DIFFERENCES IN THE REPERTOIRE OF THE LEWIS RAT T CELL RESPONSE TO SELF AND NON-SELF MYELIN BASIC PROTEINS

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Several of the naturally occurring autoimmune diseases have experimental correlates induced in laboratory animals by immunization with specific components of the target organ (1–3). The ensuing disease is proof that self-reactive cells are not always deleted from the repertoire, but circulate and can be activated in the periphery. Among those inducible diseases believed to be T cell mediated, it has been observed that clinical and histological symptoms of disease are sometimes more easily induced with heterologous antigen than with the homologous self-antigen (4–7). Studies on the specificity of the resulting autoimmune T cell populations indicate a concordantly higher degree of responsiveness to the heterologous antigens than to the self-antigen (8, 9). This lends support to the hypothesis that the autoreactive T cells that escape from the thymus and circulate in the periphery have a relatively low affinity for self and therefore may not be activated by physiological levels of self-antigen, although they may exhibit higher affinities for closely related non-self antigens.

We have investigated the T cell response of Lewis rats to the autoantigen myelin basic protein (MBP). Immunization with a single injection of MBP in CFA produces an acute episode of experimental allergic encephalomyelitis (EAE), characterized histologically by infiltration of the central nervous system with Ia+ mononuclear cells and T cells of the helper phenotype (10, 11), and clinically by an ascending paralysis of the fore and hind limbs. The disease has been shown to be T cell mediated, without dependence on B cells or antibody (12–14), and can be induced more easily with guinea pig (GP) MBP than with autologous rat MBP (6, 7). This result has been explained in previous studies as a rat MBP-induced T cell population that exhibits a heteroclitic response for the major encephalitogenic determinant of GP MBP, amino acid residues 68–88 (8). We generated cloned T cell hybridomas from both rat and GP MBP–immunized animals to assess the degree of heterocliticity as related to the above hypothesis.

We report here an unpredicted subpopulation of T cells that are reactive equally to GP and rat MBP and the 68–88 peptide, appearing more frequently in rat MBP–immunized animals than in GP MBP–immunized animals, and proving that not all self-reactive clones exhibit poor reactivity to self. Moreover,
we observed that immunization with rat as opposed to GP MBP induced a different population of responding T cells, most notably an encephalitogenic subpopulation specific for non-68-88 regions of MBP. Surprisingly, all of the clones induced by rat MBP demonstrated equal reactivity to GP and rat MBPs. Taken together, these results do not support the notion that rat MBP is poorly encephalitogenic because the responding T cells exhibit relatively low sensitivity to the self antigen. Rather, they indicate that the T cell responses to self (rat MBP) and to non-self (GP MBP) antigens may be subject to different immunoregulatory forces in the immunized animal.

Materials and Methods

Animals. Female Lewis rats were obtained from Charles River Breeding Laboratories, Wilmington, MA. All rats were housed at The Wistar Institute animal facility and were used at 2–5 mo of age.

Antigen. MBP was obtained from the spinal cords of GP and brains of rabbits and Lewis rats (rat MBP) by a modification of the method of Diebler as previously described (Happ, M. P., B. Dietzschold, P. Wettstein, and E. Heber-Katz, submitted for publication). Pig MBP was obtained from Calbiochem-Behring Corp., La Jolla, CA (Lot 475916). SDS-PAGE on a linear gradient slab gel revealed a major band at 18 kD for GP, rabbit, and pig MBPs with <5% contamination. HPLC analysis of GP MBP revealed a single sharp peak. SDS-PAGE analysis of rat MBP revealed two major bands at 18 and 14 kD, with the smaller band being more intense. HPLC analysis produced a single peak with a broad base.

We prepared a 19-amino acid peptide corresponding to the 68–88 sequence of GP MBP using the Merrifield solid-phase method as previously described (Happ, M. P., et al., submitted for publication). MBP fragments generated by limited pepsin digestion of GP MBP were kindly donated by R. Fritz (Emory University School of Medicine, Atlanta, GA). The preparation and purity of the fragments have been previously described (16). All MBPs and peptides were stored lyophilized, and portions were periodically reconstituted to a concentration of 1 mg/ml saline for use.

