Regulatory T cells in experimental allergic encephalomyelitis. I. Frequency and specificity analysis in normal and immune rats of a T cell subset that inhibits disease

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Abstract

We have shown previously that administration of myelin basic protein (MBP)-reactive T cells to naive Lewis rats induces not only autoimmune encephalomyelitis (EAE) but also a near total resistance to subsequent disease. By isolating the effector cells that are responsible for the resistance, we demonstrated that disease protection paralleled with increased numbers of a CD8+ regulatory T cell (RTC) subset and that co-injection of this RTC subset with encephalitogenic T cells aborted the pathogenic activity of the latter cells. Here, we show that a radio-sensitive splenic population of RTC also exists in naive rats that can be recruited and activated to inhibit the onset of secondary episodes of adoptive EAE. In co-transfer experiments, this protective RTC subpopulation can be isolated to neutralize the pathogenic activity of stimulatory MBP-reactive T cells in vivo. We show that the frequency of RTC with specificity for MBP-reactive T cells in naive rats is two orders of magnitude higher than the frequency of MBP-specific precursors, the activity of RTC increases substantially with age and RTC frequencies increase as a consequence of immunization with MBP-reactive cells lines. In specificity studies, we show that RTC isolated from naive rats and RTC from animals primed with one MBP-reactive cell line show cross-reactive responses to a variety of different MBP-reactive T cell lines. However, following repeated stimulation with a given MBP line, these RTC display a more limited, clonotypic response to the selecting line and assume a uniform CD8 phenotype. Finally, functional studies with RTC indicate that proliferative and lytic specificities do not necessarily correlate and that activated rat RTC are especially lytic for a Fas-sensitive murine cell line.

Introduction

Lewis (LEW) rats immunized with guinea pig myelin basic protein (MBP) in complete Freund’s adjuvant (CFA) (1) show overt signs of experimental allergic encephalomyelitis (EAE), a paralytic neuropathy caused by T lymphocytes that infiltrate the central nervous system, ~12–13 days post-immunization. Paralysis increases rapidly in severity and lasts ~1 week, at which time the animals recover spontaneously (2,3). Of particular interest, these recovered animals develop a near total resistance to further attempts to induce active EAE (4). EAE can also be transferred adoptively to normal LEW rats with T cells from MBP-immunized rats following brief re-stimulation of these cells with antigen in culture (5,6). These animals develop paralytic symptoms of adoptive EAE sooner, ~3–5 days post-transfer, disease lasts for several days, after which animals recover, and they too become resistant to further attempts to cause EAE either by subsequent active immunization or by adoptive transfer (1,6,7).

Induced EAE resistance does not necessarily require the transfer of active T cells; it also occurs following treatment of recipients with attenuated T cells (2,6). Thus, it appears that autoreactive T cells that are pathogenic in EAE can, themselves, act as protective vaccines. Similar resistance-
inducing properties of CD4+ pathogenic T cells have been described for various other experimental models of autoimmune disease, such as autoimmune thyroiditis (8), autoimmune arthritis (9,10) and graft-versus-host disease (11,12). This protective activity appears to be disease specific; MBP-specific T cells, used as vaccines, provide preferential protection against EAE, but not arthritis (10). It also appears to be antigen specific; in the graft-versus-host model, inter-strain A/B F1 animals rendered resistant to graft-versus-host disease caused by inoculation with parental strain A T cells carrying anti-MHC$^p$ specificity remain fully vulnerable to anti-MHC$^a$ graft-versus-host disease caused by T cells from strain B donors (11,12). These findings provide support for the notion that pathogenic T cells possess clonotypic, immunogenic structures, i.e. antigen-specific TCR, responsible for their ability to induce a specific protective resistance to further disease. The finding that resistance in these immune disease models is mediated by T cells (6, 7) implies anti-clonotypic/anti-idiotypic specificity of such protective T cell populations involving recognition of naturally processed TCR peptide fragments expressed in association with MHC molecules on the surface of pathogenic T cells.

