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SPECIFICITY OF T LYMPHOCYTE LINES FOR PEPTIDES OF MYELIN BASIC PROTEIN[1]

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T lymphocyte lines specific for myelin basic protein (BP) can mediate experimental autoimmune encephalomyelitis (EAE), or can protect against the active induction of the disease. To investigate the antigenic fine specificity of guinea pig (GP) BP-specific T cell lines raised from different rat strains, and to determine whether functionally different T lymphocyte lines and clones recognized the same or different regions of the BP molecule, the proliferation responses of line cells were assessed after stimulation with purified peptides of GP-BP.

Lewis rat T cell lines and clones selected for responses to whole GP-BP responded selectively to the 68-88 amino acid sequence of GP-BP, but not to the 1-37, 43-67, or 89-169 sequences. The region of GP-BP recognized by Lewis T cells was additionally defined to include the 75-80 amino acid sequence, because a T cell clone responded equally to GP and rat BP which differed by only one amino acid at position 79, but did not respond to human or bovine BP, which had a Gly-His insertion in this region. T lymphocyte lines derived from the F344 and PVG (Weizmann) rat strains shared the same selective response to peptide 68-88, but lines from BN rats responded to an epitope(s) outside of the 68-88 sequence. The functional capacity of the various T cell lines to mediate experimental autoimmune encephalomyelitis (EAE) or to induce resistance against EAE was independent of their specificity for the different GP-BP peptides; lines specific for epitope(s) within or excluded from the 68-88 sequence could be encephalitogenic depending on their strain of origin, and various lines specific for the 68-88 peptide could induce both disease and protection, disease only, or neither activity.

Experimental allergic (autoimmune) encephalomyelitis (EAE)[3] is a paralytic disease induced by immunization with myelin basic protein (BP) in a suitable adjuvant (1). In Lewis rats, the major encephalitogenic region resides within the 68-88 amino acid sequence of guinea pig (GP) or rat BP (2), and a weaker encephalitogenic region that is present on bovine BP appears to lie outside the 68-88 region (3). Other regions of BP are antigenic and can induce delayed-type hypersensitivity reactions, although they have no encephalitogenic activity (4). When BP is injected without adjuvant, it can protect the animal against subsequent encephalitogenic challenges of BP in complete Freund's adjuvant (CFA) (5). This protection can be transferred to naive recipients with peritoneal exudate cells that apparently also respond to a determinant within the 68-88 peptide (6).

The study of EAE has been aided by the development of BP-specific T lymphocyte lines. These lines have been obtained from rats immunized with BP in CFA, and were selected for their ability to proliferate specifically to BP in vitro (7). In vivo, the BP-specific lines were able to induce paralytic EAE in naive recipient rats. Encephalitogenic T cell lines possess an additional important biologic property; rats that had recovered from line-mediated EAE, or that had received attenuated (x-irradiated or mitomycin-treated) line cells, acquired resistance to subsequent attempts to actively induce EAE with BP in CFA. Resistance to EAE induced by exposure to anti-BP line cells has been termed vaccination (8). Encephalitogenic anti-BP T cell lines have been developed from strains of rats genetically resistant to actively induced EAE (BN, F344, PVG-Weizmann) (9), as well as from susceptible strains. Thus, functional T lymphocyte populations of different immune response genotypes are available for comparison. In addition, from a single line that both mediates and protects against EAE, it has been possible to derive clones of anti-BP T lymphocytes that are encephalitogenic but apparently not protective (10).

These results raised the question of whether lines or clones from different strains of rats, or with different biologic functions, recognized the same regions of the BP molecule. Specifically, we wished to learn whether recognition of a specific region of BP was associated with the encephalitogenic or vaccinating properties of an anti-BP T lymphocyte population, and whether encephalitogenic lines derived from different rat strains recognized distinct regions of the BP molecule. Accordingly, we measured the proliferative responses of a variety of BP-specific T cell lines and clones to purified peptide fragments of GP-BP.