Induction of EAE. Animals were immunized in both hind foot pads with a total of 0.2 ml of a 1:1 emulsion of the appropriate antigen in saline and H37Ra adjuvant (Difco Laboratories Inc., Detroit, MI), consisting of IFA supplemented with 1.0 mg of heat-killed and dried Mycobacterium tuberculosis per milliliter of adjuvant. Thus, each rat received a total of 100 μg of M. tuberculosis. Rats were observed daily until clinical symptoms disappeared in affected animals, or for 3–4 wk if no clinical symptoms appeared.

Proliferation Assays. In vitro proliferation assays were performed as previously described (Happ, M. P., et al., submitted for publication). Briefly, animals were killed 11–13 d after immunization and their popliteal and inguinal lymph nodes were removed. Single cell suspensions were made and passed over nylon-wool columns. The eluted cells (90–95% T cells by FACS analysis) were placed in 96-well flat-bottomed plates at 4 × 10^5/well. 10^5 irradiated (3,000 rad) syngeneic spleen cells/well were added to serve as APCs. The assays were pulsed at the time of peak proliferation (60–90 h) with 10 μl of [3H]ThiR (at a concentration of 50 μCi/ml) per well and then harvested 14–18 h later and counted.

The medium used was RPMI supplemented with 10 mM Hepes, 5 × 10^{-5} M 2-ME, 2 mM l-glutamine, 1 mM sodium pyruvate, 1% 100X vitamins for Eagle's modified MEM, 1% 100X nonessential amino acids for Eagle's MEM, 250 μg gentamicin/ml, and 10% FCS.

Generation of T Cell Hybridomas. Animals were killed 11–13 d after immunization with 50 μg GP MBP or 500 μg rat MBP. The popliteal and inguinal lymph nodes were removed, ground to single cell suspensions and washed several times with Dulbecco's PBS. 25 × 10^6 cells were cultured with 10^7 irradiated syngeneic spleen cells prepared as described above. The cultures were supplemented with 10 μg of the immunizing anti-
gen/ml and were maintained in upright 25-cm² flasks in the enriched RPMI described above. After 4 d, 2–4 × 10⁶ blast cells were fused for 2 min to 2–4 × 10⁶ BW5147 thymoma cells at a ratio of 1:2 or 2:1 using the polyethylene glycol (PEG)-mediated fusion procedure previously described (17) and 35% PEG (vol/vol). The cells were immediately plated in selective HAT medium to 96-well plates and observed for the appearance of growth-positive wells. Hybrids were picked from plates with 50% or fewer growth-positive wells and tested for the production of IL-2 in response to 50 µg/ml of the immunizing antigen as described below. Positive hybrids were recloned and maintained in enriched RPMI.

T Cell Hybridoma Assays. T cell hybridomas were plated at 1–1.5 × 10⁴/well in 96-well flat-bottomed plates. Antigen and 10⁵ syngeneic spleen cells were added to each well in enriched RPMI. After 48 h, an aliquot of the supernatant was removed to a second 96-well plate for IL-2 quantitation. 5 × 10⁵ HT2 cells were added to each well of the second plate and the assay was pulsed 16–22 h later with 10 µl of [³H]Tdr (50 µCi/ml). The wells were harvested 8 h later and counted.

Generation of T Cell Lines and Adoptive Transfer of EAE. Bulk cultures were prepared as described above for fusions and were incubated for 10 d. Live cells were separated on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and restimulated in 6-well plates at 0.4 × 10⁶ cells/ml with 1.6 × 10⁶ irradiated syngeneic spleen/ml and antigen in 5 ml of enriched RPMI. 7 d later, the Ficoll separation and restimulation were repeated. Antigen responsiveness was measured using the proliferation assay described above, substituting 2.5 × 10⁴ line cells/well in place of primed lymph node.

