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Selection of anti-myelin basic protein T-cell lines in the Lewis rat: V-β 8.2 dominance and conserved complementarity-determining-region-3 motifs are dependent on serine at position 78 of myelin basic protein

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Abstract

In the Lewis rat, the dominant T cell repertoire to myelin basic protein (MBP) is directed to the peptide 71–87 and the T cell receptors of pathogenic T cells are of the Vβ 8.2 genotype with short CDR3 sequences having a characteristic motif. However, this paradigm has been reached through analysis of long-term encephalitogenic lines and clones. We initiated the present study to examine the process of selection of the TCR Vβ 8.2 and characteristic CDR3 motifs upon immunization with guinea-pig MBP, and rat or guinea-pig 71–87 peptides. We found that the dominance of Vβ 8.2 developed progressively over 4–6 in vitro stimulations. Following immunization with rat 70–86, which differs from the guinea-pig peptide in one amino acid at position 78, the dominance of Vβ 8.2 and the characteristic CDR sequences are not seen. Thus, Vβ 8.2 dominance and specific CDR3 TCR motifs are seen with heterologous GpMBP but not with self rat MBP.

Keywords: Autoimmunity; Encephalomyelitis; Myelin basic protein; T lymphocytes

1. Introduction

The immune repertoire to myelin basic protein has been extensively studied in both rats (Ben-Nun et al., 1981; Happ et al., 1988; Burns et al., 1989; Chluba et al., 1989; Gold et al., 1991; Kim et al., 1998) and mice (Zamvil et al., 1988; Burns et al., 1989; Heber Katz and Acha Orbea, 1989). Based largely on the analysis of long term encephalitogenic T cell lines and clones, most investigators in the field have concluded that the pathogenic T cell repertoire in the Lewis rat is restricted in both the peptide reactivity and the TCR Vβ and Vα usage and CDR3 sequences (Heber Katz and Acha Orbea, 1989). Some investigators, however, have reported that the pathogenic repertoire was much more heterogenic in both Vβ TCR and CDR3 sequences (Mannie et al., 1989; Sun et al., 1992, 1993a,b, 1995; Gold et al., 1995). Some investigations also have used T cell hybridomas from freshly isolated MBP reactive T cells to examine the fine antigen specificity (Happ and Heber Katz, 1988) or the heterogeneity of co-stimulatory requirements of MBP reactive cells (Mannie and Nairn, 1992; Watkins and Mannie, 1993). The dimensions of the pathogenic T cell repertoire are of considerable importance both at the level of our understanding of the immune repertoire in autoimmune disease, and at the more practical level of planning various modes of therapy that are based on enhancing regulation of the pathogenic cells, such as T cell vaccination (Cohen, 1989; Lider et al., 1988) or TCR peptide vaccination (Vandenbark et al., 1996; Kumar et al., 1997).

More recent work has attempted to examine the predominance of Vβ 8.2 in the immune response of Lewis rats to GpMBP. In the draining popoliteal lymph nodes the percentage of Vβ 8.2 bearing cells was found to be only 5–10%, while in the CNS infiltrate the percentage of Vβ 8.2 was higher: 15–20% (Offner et al., 1993; Tsuchida et al., 1993). When direct sequencing of Vβ 8.2 cells was done from the CNS lesions, characteristic CDR3 sequences were detected (Buenafe et al., 1994). When the primed lymph node cells were double stained with antibodies to Vβ 8.2 and IL-2 receptor alpha chain to detect activated T cells, both GpMBP and peptide 63–88 were found to induce a detectable enrichment of the Vβ 8.2 population (Weissert et al., 1998). In addition to the T-cell repertoire

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directed against the immuno-dominant peptide, revealed by immunization with whole GpMBP, there is a diverse pathogenic cryptic T-cell repertoire to MBP determinants including peptide 87–99 (Vandenbark et al., 1989), and the peptides 11–30, 51–70 and others (Mor and Cohen, 1995). The T-cells reactive to these cryptic determinants are not restricted in terms of their V-β T cell receptors (Mor and Cohen, 1995).

We initiated the present work to examine the process of Vβ 8.2 accumulation during in vitro culture, and the role played by the conditions in culture and the MBP antigen used for immunization and line expansion. We found a progressive enrichment of Vβ 8.2 cells that increased with successive stimulations with GpMBP and decreased during the rest period in propagation medium. The Gp71–87 peptide was similar to GpMBP in its ability to expand the Vβ 8.2 population. The rat 70–86 peptide, which differs from the guinea-pig sequence in a single amino acid at position 78, was ineffective in Vβ 8.2 selection.