The autoreactive T cells that are pathogenic in EAE, and which induce EAE resistance, belong to the CD4+$^+$ subset, react to the dominant encephalitogenic epitope of MBP encompassed in peptide 68–86, and display surface TCR that preferentially use the $\nu_{8.2}$ and $\nu_{8.2}$ gene segments (13,14). These TCR $\alpha$ chains also preferentially express Asp and Ser as the first two amino acid residues of the third complementarity-determining region (CDR3) with minimal, if any, non-germline encoded N-region additions (15). The findings in these immune resistance models that pathogenic T cells were immunogenic, employed a common TCR usage and that resistance was mediated by T cells led to an important series of studies showing that rats vaccinated with peptide fragments of the CDR2 and the junctional CDR3 region of TCR $\nu_{8.2}$ chains developed resistance to active EAE (16,17). This, in turn, led to the possibility that immunization with MBP-reactive whole cell preparations or TCR peptides to induce immune network regulatory responses might be a useful approach in the treatment of some human autoimmune diseases, such as multiple sclerosis, that may involve oligoclonal populations of pathogenic T cells. Several clinical studies have explored this possibility with some degree of success (18–23). Much remains unknown, however, concerning the immunobiology of regulatory T cells; in particular, their frequency in normal and disease-resistant animals, how they are activated, details of their specificity and mode of action, and whether they exist as a complex of multiple subsets. In recent studies, for example, we observed that not all MBP-reactive T cell populations, sharing similar surface phenotypic features, have a similar ability to recruit regulatory T cells (RTC) and to provide disease protection. Only three out of a total of 10 pathogenic MBP-reactive T cell clones tested were able to stimulate RTC and generate protection against EAE in vivo (24).

To determine the mechanism by which adoptive transfers of subpathogenic numbers of autoreactive T cells immunize and protect recipients against autoimmune disease and to determine how some autoreactive T cell clones induce better protection than others, we considered the possibility that the frequency of RTC subsets may differ in the T cell repertoire and that individual RTC subsets may have distinctive activation requirements. In the current investigation, we examined the possibility that subsets of RTC exist in naive animals, how such cell populations expand under specific and non-specific immune conditions, and whether there are differences in RTC frequency in young and aged animals. Our results show that RTC reactive to MBP-specific T cells are found not only in immunized animals but also in naive rats. Such cells expand at varying rates, in vivo and in vitro, in clonotypic or cross-reactive fashion in immunized animals. Individual MBP-reactive T cell lines and clones differ greatly in their ability to induce proliferation and expansion of RTC. The possibility that clonotypic and cross-reactive subsets of RTC may be responsible for long-term disease resistance in recovered animals is discussed.

**Methods**

**Animals, cells and reagents**

Inbred LEW rats were obtained from Harlan-Sprague-Dawley (Indianapolis, IN). All cells, lines and clones were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Gibco) and 5 x 10$^{-5}$ M 2-mercaptoethanol in a humidified atmosphere of 5% CO$_2$ at 37°C. Media used for the growth and expansion of all lines and clones was supplemented with 10% supernatant derived from bulk cultures of concanavalin A (Con A)-stimulated rat spleen cells (CASUP). MBP was prepared from guinea pig spinal cord as described previously (32). Synthetic peptides were produced by the Molecular Resource Center at St Jude Children’s Research Hospital. mAb specific for rat CD4 (OX-35) and CD8 (OX-8) were gifts from Dr A. F. Williams (29,33). MBP-specific S1 (6), S22, S26, S28 and S40 lines (34) and C10 clone (6,29) have been described previously. A20 is a murine lymphoma line which is sensitive to Fas ligand-mediated cell death (35).

**Preparation of T cells**

T cells were enriched by passing whole suspensions of spleen or lymph node cells through a nylon wool column. Cell populations recovered from these columns after 1 h of incubation at 37°C consist of ~98% T cells (24). Enrichment was confirmed by flow cytometry following staining with a standard panel of mAb (see below).