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[3] Abbreviations used in this paper: BP, myelin basic protein; EAE, experimental autoimmune encephalomyelitis; GP-BP, guinea pig basic protein; BN, Brown Norway.

The results indicated that a restricted region of BP was recognized preferentially over other available determinants when the T lymphocytes were selected for their response to the whole BP molecule. The identity of the dominant epitope(s) appeared to be influenced by the genotype of the rat from which the lines were derived. Moreover, different lines or clones that recognized the same peptide were found to vary in their ability to induce EAE or to protect against EAE.

MATERIALS AND METHODS

Animals. Animals used in these studies were inbred 2- to 3-mo-old Lewis, BN, F344, and PVG rats obtained from the Weizmann Institute (Rehovot, Israel) animal facility.

T Cell Lines. T cell lines were selected from the draining (popliteal) lymph nodes of animals immunized in the hind foot pads (0.2 ml total) with 25 μg GP-BP emulsified in CFA containing 500 μg/rat *Mycobacterium tuberculosis* (7). The lymph nodes were removed and were trimmed free of connective tissue and fat, and were minced through a 60-mesh wire screen. The cell suspension was washed 2× in phosphate-buffered saline and was resuspended at 6.6×10^6/ml in "stimulation medium" consisting of Dulbecco's modified minimum essential medium containing antibiotics, 1 mm L-glutamine, 5×10^{-5} M 2-mercaptoethanol (2-ME), and 1% fresh autologous rat serum. Six milliliters of cells were stimulated for 3 days with 200 μg BP per 6 cm Petri plate incubated at 37°C and 10% CO_2. The stimulated cell suspension was then additionally expanded in "growth medium" containing 10% heat-inactivated horse serum, antibiotics, glutamine, and 2-ME as above, and 5% supernatant taken after 24 hr from concanavalin A (5 μg/ml)-stimulated rat spleen cultures (containing 5×10^6 spleen cells/ml of the stimulation medium described above). The T cell lines were maintained in growth medium until the rate of division slowed (usually 4 to 7 days, initially). The cells were then restimulated with BP presented on x-irradiated thymocytes (2500 R). Line cells (5×10^6) were added to 1.25×10^8 thymocytes in 10 ml "stimulation medium" containing 200 μg BP, and the suspension was incubated for 3 days. The T cell lines were alternately stimulated and expanded until proliferation was restricted to BP. T cell clones were derived by limiting dilution of activated Zla line cells on a 96-well plate. The Zla line and each clone were phenotyped by using the W 3/13 (anti-T total), W 3/25 (anti-T helper), OX-8 (anti-T nonhelper) monoclonal antibodies, and a fluorescence-activated cell sorter, as described (7).

Proliferation assay. The proliferation response of the T cell lines was measured by incorporation of [^{3}H]methyl-thymidine. Viable T cells (2×10^4) were cultured with 2×10^6 x-irradiated thymocytes (2500 R) per well of a 96-well microtiter plate (Costar Data Packaging, Cambridge, MA or Nunc, Copenhagen, Denmark). Ten microliters of antigen or saline as a control were added in triplicate wells to give a total vol of 210 μl/well, and the plates were incubated for 72 hr at 37°C in 10% CO_2, the last 18 hr with 1 μCi [^{3}H]Tdy (43 Ci/mM, New England Nuclear, Boston, MA). The cells were harvested on glass fiber filters, and [^{3}H]Tdy uptake was assessed by standard liquid scintillation procedures.

Activity of T cell lines in vivo. Various T cell lines were evaluated for their ability to induce clinical EAE or to vaccinate against the induction of active EAE by using GP-BP in CFA. Recipient rats received an i.v. injection of 5×10^4 to 10^7 viable T cells from various lines or clones 3 days after stimulation with antigen presented by x-irradiated thymocytes. Clinical signs scored as no signs (0), limp tail (1+), ataxia (2+), severe paralysis (3+), or death (4+), and were evident from 3 to 7 days after injection. The ability of the T cell lines or clones to vaccinate was evaluated 10 days after recovery by a second injection of 25 μg GP-BP in CFA in the hind footpads (0.1 ml per injection site). Protection was measured by comparing the severity of actively induced EAE in the T cell lines of recipient rats vs normal control rats [8].