Cells were harvested and Ficoll separated for adoptive transfer 3 d after restimulation with antigen and APC. After washing, the cells were injected in a volume of 1.0 ml saline into naive Lew recipients. Because we have found that lightly irradiating recipients increase the efficiency of adoptive transfer (unpublished results), recipients were given 350 rad whole-body irradiation immediately before transfer.

Results

Induction of EAE. Guinea pig and rat MBPs were compared for their ability to induce EAE in Lewis rats using adjuvant supplemented with only 100 µg of M. tuberculosis. In agreement with previous reports (6, 7), GP MBP was ~10-fold more potent at inducing clinical symptoms of EAE than was rat MBP (Table I). A synthetic peptide encompassing amino acid residues 68–88 of GP MBP was found to be equivalent to the whole GP MBP on a molar basis for induction of clinical EAE, again demonstrating that this determinant retains the encephalitogenic potential of the whole MBP (7, 16, 18) and affirming that the peptide preparation was a good one.

In Vitro Proliferation to MBP. After 11–13 d, lymph node cells from rats immunized with 50 µg of GP MBP or 100 µg of rat MBP were assayed for proliferation in response to the various MBP antigens listed in Table II, showing their 68–88 amino acid sequences.

Rats immunized with GP MBP responded best to GP MBP, less well to the GP 68–88 peptide and rat MBP, and minimally to rabbit and pig MBP (Fig. 1A). Animals immunized with rat MBP produced the response pattern illustrated in Fig. 1B, although they did not always exhibit clinical symptoms at the time of sacrifice. In contrast to the GP MBP–immunized animals, rat MBP produced nearly equipotent responses to GP and rat MBPs, as well as significant responses to rabbit and pig MBPs. To determine the basis for the different response patterns elicited by the autoantigen and its more potent heterologous counterpart, we examined the specificities of individual clones within these populations.
TABLE I
Summary of Induction of EAE by MBP and Encephalitogenic Peptide in Lewis Rats

| Antigen       | Dose*   | Number sick/total | Average day of onset‡ | Average maximum clinical symptom§ |
|---------------|---------|-------------------|------------------------|-----------------------------------|
|               | µg      | nmol              |                        |                                   |
| GP MBP        | 50      | 2.5               | 16/16                  | 11.2 ± 0.54                      | 3.0 ± 0 |
|               | 20      | 1.0               | 6/7                    | 11.7 ± 0.51                      | 3.0 ± 0 |
|               | 10      | 0.5               | 5/5                    | 13.6 ± 0.89                      | 2.0 ± 0.7 |
|               | 5       | 0.25              | 0/7                    | —                                 | —       |
| GP 68-88      | 20      | 10.0              | 5/5                    | 11.4 ± 0.54                      | 3.0 ± 0 |
|               | 5       | 2.5               | 5/5                    | 12.0 ± 0.70                      | 3.0 ± 0 |
|               | 1       | 0.5               | 5/5                    | 11.8 ± 0.83                      | 3.0 ± 0 |
| Rat MBP       | 250     | 12.5              | 8/9                    | 12.2 ± 0.70                      | 2.6 ± 0.62 |
|               | 100     | 5.0               | 8/9                    | 14.4 ± 0.74                      | 1.4 ± 0.51 |
|               | 50      | 2.5               | 0/5                    | —                                 | —       |

* Antigen was administered in 0.2 ml adjuvant containing 100 µg of Mycobacterium tuberculosis.
‡ Average day of onset indicates the average number of days ± SD before the appearance of the first clinical symptom and excludes those animals within the group that did not develop any symptoms within 3 wk of immunization. Those animals are also excluded from the calculation of average maximum clinical symptom.
§ Clinical symptoms were scored as follows: 1, floppy tail; 2, partial paralysis of the hind limbs; 3, full paralysis of both hind limbs; 4, full paralysis of the fore and hind limbs.