**Proliferation assay for RTC**

Responder splenic T cells were prepared either from normal LEW rats or from animals that were immunized with a MBP-reactive T cell clone 2–3 weeks earlier. Proliferation assays were performed in 96-well flat-bottom microtiter plates. Each well contained 2 x 10$^4$ nylon wool-enriched splenic T cells and 4 x 10$^4$ irradiated (2000 rad) resting MBP-reactive T cells. Cultures were incubated for 2 days and cells were labeled with 0.5 µCi (18.5 kBq) $[^3]$H]thymidine/well for 6 h, harvested and examined for incorporation of $[^3]$H]thymidine. To insure that MBP-reactive stimulatory cell populations do not contribute to the observed proliferative responses in these cultures, we determined in preliminary experiments that as little as 500
rad of irradiation obliterates the specific response of these cells to MBP.

**Bulk cultures of RTC**

Forty million splenic responder T cells were co-cultured with $8 \times 10^6$ irradiated, MBP-reactive S1 cells in a 100 mm Petri dish. After 2 days, proliferating cells were isolated by Lymphoprep gradient centrifugation and cultured in media supplemented with CASUP.

**Flow cytometric analysis**

Indirect immunofluorescence was performed by incubating $1 \times 10^6$ cells with appropriate concentrations of a standard panel of mAb consisting of OX-35 (CD4) and OX-8 (CD8) for 30 min. After the cells were washed twice in PBS containing 1% BSA and 0.1% sodium azide, FITC-conjugated goat anti-mouse Ig was added to the culture for 30 min. The cells were then washed twice and stained cells were analyzed with a FACScan ($4 \times 10^4$ cells counted in each sample). The results are presented as cell number versus fluorescence intensity.

**Limiting dilution assay (LDA) analysis of RTC and MBP-reactive T cell frequencies**

For analysis of RTC frequencies LEW rats were immunized by i.v. injection with $5 \times 10^6$ MBP-reactive S1 cells or left untreated. The spleen and draining lymph nodes were removed 14–21 days later and single-cell suspensions were prepared. These responder T cells were enriched by passage through nylon wool and seeded at $3 \times 10^4$ to $1 \times 10^5$/well in replicates of 24 in 96-well flat-bottomed culture plates that had previously been seeded with irradiated (3000 rad) S1, S22, S40 or C10 cells ($2 \times 10^4$). Forty-eight hours later, these plates were pulsed with 0.5 µCi [³H]thymidine/well for 6 h, harvested and the cultures assessed for isotope incorporation.

Similarly, the frequencies of T cells reactive to MBP from normal and immune animals were established by LDA using 24 replicate dilutions of $6 \times 10^4$ to $3 \times 10^5$ cells/well in cultures seeded with irradiated spleen cells as antigen-presenting cells with or without MBP (20 µg/ml).

**Cytolytic assays**

$^{51}$Cr-release assays to quantify specific cytotoxicity were performed as described previously (36). Briefly, $2 \times 10^6$ target cells were incubated for 50 min in 0.3 ml RPMI medium plus 10% FCS containing 150 µCi $^{51}$Cr and washed 3 times in PBS prior to use in lytic assays. Calculated numbers of effector cells were cultured in 96-well flat-bottomed microtiter plates with $10^4$ $^{51}$Cr-labeled target for 18 h. Percentage cytotoxicity was calculated according to the formula: percent cytotoxicity = [(c.p.m. supernatant/(c.p.m. sediment + c.p.m. supernatant)] x 100.

**Evaluation of EAE**

Animals were examined daily for weight loss and evidence of clinical disease. Results were scored as follows: 0 = no EAE; 1 = partial loss of tail tonicity; 2 = loss of tail tonicity; 3 = unsteady gait and mild paraparesis; 4 = hind-limb paralysis and incontinence; and 5 = death.