Preparation of GP-BP Fragments. GP-BP fragments 1-37, 43-88, and 89-169 were prepared by using a limited pepsin digestion method as described (2). GP-BP (100 mg) was incubated with 0.2 mg of pepsin at pH 3.0 for 1 hr at 40°C, and the lyophilized digest was chromatographed on CM-52 with a linear NaCl gradient in 0.02 M ammonium bicarbonate, pH 7.5. The fragments were additionally purified on Sephadex G-50 in 0.01 N HCl. Approximately 10 mg of each fragment were obtained from 50 mg BP.

Fragments 43-67 and 68-88 were obtained by splitting the 43-88 fragment at tyrosine 67 with a-chymotrypsin (2). Ten milligrams of the 43-88 fragment were dissolved in 10 ml 0.02 M ammonium bicarbonate, pH 8.0, and were stirred gently at 25°C for 1 hr with 10 μg a-chymotrypsin. The reaction was stopped by lyophilization, and the digest was chromatographed on CM-52 in 0.02 M ammonium bicarbonate, pH 6.5, at 25°C, with a linear NaCl gradient. The two major peaks were pooled separately, were lyophilized, and were purified additionally on Sephadex G-50.

The purity of the fragments was evaluated by amino acid and tryptic peptide map analysis (2). Tryptic digests were subjected to two-dimensional paper chromatography and electrophoresis at pH 6.5. The papers were stained with trinitrobenzene sulfonic acid and were viewed against UV light. Peptide spots were eluted from the paper in 0.01 N HCl and were subjected to amino acid analysis. Samples for amino acid analysis were hydrolyzed in 6 N HCl in sealed, evacuated tubes at 110°C for 48 hr, and were analyzed on a Beckman Model 120C Amino Acid Analyzer with a 2 hr accelerated mode.

RESULTS

BP-specific T cell line from Lewis rats responds selectively to peptide 68-88. In previous studies, we have shown that the BP-specific T lymphocyte line Zla, derived from Lewis rats, was found to possess both encephalitogenic and vaccinating activities (8). However, when tested similarly, clones derived from the Zla line could induce EAE but could not induce protection, suggesting that encephalitogenicity and vaccination are produced by different cells or combinations of cells within the line. To evaluate whether the Zla line could recognize multiple determinants within the BP molecule, the line cells were stimulated with whole GP-BP, with the GP-BP peptides 1-37, 43-88, 43-67, 68-88, and 89-169, and with bovine BP. As is shown in Figure 1, the Zla line responded selectively to whole GP-BP and to the 43-88 and 68-88 peptides at the lower concentrations, but not to bovine BP. Responses observed to other GP-BP fragments at the higher concentrations may be explained by a 1% contamination with the 68-88 fragment.

T cell clones derived from the Zla line respond selectively to the 68-88 peptide. To additionally study whether the Zla line responded selectively to the 68-88 region of GP-BP, the responses of individual clones derived from the line were tested with the five GP-BP peptides. As is shown in Table I, the Zla line and each of the six clones responded selectively to the 43-88 and 68-88 peptides, regardless of the differences in their functional abilities in vivo. The Zla line both induced and vaccinated against EAE, clone 2 had neither activity, and clones G6 and D9 induced EAE but had no vaccinating ability.

To determine whether multiple subpopulations within the Zla line could be detected by cell surface phenotyping, the line and each of the clones were phenotyped by using