TABLE II
Amino Acid Sequence of the 68–88 Region of MBP from the Various Species Used to Determine Fine Specificity

| MBP       | 68 | 75 | 80 | 85 | 88 |
|-----------|----|----|----|----|----|
| Rat       | G-S-L-P-Q-K-S-Q---R-T-Q-D-E-N-P-V-V-H-F |
| GP        | G-S-L-P-Q-K-S-Q---R-S-Q-D-E-N-P-V-V-H-F |
| Rabbit    | G-S-L-P-Q-K-S---G-H-R-P-Q-D-E-N-P-V-V-H-F |
| Pig       | G-S-L-P-Q-K-A-Q-G-H-R-P-Q-D-E-N-P-V-V-H-F |
* Indicates residues displaying interspecies heterogeneity. Sequences taken from reference 19.

The Clonal Response to GB MBP. We generated T cell hybridomas as a source of clonable, antigen-specific T cells, assuming that the hybridization event is a random one and does not discriminate on the basis of receptor specificity. T cell fusion should allow cloning of weakly represented specificities that may become lost or “overgrown” in the generation of long-term lines. We fused bulk cultures of GP MBP–immune lymph node cells to the BW5147 fusion partner, then selected and cloned, from three different fusions, 30 hybridomas specific for GP MBP. Eight of these hybridomas responded to GP MBP alone, apparently to determinants not encoded within the 68–88 peptide (Fig. 2A). Cleavage fragments of GP MBP were used to localize the antigenic determinants seen by these clones. Two of the seven tested responded to the 43–88 fragment but not the 68–88 peptide, while the remaining five responded to none of the fragments.
(Table III), suggesting that the determinant they recognize may have been destroyed by cleavage events.

The majority of the hybrids (22 of 30) responded to both GP MBP and the 68–88 peptide with varying degrees of crossreactivity on rat and rabbit MBPs, enabling us to group these hybrids into three sets of response patterns, illustrated in Fig. 2, B–D. Although each pattern gave maximal responses to GP MBP at concentrations between 0.3 and 0.03 nmol/ml, only pattern 68–88.1 displayed similar sensitivity to the GP 68–88 peptide and rat MBP. This was also the only pattern to display a moderate response to rat MBP. Pattern 68–88.2 required up to 50 times more GP 68–88 peptide and ~1,000 times more rat MBP to...
induce the same level of stimulation as GP MBP, while pattern 68–88.3 required 1,000 times more rat MBP or GP 68–88 peptide to induce the same level of IL-2 production as 0.003 nmol/ml of GP MBP. Table IV summarizes the distribution of the various reactivity patterns among the hybrids. Patterns 68–88.1, 68–88.2, and 68–88.3 appear almost equally among the 68–88–specific hybrids.

**The Clonal Response to Rat MBP.** Of 15 hybrids generated from rat MBP–immune animals (two different fusions), 8 hybrids responded to the 68–88 determinant of GP MBP (Fig. 3A) and all of these possessed a reactivity pattern of 68–88.1. Not surprisingly, this was the pattern displaying the best reactivity to rat MBP (equipotent with GP MBP).

The remaining seven hybrids displayed equal reactivity to a non–68–88 determinant of GP, rat, rabbit, and pig MBPs (Fig. 3B). This pattern was absent among the GP MBP–induced hybrids. To localize this determinant, we tested six of these hybrids with the cleavage fragments of GP MBP. Four of the hybrids responded only to the 89–169 fragment, one only to the 1–37 fragment and one to none of the fragments (Table V). Table VI compares the reactivity patterns of all of the rat MBP–induced hybrids to those of GP MBP–induced hybrids.