2. Materials and methods

2.1. Animals

Inbred female Lewis rats were supplied monthly by Harlan Olac (Bicester, UK) and were used at 2–3 months of age.

2.2. Antigens and antibodies

Myelin basic protein (MBP) from the spinal cords of guinea pigs was prepared as described by Hirshfeld et al. (1970). *Mycobacterium tuberculosis* H37Ra was purchased from Difco (Detroit, MI, USA). Peptides were synthesized using the F-MOC technique with an automatic multiple peptide synthesizer (AMS 422, Abimed, Langenfeld, Germany): G71–87 is SLPQKSQRSQDENPVVH, and Rat70–86 is SLPQKSQRTQDENPVVH (the difference is underlined). The purity of the peptides was analyzed by HPLC and amino acid composition. Monoclonal antibodies anti Vβ 8.2, 8.5, 10 and 16 were purchased from Pharmingen (San Diego, CA, USA). FITC conjugated goat anti-mouse antibody was obtained from Jackson Immuno Research (West Grove, PA, USA).

2.3. Immunizations

Peptides were dissolved in PBS (1 mg/ml), and an oil emulsion was prepared (1:1 ratio) with IFA containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra. Naive female Lewis rats were immunized in both hind foot pads with 50 μl of the emulsion; each rat was injected with 50 μg of peptide. Draining popliteal lymph node cells were removed on day 12 after injection and a single cell suspension was prepared by pressing the organs through a fine wire mesh. T cell proliferation assay was performed in 96 microtitter wells (Greiner, Nurtingen, Germany).

2.4. T-cell proliferation assay

T-cell proliferation assays of popliteal lymph node cells from animals primed 12 days previously with the specific peptide in CFA was performed by seeding 2×10^5 cells in stimulation medium for 3 days. Stimulation medium was composed of Dulbecco's Modified Eagle's Medium supplemented with 2 mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), non-essential amino acids (1 ml/100 ml, Bio-Lab, Jerusalem, Israel), and autologous serum 1% (v/v) (Mor et al., 1990). The cultures were incubated in quadruplicate for 72 h at 37°C in humidified air containing 7% CO_2. Each well was pulsed with 1 μCi of [^{3}H] thymidine (10 ci/mmol sp. act. Nuclear Research, Negev, Israel) for the final 18 h. The cultures were then harvested using a MicroMate 196 cell harvester and cpm were determined using a Matrix 96 direct beta counter using avalanche gas (98.7% helium–1.3% C_4H_{10}) ionization detectors (Packard, Meriden, CT, USA). The results of proliferation are expressed as cpm.

2.5. T-cell lines

Antigen-specific T-cell lines were established from lymph node cells that had been stimulated with GpMBP (10 μg/ml) or peptide (5 μg/ml) for 3 days in stimulation medium as described above. Following stimulation, the T-cell blasts were isolated on Lympho-prep (Nycomed Pharma, Oslo, Norway) and seeded in propagation medium: identical to stimulation medium without autologous serum, but supplemented with fetal calf serum 10% and T-cell growth factors from the supernatant of Con A stimulated spleen cells 10% (Mor et al., 1990). Five days after seeding, the cells (5×10^5/ml) were re-stimulated with MBP (10 μg/ml) or peptide (5 μg/ml), and irradiated thymocytes as antigen presenting cells (10^7/ml) for 3 days in stimulation medium. T-cell lines were expanded by repeated stimulation with antigen and irradiated thymocytes as antigen presenting cells every 10–12 days (Mor and Cohen, 1993). Following four rounds of stimulation, the cells were analyzed for their specificity to the immunizing peptides in a proliferation assay. When T-cell lines reached adequate numbers at the end of a rest phase, 5×10^4 line cells were seeded in 96 round-bottomed microtiter wells (Greiner) with 5×10^5 irradiated (2500R) thymocytes.

2.6. Induction of EAE

Groups of rats were injected in both hind foot pads with a 50-μl emulsion of mineral oil (IFA) containing 50 μg of

peptide or 250 μg of rat MBP and 200 μg of *Mycobacterium tuberculosis* (complete Freund's adjuvant; CFA) per rat. Adoptive EAE was transferred by intraperitoneal injection of 2×10^7 peptide-activated cells of the lines as described (Mor and Cohen, 1995). Clinical EAE was observed in immunized rats 10–14 days after peptide/CFA induction and 4–7 days following administration of T cell lines. Clinical scoring was: +1, paralysis of tail; +1.5, paresis of posterior paws and ataxia; +2, paraplegia; +3, paralysis extending to thoracic spine; +4, a moribund state (Mor and Cohen, 1995).