![Graph](image.png)

**Fig. 1.** Lymphocyte populations from naive rats contain a subpopulation that can be recruited during adoptive EAE to provide resistance to further attempts to cause adoptive EAE. Among the three groups (n = 4) of LEW rats, two (middle and bottom panels) received a single dose of whole body irradiation (750 rad). The third group of the animals were also injected with $5 \times 10^7$ nylon wool-enriched splenic T cells. All the animals were challenged with two doses of activated S1 cells ($3 \times 10^6$) 20 days apart (day 0 and day 20). Animals were examined daily for weight loss and clinical disease.
To analyze the LDA data acquired from the in vitro experiments.

In contrast, previously irradiated, but non-reconstituted, subsequent EAE caused by the second injection on day 20. As shown, the naive animals of group 1 developed EAE after the first injection but were resistant to the second. This result demonstrates the existence in normal animals of a radio-sensitive T cell population that can be recruited, during the course of adoptive EAE, to inhibit the onset of secondary episodes of adoptive EAE.

Statistical analysis

The frequencies of responding RTC were estimated by comparing proliferation of calibrated numbers of T cells in the presence and absence of stimulator MBP-reactive T cell lines/clones. Twenty-four replicated wells were used for each cell density. The positive threshold was set as the mean negative control + 3 SD (37–39). A computer program was developed to analyze the LDA data acquired from the in vitro experiments. This program uses the Poison distribution to calculate the frequency of responder T cells with 99% confidence limits from the fraction of negative wells of each cell density seeded to the 96-well culture plates.

Results

T cell populations of normal rats contain a radiosensitive subset of RTC which can be recruited to protect against adoptive EAE

Figure 1 shows the results of a protection assay conducted in three groups (n = 4) of LEW rats. The first group (Fig. 1, top panel) consisted of naive animals; the second (middle panel) and the third (Fig. 1, lower panel) groups of animals received whole-body irradiation (750 rad). To determine whether the peripheral immune system of normal rats contains RTC, the irradiated animals in the third group were reconstituted with 5 × 10⁷ nylon wool-enriched splenic T cells derived from naïve donors. All the animals received two injections (3 × 10⁶) of activated S1 cells, the first on day 0 and the second on day 20. As shown, the naïve animals of group 1 developed EAE after the first injection but were resistant to subsequent EAE caused by the second injection on day 20. In contrast, previously irradiated, but non-reconstituted, animals in group 2 developed paralytic symptoms of EAE following both injections of the MBP-specific T cell line. Irradiated, reconstituted animals of group 3 developed EAE following the first injection with the MBP-specific T cell line, but were resistant to the second. This result demonstrates the existence in normal animals of a radio-sensitive T cell population that can be recruited, during the course of adoptive EAE, to inhibit the onset of secondary episodes of adoptive EAE.

Detection of T cell populations in naïve donors specific for MBP-reactive T cell clones

Two hundred thousand nylon wool passed splenic T cells from normal LEW donors were exposed, at a ratio of 10:1, to irradiated cells of the MBP-reactive S1 line (2 × 10⁷) in 96-well flat-bottom plates, in the absence of additional APC and antigen. Forty-eight hours later cultures were pulsed with [³H]thymidine for 6 h and then harvested. As demonstrated in Fig. 2, appreciable proliferation was detected in cultures where irradiated MBP-reactive stimulator T cells were added. The extent of the response differed, depending on T cell line or clone used for stimulation. Thus, T cell lines S1 and S22 cells caused strong proliferative responses, whereas C10 cells showed no such stimulatory effects.

The possibility that [³H]thymidine incorporation by the irradiated stimulatory contributes to the overall proliferative response in these cultures was excluded in two ways. First, as little as 500 rad irradiation completely abolishes the response of these resting T cells to antigen (data not shown); stimulatory cell populations in these experiments received 2000 rad irradiation. Secondly, addition of irradiated thymocytes as antigen-presenting cells, along with antigen, to cultures of irradiated MBP-reactive cells provided no proliferative activity (Fig. 2), indicating that the macrophage/dendritic cells present in the responder splenic cell populations do not cause responses by the MBP-reactive stimulator population.