**Encephalitogenicity of Non–68–88 Determinants.** To determine if the non–68–88 specificities could contribute to the development of EAE, we developed
specific short-term T cell lines and used them to adoptively transfer EAE. The lymph node cells from a single rat immunized with 500 µg of rat MBP were divided into two equal aliquots. One was stimulated in vitro three times with 5 µg GP 68–88/ml and the other was cultured in parallel with 40 µg of pig MBP/ml. These two antigens were chosen because hybrids specific for the 68–88 determinant never showed crossreactions on pig MBP, while all of the crossreactive non–68–88–specific hybrids induced by immunization with rat MBP reacted well to pig MBP. The two different lines displayed mutually exclusive reactivities as shown in Fig. 4 after three rounds of in vitro stimulation. At this time, they were tested for their ability to transfer EAE to naive syngeneic
TABLE VI
Summary of Fine Specificities of Rat MBP-induced Hybridomas and Comparison to GP MBP-induced Hybridomas

| Specificity pattern | GP MBP-induced | Rat MBP-induced |
|--------------------|----------------|-----------------|
|                    | Number of hybrids | Percent of total | Number of hybrids | Percent of total |
| Non-68-88, GP only | 8 | 26 | — | — |
| Non-68-88, crossreactive | 0 | 0 | 8 | 53 |
| 68-88.1 | 7 | 24 | 7 | 47 |
| 68-88.2 | 8 | 26 | 0 | 0 |
| 68-88.3 | 7 | 24 | 0 | 0 |

TABLE VII
Adoptive Transfer of EAE by Short-term T Cell Lines from Rat MBP-immunized Donors

| In vitro stimulant | Cell number transferred* | No. sick/total | ẍ Day of onset | ẍ Maximum clinical symptom |
|--------------------|-------------------------|----------------|----------------|---------------------------|
| GP 68-88 | 15 x 10⁶ | 3/3 | 4.3 ± 0.57 | 3.0 ± 0 |
| 3 x 10⁶ | 3/3 | 6.0 ± 0 | 2.7 ± 0.57 |
| Pig MBP | 15 x 10⁶ | 3/3 | 5.0 ± 0 | 3.0 ± 0 |
| 3 x 10⁶ | 3/3 | 5.7 ± 0.57 | 2.0 ± 0 |

* Cells were harvested 3 d after the third in vitro stimulation, washed, and administered intravenously in saline; recipients were given 550 rad whole-body irradiation immediately before transfer.

1 Average day of onset indicates the average number of days ± SD before the appearance of the first clinical symptom.

2 Clinical symptoms were scored as follows: 1, floppy tail; 2, partial paralysis of the hind limbs; 3, full paralysis of both limbs; 4, full paralysis of the fore and hind limbs.

FIGURE 4. (A and B) Proliferative response of T cell lines generated from a rat MBP-immunized animal. One subline was raised against pig MBP (A) and the other against the 68–88 peptide of GP MBP (B). Proliferation was measured at the time of the third in vitro stimulation. The antigens used were rat MBP (●), the 68–88 peptide of GP MBP (○), pig MBP (■), and PPD (□).
recipients. As illustrated in Table VII, both T cell lines were able to transfer clinical symptoms of EAE in a dose-dependent fashion.

Discussion

We began these studies by asking why GP MBP is so much more potent than rat MBP at causing EAE in Lewis rats. This is especially important, considering that it is rat MBP that is seen in vivo and its recognition is most likely responsible for disease. We expected that the difference between these two MBPs seen in disease induction would also be reflected in the antigenic potency of these molecules for T helper cell populations which both proliferate and produce IL-2 in response to antigen, since it is these populations that have been shown to transfer disease. In fact, in support of this notion, previous studies by Kibler et al. (8) had shown that immunization of Lewis rats with the rat MBP 68–88 peptide cleavage fragment resulted in a proliferative T cell response that at the whole population level was greater to GP fragment than to rat MBP fragment.

Thus, we proposed a model in which GP MBP and rat MBP induced the same population of disease-causing T cells with a higher affinity for GP MBP than for rat MBP, and therefore a lower concentration of GP MBP than of rat MBP would be needed to give the same level of disease.