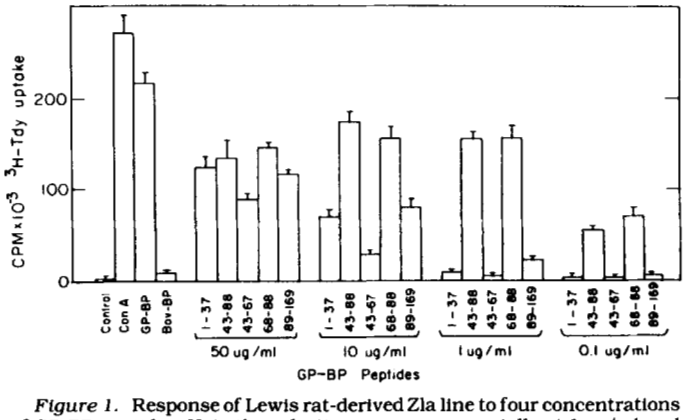

Figure 1. Response of Lewis rat-derived Zla line to four concentrations of GP-BP peptides. Note the selective response, especially at 1 μg/ml and 0.1 μg/ml, to the 43-88 and 68-88 peptides, and the lack of response to bovine BP (Bov-BP).

TABLE I

T cell clones derived from the Zla line respond selectively to the 68-88 peptide

T cells	Activity In Vivo		Response[c] (cpm × 10^{-3}) to							
	Passive EAE[a]	Protection[b]	No Antigen	BP	PPD	1-37	43-88	89-109	43-67	68-88
Zla line	26/26	15/20	1	214	3	10	153	24	4	153
Zla clone										
2	0/4	0/4	1	103	1	3	82	4	1	92
4	5/5	ND	1	194	2	12	269	9	5	295
5	5/5	ND	3	191	1	14	238	14	7	257
G6	16/16	0/12	2	283	2	5	217	4	4	236
7	5/5	ND	1	162	2	14	151	32	4	149
D9	30/30	0/10	1	207	2	20	199	15	10	198

[a] Incidence of clinical EAE produced by i.v. inoculation of 5 to 10 × 10^6 activated T lymphocytes into naive Lewis rats as described (7).

[b] Vaccination-induced resistance to active EAE. Rats were inoculated i.v. with 5 to 10 × 10^6 irradiated (1500 R) or intact activated T lymphocytes to vaccinate them against active EAE. Ten to 14 days later, they were challenged with BP/CFA and the incidence of rats resistant to development of EAE was recorded. ND, not done. (8).

[c] T cells (2 × 10^4) were incubated with 2 × 10^6 irradiated thymocytes and 1 μg/ml of each peptide in triplicate cultures for 72 hr. Replicate cultures varied less than 10% from the mean value. PPD, purified protein derivative.

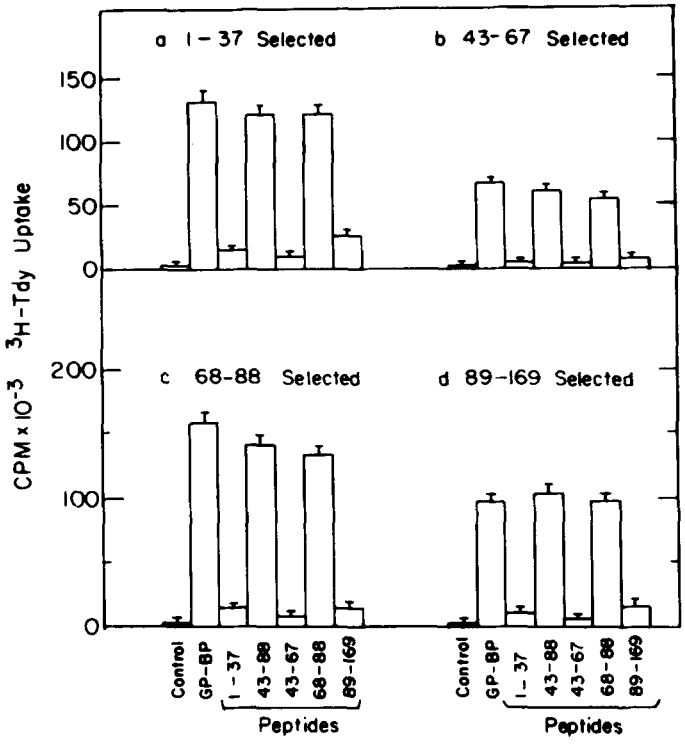

Figure 2. Response of Zla line cells to GP-BP peptides after two rounds of subselection with each respective GP-BP peptide. Note that in spite of attempts to subselect for clones that respond to non68-88 peptides, all cultures retained specificity for only the 68-88 peptide.

monoclonal antibodies to the T helper (W3/25) and the T nonhelper (OX-8) determinants. The Zla line and all of the clones were more than 95% positive for the W 3/25 marker, and were uniformly negative for the OX-8 marker.