In other experiments, we determined that a number of newly prepared MBP-reactive T cell lines, maintained in culture for <3 weeks with two rounds of antigen stimulation, caused similar strong proliferative responses by normal T cells from naïve donors (data not shown). This raises the possibility that the variable stimulatory effects of different MBP-reactive T

Table 1. Proliferating T cells derived from naïve rats stimulated with MBP-reactive S1 cells protect against EAE caused by adoptive transfer of 5 × 10⁶ S1 cells

<table>
<thead>
<tr>
<th>Treatment of LEW rats</th>
<th>Incidence of clinical EAE</th>
<th>Day of onset</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>4/4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>15 × 10⁶ anti-S1 cells</td>
<td>0/4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15 × 10⁶ Con A blasts</td>
<td>4/4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

₃Rats were injected with 5 × 10⁶ newly activated, MBP-reactive S1 cells (6) alone (group 1), S1 cells plus 15 × 10⁶ T cells from normal donors stimulated in bulk cultures with irradiated S1 cells (group 2), or with S1 cells and 15 × 10⁶ Con A-activated T syngeneic T cell blasts.

₅Animals were examined daily for weight loss and overt signs of disease as described in Methods.
cell clones (Fig. 2) are not affected by the prior history of a given clone, i.e. how long it had been maintained in culture, a circumstance which may alter the expression of surface molecules and functions of these T cell populations.

RTC and protection against EAE

T cells from naive donors that react to MBP-reactive stimulator cells in culture are functionally antagonistic to MBP-reactive T cells and capable of affording protection against adoptive EAE. Given the capacity of these cell populations to regulate the severy of EAE in vivo we refer to them hereafter as regulatory T cells (RTC). Table 1 shows the result of an experiment with three groups of LEW rats injected with 5 x 10^6 MBP-reactive S1 cell clones to cause adoptive EAE. The control group received MBP-reactive S1 cells only; the second group was co-injected with 15 x 10^6 cells from of S1-stimulated bulk cultures derived from naive donors; and the third group was co-injected with a similar number of Con A-activated T cell blasts. Only the cells, mostly CD8+ (see below), from S1-stimulated bulk cultures were capable of abolishing adoptive EAE completely.

Comparison of RTC proliferative activity in young and aged naive rats

Data summarized from over 30 naive rats showed that the proliferative activity of RTC increases substantially with age. Splenic cells from aged rats (>16 weeks old) consistently showed proliferative responses 2–4 times greater than those generated by T cells from younger (4–6 weeks old) donors (Fig. 3). This increased RTC activity with age may correlate with the possibility that the symptoms of active EAE are less severe in older animals.

RTC frequency comparisons in naive and immune rats

Table 2 shows the results of LDA comparing the frequencies of T cells with RTC activity in naive animals and in animals immunized with a panel of MBP-reactive cell lines. Prior to immunization these MBP-reactive cell lines the frequency of responding RTC ranged from 50 to 150/10^6. After immunization this frequency shows a 10- to 15-fold increase for the S1 and S22 cell lines. In contrast, immunization with C10 or S40 MBP-reactive T cells showed no increase in responding cell frequency.

Table 2. LDA of frequencies of RTC in spleens of naive and immunized LEW rats responsive to MBP-reactive T cells

<table>
<thead>
<tr>
<th>T cells for immunization and stimulation^a</th>
<th>Before immunization (/10^6)</th>
<th>After immunization (/10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>155 ± 11</td>
<td>1582 ± 1357</td>
</tr>
<tr>
<td>S22</td>
<td>108 ± 5</td>
<td>2294 ± 1815</td>
</tr>
<tr>
<td>C10</td>
<td>46 ± 3</td>
<td>47 ± 5</td>
</tr>
<tr>
<td>S40</td>
<td>80 ± 11</td>
<td>77 ± 13</td>
</tr>
</tbody>
</table>

^aLEW rats, randomly assigned to four groups (n = 4), each received a single immunizing dose of 3 x 10^6 cells of the indicated MBP-reactive cell lines. Fifteen days later, normal and immune, nylon wool-passed, splenic T cells were seeded at 3 x 10^6 to 1 x 10^7/well, each dilution in 24 wells of 96-well flat-bottomed culture plates. Plates were previously seeded with irradiated (3000 rad) S1, S22, S40 or C10 cells (2 x 10^6). Forty-eight hours later, the plates were pulsed with 0.5 mCi (3H)thymidine/well for 6 h, harvested and assessed for isotope incorporation. Results of a single assay from eight independent experiments are shown and expressed as number of RTC per 10^6.