To resolve this at the clonal level, we made T cell hybridomas from immunized Lewis rats. Animals immunized with GP MBP, the heterologous antigen, generated a large number of T cell clones (75%) that reacted to GP MBP, rat MBP, and the encephalitogenic determinant, 68–88. Two-thirds of the 75% were more reactive to GP MBP than to rat MBP, as we predicted. Interestingly, one-third of the 75% reacted equally well to GP MBP and to rat MBP. Thus, GP MBP was not only good at inducing T cells with anti-rat MBP reactivity but could also induce highly reactive rat MBP-specific T cell clones.

When we examined the rat MBP–immunized Lewis rats, we found a picture very different from what we expected. All of the T cell hybridomas were high responders to rat MBP and these were of two major specificities. Half of the hybridomas were reactive to 68–88, equally reactive to GP and rat MBP, and had the same antigen concentration requirements seen with the most potent anti–rat MBP T cells from GP MGP-primed animals. The remaining 50% were reactive to other determinants outside 68–88. Using MBP cleavage fragments, the determinants were mapped to the 1–37 and 89–169 regions and one in a region destroyed by pepsin cleavage (perhaps the same encephalitogenic determinants at 89–115 and 37–42 previously mapped for bovine BP [18, 20]). A clear demonstration as to the potency of these cells with such specificities was seen in the ability of cell lines from rat MBP primed animals with the same two specificity patterns to be highly encephalitogenic when injected into Lewis rats.

The ability of rat MBP primed T cells to respond well to rat MBP in vitro, even to respond better to rat MBP than do GP MBP primed T cells, and to transfer disease regardless of the specificity, does not reflect the poor immunogenicity of rat MBP in vivo and presents us with a paradox. A simple explanation, however, is that differential regulation of the immune response is occurring in vivo.

The fact that GP and rat MBPs were equally capable of stimulating the rat--
induced clonotypes indicates that the respective target determinants were equally antigenic on both MBPs. We feel that this indicates that both MBPs can be processed and presented identically by APC, despite minor amino acid sequence differences and the presence of the truncated form of rat MBP. However, immunization with the two different MBPs induced two T cell populations differing not only in the clonotypes that appeared, but also in the frequencies with which they appeared, indicating that some component of the immune system can discriminate between these two antigens. We propose here that it is neither the T helper cell nor the APC, but must be a regulatory T cell or MBP-specific antibody.

Summary

We have examined the fine specificity of a panel of cloned T cell hybridomas generated from Lewis rats immunized with guinea pig (GP) or Lewis rat myelin basic protein (MBP) to determine the autoimmune T cell repertoire that develops in experimental allergic encephalomyelitis (EAE). This analysis has demonstrated that GP MBP, which was ~10-fold more potent for EAE induction than the autologous rat MBP, produced a population in which almost one-fourth of the members responded to GP-unique determinants and displayed no crossreactivity on the self antigen. The remaining majority of GP MBP–induced clones were specific for the 68–88 encephalitogenic determinant and could be subdivided into three groups based on their varying responses to the 68–88 peptide and rat and rabbit MBPs. Surprisingly, one of these groups showed equal reactivity to GP and rat MBPs.

In contrast, the clonotype composition of the T cell population induced by rat MBP was quite different. One-half of these clones comprised a single group responding to the 68–88 determinant, reacting equally with GP and rat MBP. All of these responded to the same range of antigen concentrations as their GP-induced counterparts. The remaining half of that population contained a collection of clones that was nearly as encephalitogenic as the 68–88 population after propagation as a short-term T cell line. These clones were specific for at least three distinct antigenic determinants, all displaying extensive cross-species reactivity, and required as little or less rat MBP for maximal stimulation as did the 68–88–reactive clones. We therefore conclude that the T cell repertoire for MBP does include clones with reactivity to both 68–88 and non–68–88 determinants of GP and rat MBPs, and that both MBPs appear to be equally capable of stimulating these clones in vitro. However, the differences in the clonotype composition of the populations induced by immunization with these two antigens suggest that rat and GP MBPs are subject to different immunoregulatory constraints in the animal and may account for the difference in the encephalitogenic potential of these two antigens.

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