In an attempt to rescue silent clones responsive to non-68-88 peptides, Zla line cells were stimulated twice with the 1-37, 43-67, 68-88, and 89-169 peptides, and the responding cells were expanded in the growth medium. This method of subselection should allow responses to minor determinants to be detected. However, as is shown in Figure 2, the Zla sublines still responded selectively to the 68-88 peptide, and no new specificities could be observed. After the second stimulation, the sublines could not be additionally expanded, and were discarded due to the lack of viable cells.

To additionally define the encephalitogenic determinant for Lewis T lymphocytes, the D9 clone derived from the Lewis Zla line was stimulated with BP of known amino acid differences within the 68-88 amino acid sequence (Table I). The D9 clone responded equally to GP and rat BP that differed only at residue 79, but did not respond at all to human or bovine BP, which had a Gly-His insertion as well as other amino acid differences (Table II). Additionally, clone D9 did not respond to the 79-88 sequence of GP-BP (data not shown).

Lewis rat T cell line recognition of non-68-88 peptides. Although the 68-88 sequence appeared to contain the dominant epitopes of the whole GP-BP in Lewis rats, it was of interest to determine whether other fragments could be recognized by Lewis T cell lines. Responses to other regions of GP-BP could be obtained when the whole GP-BP molecule was not used for immunization. By immunizing Lewis rats with the 43-67 peptide in CFA, and by stimulating the draining lymph node cells with only the 43-67 peptide in vitro, a T cell line that responded selectively to the 43-67 region was produced (Table III). The injection of 10 × 10^6 43-67 peptide-activated T cells into four recipients failed to induce clinical EAE or protection against actively induced EAE.

PVG and F344 rat strains respond to the 68-88 determinant. Encephalitogenic T cell lines were derived from the F344 (Fisher) and the PVG (Weizmann) rat strains, even though these strains resist active induction of EAE. When these lines were evaluated for their re-

TABLE II

Response of the D9 T cell clone to basic proteins with known amino acid sequence differences in the major encephalitogenic region for Lewis rats

Antigen in Culture[a]	BP Amino Acid Sequence[b]	Response[a] (cpm × 10^{-3})
None		2 ± 0
	Position 79	
GP-BP	-Lys-Ser-Gln-(−)-(−)-Arg-Ser-Gln-	87 ± 2
Rat-BP	-Lys-Ser-Gln-(−)-(−)-Arg-Thr-Gln-	83 ± 2
Human-BP	-Lys-Ser-(−)-Gly-His-Arg-Thr-Gln-	3 ± 1
Bovine-BP	-Lys-Ala-Gln-Gly-His-Arg-Pro-Gln-	2 ± 1

[a] D9 T cell clone cells (2 × 10^4) were incubated with 50 μg/ml BP and 2 × 10^6 irradiated thymocytes for 3 days, the last 20 hr with 0.5 μCi ^{3}H-Tdy.

[b] Underlined sequence positions indicate differences from GP-BP used for immunization and selection of the parent Zla line and the D9 clone. Sequences are based on information in Reference 10.

TABLE III

Selective response can be produced to the 43-67 peptide in the Lewis rat

T Cell Line Selection[a]	Response[b] (cpm × 10^{-3})							
	No Antigen	GP-BP	PPD	1-37	43-88	89-169	43-67	68-88
Immunized and selected with 43-67 peptide	5	79	11	17	95	11	92	8

[a] 43-67 peptide (100 μg) emulsified in CFA was injected into four donor rats, and the line selected as described above, by using only the 43-67 peptide to stimulate the cultures.