Table 3. Comparative frequencies of anti-S1 RTC and MBP-reactive T cells

<table>
<thead>
<tr>
<th>Immunization of LEW rats^a</th>
<th>MBP-reactive T cells (/10^6)</th>
<th>RTC reactive to S1 (/10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>&lt;1</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>MBP/CFA (15 days)</td>
<td>43 ± 10</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>S1 cell line (21 days)</td>
<td>&lt;1</td>
<td>1451 ± 328</td>
</tr>
</tbody>
</table>

^aLEW rats were randomly assigned to three groups (each n = 4): (i) untreated, (ii) immunized with MBP/CFA and (iii) immunized with 5 x 10^6 resting S1 cells. Splenic and lymph nodes were recovered at the indicated times and nylon wool-passed suspensions prepared for LDA. For assessing the frequencies of MBP-reactive T cells, responder populations were seeded at 3 x 10^6 to 6 x 10^6/well in 24 replicate wells containing irradiated (3000 rad) spleen cells as APC (2 x 10^5) with or without MBP (20 µg/ml), RTC frequencies were established as described in Table 2.
Regulatory T cells and EAE

Fig. 4. LEW rats immunized with resting S1 cells have greatly increased proliferative responses against the immunizing S1 cells. For descriptions see Fig. 2 legend.

Fig. 5. RTC have a range of clonotypic and cross-reactive specificities. Splenic T cells from an S1 immunized rat show strong responses to the immunizing S1 line and significant cross-reactive responses to a second MBP specific line, S22 (left panel). In contrast, RTC from an S1 immunized animal that has received four consecutive rounds of re-stimulation with irradiated S1 cells lose much of their cross-reactivity to S22 cells (right panel). For descriptions see Fig. 2 legend.

epitopes characteristic of activated T cells (25). To avoid this, groups of rats \( (n = 4) \) were immunized with a panel of MBP-reactive cells \( (3 \times 10^6) \) that had been rested for 7 days after the previous round of stimulation prior to their use as immunogens. Control animals were left untreated. Splenic T cells were prepared for proliferation assays 2–3 weeks later. Figure 4 shows that T cells from young rats immunized with resting S1 cells have proliferative responses nearly 10 times greater than naive T cells from young non-immune donors and at least twice the level of responses by normal cells from aged donors (see Fig. 3).

Clonotypic and CR-RTC

To explore the specificity of RTC, cells from animals immunized with the S1 line were stimulated in culture with a panel of different MBP-reactive cell lines. Splenic cells from rats immunized with the S1 line showed strong proliferative responses to the immunizing S1 line and significant cross-reactive responses to the S22 line as well, but no response to the C10 line and, as expected, to thymocyte stimulators (Fig. 5, left panel). In contrast, when cells from S1 immune animals have been re-stimulated with S1 cells in bulk cultures for three rounds, their cross-reactive response to other lines is minimal (Fig. 5, right panel).

Surface phenotype of RTC

Phenotypic comparisons of an early RTC line and a repeatedly stimulated RTC line are shown in Fig. 6. The early line expresses varying levels of CD8 and contains a minor population of CD4+ cells (Fig. 6A). After three rounds of further stimulation with the immunizing S1 cells, the surviving RTC population is exclusively CD8+ (Fig. 6B).

Comparison of proliferative and lytic specificity of RTC from naive donors

Figure 7 shows the results of a comparison of the proliferative response of RTC from naive donors against various cell lines and their ability to lyse these cells following activation; proliferative activity does not correlate with lytic activity. In this example, the S1 cell line was most effective in stimulation of a proliferative response; however, RTC from these cultures showed only minimal lysis of S1 and C10 target cells. In contrast, these S1 activated RTC showed extensive lysis of A20 target cells, a murine lymphoma line highly susceptible to Fas mediated lysis, despite the fact that A20 cells provoke no proliferative response.

