitogenic to PVG/c rats, because PVG/c rats immunized with GPBP/conv. CFA did not develop EAE even though the frequencies of GPBP-reactive T cells were almost the same as those of PVG/c rats showing clinical EAE after immunization with GPBP/suppl. CFA. Finally, the frequencies of GPBP-reactive or GP68–88-reactive T cells of PVG/c rats with EAE were still lower than the frequency of LEW rat with full-blown EAE (no. 4). Taken together, PVG/c rats show intermediate susceptibility to EAE, and the development of EAE depends upon appearance of GP68–88-reactive T cells. Therefore, the mechanism of EAE development is fundamentally the same as that in LEW rats, but PVG/c rats need stronger stimulation probably because of impairment in the process of antigen recognition and/or antigen presentation. The third pattern seen in BN rats, which were all asymptomatic, is that the frequencies of both GP68–88-reactive and GPBP-reactive T cells were much lower than those of asymptomatic LEW and PVG/c rats.

Little is known about the immune mechanisms which regulate resistance to EAE. Based on the data obtained from this study, it has been suggested that there are at least two types of resistance. One is that as seen in PVG/c rats, resistance (to conventional active immunization) is overcome by the stronger immunization protocol. The increase of GP68–88-reactive T cells is the key step for the development of EAE. The other type of resistance is seen in BN rats. Both GPBP-reactive and GP68–88-reactive T cells are extremely few, even when using the stronger immunization protocol. The latter type of resistance is usually explained as follows. When immunized with GPBP, BN rats respond to immunization and GPBP-reactive T cells emerge. In contrast to LEW T cells, which preferentially recognize the highly encephalitogenic GP68–88 sequence, BN T cells recognize weakly encephalitogenic epitopes outside the 68–88 sequence. Thus, BN rats are resistant to active immunization (Beraud *et al.*, 1986; Vandenbark *et al.*, 1985b). Limiting-dilution analysis done in the present study revealed two interesting findings which provide further information to the mechanism of BN resistance. One is that BN rats developed a very small number of GP68–88-reactive T cells after immunization with either GPBP/conv. CFA or GPBP/suppl. CFA. This may be the results of impairment in the presentation of the 68–88 sequence by BN APC for two reasons. First, established GPBP-reactive T-line cells from BN rats do not respond to the 68–88 sequence of the GPBP molecule (Vanderbark *et al.*, 1985b; our observation). Second, LNC from the LEW.1N strain, which is a congenic rat with RT1^n haplotype on LEW background, also does not respond to GP68–88 after immunization with GPBP or GP68–88 (Happ *et al.*, 1988; our unpublished observation). Another interesting finding was that lymph node T cells from immunized BN rats responded very poorly to the whole GPBP molecule. As shown in this study, the immunodominant epitope for BN rats lies within the 89–169 sequence of GPBP, and BN line cells responded strongly to the immunodominant epitope (GP89–169) in the presence of syngeneic APC. These findings indicate that there is neither a hole in the T-cell repertoire nor impairment in the presentation of GP89–169. Low responsiveness of lymph node T cells to GPBP may, thus, be the result of the small clonal size. Although it was previously speculated that BN resistance to active immunization was due to appearance of T cells reactive with the less encephelitogenic sequence of GPBP, the present study has clearly demonstrated that the resistance is primarily attributable to the poor response to the whole GPBP molecule.

In the present study, we re-evaluated susceptibility to EAE of three strains of rats by four methods and found that these strains showed a different pattern of EAE development to each other. More importantly, such a difference in susceptibility to EAE is closely related to the frequency of GPBP-reactive T cells.

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