[b] Line cells (2.5 × 10^4) were incubated with 2 × 10^6 antigen-presenting cells and 2 μg 43-67 peptide per well of a microtiter plate. Replicate cultures varied by less than 10% from the mean value. PPD, purified protein derivative. The injection of 10 × 10^6 43-67 peptide-activated T cells into four recipients failed to induce clinical EAE or protection against actively induced EAE.

sponses to GP-BP peptides, both the F344 and PVG T cell lines were again selective for the 68-88 determinant (Figure 3). Lewis and F344 rat strains have common alleles at the *RT1* locus of the major histocompatibility complex (MHC). PVG (Weizmann) and Lewis rats mutually reject skin grafts, but it is likely that they share I region alleles, because their accessory cells can reciprocally present antigen to the T lymphocytes of the other strain (9).

Brown Norway (BN) rat T cell lines respond to a non-68-88 determinant. The BN rat strain is resistant to actively induced EAE unless a pertussis vaccine is included in the inoculum. Although the first BN T cell line selected for response to GP-BP was inactive in vivo, the second line was encephalitogenic. When responses to GP-BP peptides were evaluated in these two BN lines, neither line recognized the 68-88 sequence, but both reacted strongly to the 43-88 peptide and weakly to the 43-67 peptide (Figure 4). Thus, as with T cell lines from Lewis rats, different BN lines recognized the same region of GP-BP, but no association could be made between the selective recognition of these peptide fragments and T cell functions in vivo.

DISCUSSION

These data show that in spite of functional heterogeneity, the Lewis rat T cell line Zla responded selectively to the 68-88 fragment of GP-BP. Four findings support this conclusion. Proliferation of line Zla to the 43-88 and 68-88 fragments of GP-BP was similar to the response to the whole GP-BP molecule, and at low concentrations of peptides, little response was observed to other fragments of GP-BP (Figure 1). All six clones derived from the Zla line showed the same selective response for the 68-88 peptide (Table I). The Zla line did not respond to human or bovine BP (Table II), which differs antigenically from GP-BP in the 68-88 region. Finally, selectively subculturing the Zla line with non-68-88 peptides failed to reveal clones with other specificities within the line (Figure 2).

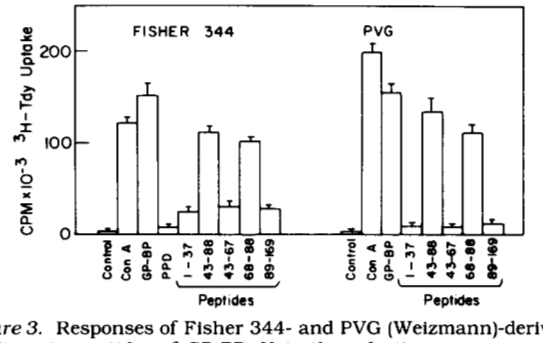

Figure 3. Responses of Fisher 344- and PVG (Weizmann)-derived rat T cell lines to peptides of GP-BP. Note the selective responses of both lines to the 43-88 and 68-88 peptides.

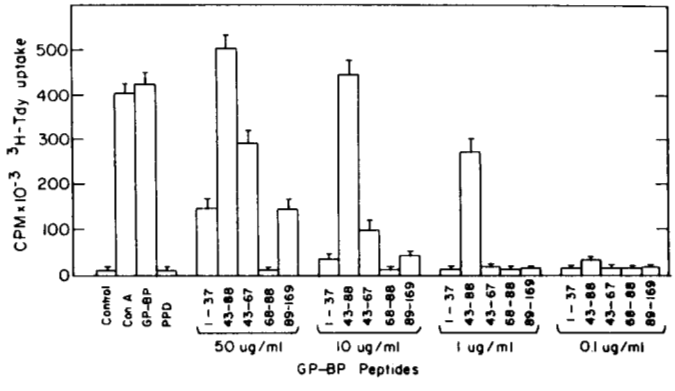

Figure 4. Response of an encephalitogenic T cell line derived from BN rats to peptides of GP-BP. Note the complete lack of response to the 68-88 peptide at all concentrations tested, the complete response to the 43-88 peptide, and a partial response to the 43-67 peptide at 50 and 10 μg/ml.