Discussion

LEW rats pretreated with a subpathogenic dose of MBP-reactive T cells develop a near total resistance to further attempts to cause EAE following adoptive transfer with a pathogenic line of MBP-reactive T cells. This resistance to adoptive EAE is accompanied by an increased number of RTC that are functionally antagonistic to the immunizing auto-reactive T cells. Co-injections of RTC and pathogenic anti-MBP T cell lines prevents adoptive EAE (6,7). These observations indicate that certain cell surface molecules on autoreactive T cells are immunogenic and able to provoke a protective immune response mediated by a subset of RTC.

In previous studies we have also shown that not all the MBP-reactive T cells are similarly effective in stimulating RTC and inducing EAE protection. Among 10 tested, only three independently derived MBP-reactive T cell lines have significant RTC-stimulatory activity. In this study we explore the most strongly immunogenic anti-MBP cell lines, S1 (6), and subclones of this line (26–28) that have been characterized for TCR sequence, cytokine production and their ability to interact with autoimmune parenchymal cells, to determine some of the features of the mechanism whereby whole cell vaccines of pathogenic cells provoke an effective immune resistance to EAE.

EAE-RTC are readily found in naive LEW rats

Here we show that CD8+ EAE-RTC are readily detectable in naive animals by (i) their ability to inhibit secondary adoptive
Regulatory T cells and EAE

Fig. 6. Surface phenotypes of CR-RTC and CT-RTC. The phenotypes of the responding T cells described in Fig. 4 are shown. CR-RTC were obtained directly from a 4-day culture of naive splenic T cells stimulated with irradiated S1 cells (A). CT-RTC were obtained from the regulatory T cell lines that received three additional subsequent rounds of re-stimulation with irradiated S1 cells at weekly intervals (B).

Fig. 7. Comparison between the proliferative response and cytotoxic effects of RTC on A20, S1 and C10 cells. T cells from naive donors show strong responses to the S1 line, but when these anti-S1 cultures are assessed for lytic ability on various target cell lines, they show minimal cytotoxicity against the stimulating S1 anti-MBP cell line and extensive lysis on the murine A20 lymphoma line which is sensitive to Fas-mediated lysis.

EAE episodes in irradiated animals (Fig. 1), (ii) their response to MBP-reactive cell lines in culture (Fig. 2) and (iii) their ability to protect normal animals from adoptive EAE in co-transfer experiments (Table 1). RTC activity increases in older rats (Fig. 3), and the numbers (Table 2) and proliferative activity (cf. Figs 3 and 4) of these cells are markedly increased following deliberate pre-immunization with pathogenic cells.

RTC that inhibit EAE significantly outnumber T cells precursors reactive to MBP

Frequency analyses of MBP-specific T cells and of RTC that react to MBP-specific cells indicate a marked difference in the proportion of these two populations in the peripheral lymphocyte pool. In normal, non-immune rats, T cells responsive to MBP number <1/10^6 and RTC capable of responding
to MBP-reactive T cells outnumber the latter by 100-fold (50–150/10⁶). This disproportionate ratio of EAE-RTC and MBP-reactive T cells is somewhat puzzling. It is tempting to speculate that these high RTC frequencies may reflect the existence of ongoing network interactions involving T cells (RTC) reactive to TCR peptide–MHC complexes on other T cells; such a network might reduce the chances of spontaneous autoimmune activation of potentially autoreactive T cell clones. If so, it can be predicted that RTC represent clonally expanded populations perhaps expressing differentiation surface markers and features typical of memory T cell populations. In this regard recent studies indicate a markedly prolonged expression of Fasl on S1 activated RTC in comparison to T cell populations activated with Con A, MBP or with alloantigens in mixed lymphocyte cultures (D. Sun and D. B. Wilson, in preparation).

Following immunization with pathogenic cell lines, RTC show an additional 20-fold increase in frequency (Tables 2 and 3). These dramatically increased levels of T cells with RTC activity continue for several months following immunization (data not shown) a finding which may account for the prolonged resistance to further attempts to cause paralytic disease in animals once they have recovered from actively or adoptively induced EAE (6,29).

In this context, it is of interest that normal rats of the Fischer 344 (F344) strain, which express an MHC haplotype identical to that of LEW rats except for a single gene in the non-classical region corresponding to the mouse Qa-Tla locus (30), show a near complete resistance to active EAE following immunization with MBP/CFA but, in contrast, they develop severe disease following adoptive transfer of syngeneic cells from immune animals following re-stimulation in culture. This observation suggests the possibility that RTC may exist in the F344 strain in substantially higher frequencies or that they are more active on a cell-for-cell basis against potentially pathogenic MBP-reactive T cells. In fact, recent analyses indicates a frequency of 2000–5000 RTC/10⁶ in the normal F344 rat, a number substantially higher than in the LEW rat. In addition, MBP-reactive cell lines such as C10 and S40 that are non-immunogenic in LEW rats provoke strong RTC responses in F344 rats (D. Sun and D. B. Wilson, in preparation).

RTC have a range of clonotypic and cross-reactive specificities

Splenic T cells from an S1 immunized rat show strong responses to the immunizing S1 line and significant cross-reactive responses to a second MBP-specific line, S22 (Fig. 5, left panel). In contrast, RTC populations from an S1 immunized animal that have received four consecutive rounds of re-stimulation with irradiated S1 cells lose much of their cross-reactivity to S22 cells (Fig. 5, right panel). Thus RTC populations, initially with broad cross-reactive specificity (CR-RTC), become more clonotypic (CT-RTC) as they undergo repeated rounds of selection on a particular MBP-reactive T cell line. During this selection process, as CR-RTC populations assume a more clonotypic specificity, they also become uniformly CD8⁺ (Fig. 6).

The precise identity of stimulatory molecules on MBP-reactive T cells that activate RTC populations remains elusive. While it is clear that they have a clonotypic distribution, thus TCR peptide–MHC complexes are obvious candidates, other factors appear to be involved. For example, we find that whole cell vaccines are far more effective at inducing disease resistance in EAE and graft-versus-host disease than are TCR peptide vaccines (D. Sun and D. B. Wilson, unpublished results). In view of the findings here that T cells with regulatory activity are CD8⁺ and given that this subset recognizes endogenously processed peptides most effectively, one factor in the differential immunogenicity of whole cells and TCR peptides as vaccines for inducing disease resistance is that exogenous peptide immunogens may be poorly handled by the endogenous class I MHC antigen presentation pathway.

Studies on the RTC in EAE-recovered animals also revealed that those recovered from active EAE do not consistently show increased regulatory cell activity against the autoreactive T cells tested (Table 3). We believe this is because the complex nature of regulatory T cells in active EAE. Conceivably, active EAE involves hundreds of autoreactive T cell clones, which in turn, elicit diverse regulatory cell subsets. As a result, those reactive to MBP-reactive T cells selective tested may, or may not, increase.

Finally, assessments of the functional specificity of RTC (Fig. 7) indicate that proliferative and lytic specificity of RTC do not necessarily correlate. RTC showing good proliferative responses to S1 show only minimal lytic activity on S1 target cells; however, they are very effective in killing the Fas-sensitive murine A20 cell line. It remains to be determined whether Fas–Fas ligand interactions is one of the regulatory mechanisms by which RTC become functionally active.

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Abbreviations

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CASUP Con A-stimulated rat spleen cells
CDR complementarity-determining region
CFA complete Freund's adjuvant
Con A concanavalin A
CT-RTC clonotypic regulatory T cell
CR-RTC cross-reactive regulatory T cell
EAE experimental autoimmune encephalomyelitis
LDA limiting dilution assay
LEW Lewis rat
MBP myelin basic protein
RTC regulatory T cell

References