The functional differences within the Zla line and its clones could not be explained by selective responses to different peptides of the GP-BP molecule (Table I). Thus, the Zla line, which can both induce or vaccinate against active EAE, responded to the same region as did clone D9, which had potent encephalitogenic activity but to date had no protective activity, and clone 2, which had neither biologic property. It has been reported that the 68-88 fragment of GP-BP contains both encephalitogenic and protective epitopes (2, 5), and it is conceivable that the Zla line, but not its clones, contained subpopulations that responded to each epitope. Multiple subpopulations were not discernible by phenotyping either the line or its clones, however, because all of the lines and clones were exclusively W3/25 (T helper/inducer) positive.

The encephalitogenic site recognized by Lewis rat T cells was additionally defined by comparing the response of the D9 clone to BP that have known sequence differences in the 68-88 peptide (11). The D9 clone responded equally to GP and rat BP, which vary by a Thr for Ser substitution at position 79 (Table II). This result is somewhat surprising, because rat BP is less encephalitogenic on a molar basis (12) than GP-BP, and suggests that the Thr substitution in rat BP does not alter recognition of the encephalitogenic determinant by the D9 clone. Because D9 cannot distinguish this minor sequence difference, other factors such as suppressor determinants (6) must contribute to the encephalitogenic differences between GP and rat BP. Clone D9 also failed to respond to GP-BP peptide 79-88, suggesting that the reactive epitope included portions of the 68-78 peptide. Both human and bovine BP have more extensive amino acid differences in the critical region of the 68-88 peptide, notably a Gly-His insertion (11). These differences undoubtedly contribute to the lack of response by clone D9, because the remainder of the sequence is identical in the various BP. Bovine BP also has mild encephalitogenic activity in Lewis rats, but this activity is mediated through a distinct epitope (3).

Despite the antigenic dominance of the 68-88 region of whole BP in Lewis rats, other epitopes on the GP-BP molecule could stimulate Lewis T cells if exposure to the 68-88 epitope was prevented. Thus, it was possible to make a Lewis T cell line specific for the 43-67 fragment by immunizing rats and selecting a line with a purified preparation of the 43-67 peptide (Table III). It is important to note that the 43-67-specific T cell line had neither encephalitogenic nor protective ability, a finding that supports the association between immunodominance and clinically relevant activity.

The region of the BP molecule that was recognized by the T cell appeared to be influenced by genetic factors. A role for the MHC in choosing the immunodominant region of BP was suggested by the observation that Lewis, F344, and PVG (Weizmann) T cell lines all responded preferentially to the 68-88 fragment (Figures 1 and 2). The F344 rat strain has the same MHC type as Lewis, and the PVG (Weizmann) and Lewis probably share some MHC alleles, because accessory cells from either strain can mutually present BP and other antigens (9). In con-

trast, the pattern of response of T cell lines originating from the BN rat was different from that of Lewis, F344, and PVG (Weizmann). The 68-88 determinant did not induce proliferation in either of the BN lines. The partial response of the BN T cell lines to the 43-67 fragment, but not to the 68-88 fragment (Figure 4), suggests that the 43-67 sequence might contain only a portion of the complete determinant present in the 43-88 sequence. Alternatively, a dominant epitope within the 43-67 fragment may require the 68-88 moiety to retain its complete antigenic properties. As was observed in the Lewis Zla line and clones, BN lines with or without encephalitogenic activity had an identical pattern of response to the GP-BP peptides. These data confirm the notion that diverse biologic functions can be induced with the same, or closely related, determinants.

In another article, we will present results that suggest that the antigen-presenting cell from a given rat strain plays the key role in determining which part of the BP molecule is recognized preferentially. This finding implies that the association of BP epitopes with different MHC products (such as the Ia-like molecules) expressed by different antigen-presenting cells is an important factor in antigen recognition by T cells. Because different strains of rats recognized distinct dominant but encephalitogenic regions of the BP molecule, it seems likely that the ability of BP to induce EAE is related to the immunodominance of the epitopes, rather than their topography or function in situ.

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