2.7. Flow cytometry

Line cells were incubated at 4°C for 45 min with an anti Vβ 8.2, 8.5, 10 or 16 antibodies that was obtained from Pharmingen (San Diego CA, USA). Secondary rabbit anti-mouse FITC conjugated antibodies were incubated at 4°C for 30 min. The cells were then washed and fluorescence was measured using the Facscan (Becton Dickinson). Analysis of the results was carried out using LYSIS II software.

2.8. Reverse-transcription polymerase chain reaction (PCR) analysis

Total cellular RNA was isolated by the single-step method using the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). A 1-μg amount of total RNA was used for the reverse transcriptase reaction. RNA was incubated with oligo-$(dT)_{(12-18)}$ (200 ng) for 5 min at 65°C and left to cool to 42°C. The reverse transcriptase reaction contained: dNTPs (0.25 mM each), RNAsin (3 units), dithiothreitol (10 mM), sodium pyrophosphate (4 mM), MMLV reverse transcriptase (200 units, RT, BRL, USA) and RT buffer. The mixture was incubated for 120 min at 42°C and then heat inactivated for 5 min at 95°C. The PCR reaction contained 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, 50 mM KCl, 1 μM of each of the primers and 1 unit of Taq polymerase for each 100 μl reaction.

2.9. Primers and sequencing

Two control primers residing in the constant region of the rat TCRβ gene were used as a positive control for rat TCR cDNA: OLCBRM-53 5′-CTG CTA CCT TCT GGC ACA ATC C-3′ OLCBHRM-355′-TCT CAT AGA GGA TGG TGG CAG ACA-3′ A constant primer was used as a counterpart to the Vβ specific primer in all reactions.

OLCONS5′-GAT CTA AGC TTC TGA TGG CTC AAA C-3′

The panel of rat Vβ specific primers:

RBV1	5′-GTA TAA ACA GAG CAC CAA GAA GCT C-3′
RBV2	5′-ACA CCA AGG TGG CGT CTG GTA CCA C-3′
RBV3.3	5′-GTA TTG CTG AAA AGG GAC ATA CTT T-3′
RBV4	5′-CTG GTG GCA GGT CCA GTC GAC CCT AAA A-3′
RBV5.1	5′-ATT CCC ATC TCT GGA CAT AGA AGT G-3′
RBV6	5′-TCA GAC ACC CAA ATT CCT GAT TGG T-3′
RBV7	5′-ACC CAG ATC TGG GGC TAC GGC TGA TTT A-3′
RVB8const	5′-ACA TGT ACT AGT ATC GAC AGG ACA T-3′
RBV8.1	5′-ATT GAG CTG TCG CCA GAC TA-3′
RBV8.2	5′-TGA GTT GTA AGC AGA ATA AT-3′
RBV8.3	5′-GTT CAA CTG TCA CCA GAC TG-3′
RBV9	5′-GTT ATG CAG AAC CCA AGA TAC-3′
RBV10	5′-AGA CTC TAG ACA ATT GCT GAA GGT T-3′
RBV11	5′-TCC TGA TCA ACT TTA ACA ACC AAG C-3′
RBV12	5′-GAG CCA GCT GCA GGC CAC AAT GAT C-3′
RBV13	5′-GTC TGA AAC TGC AGT CAC CCA GTC C-3′
RBV14	5′-GAC TAT CCA TCA ATG GCC AGC TGT C-3′
RBV15	5′-CTG GCT ATG GGG TTG GAG CTC TCG T-3′
RBV16	5′-CCT GAC TGC AGG ACA CAC AGG ACC C-3′
RBV17	5′-TAT GAG CTC TAG TCC TGA AAA GGC G-3′
RBV18	5′-AGT GTG TTG TTG ATA GTC AAG TTG C-3′
RBV19	5′-AAA AGA ACA GCT AGC CAG AAT GCA G-3′
RBV20	5′-GAC GCC AAG ACA TTT GAT CAA AAT G-3′

PCR cloning was done using Phagescript SK(+) cloning kit (Stratagene, La Jolla, CA, USA) and the PCR-Script SK(+) plasmid. Sequencing was done using the T3 and T7 primers at the Sequencing unit of the Weizmann Institute using Applied Biosystems DNA Sequencer. The method of sequencing utilized the Taq dye dideoxy terminator Cycle.

The sequencing Kit was purchased from ABI (Perkin Elmer, Foster City, CA, USA).

3. Results

3.1. Selection for Vβ 8.2 in a GpMBP reactive T-cell line

The commonly accepted paradigm is that the TCR of T cells reactive to MBP in the Lewis rat preferentially use a Vβ 8.2 TCR and express a short CDR3 sequence featuring a common DS motif. This observation has been made from the study of T cell lines and clones that were cultured in vitro for prolonged periods of time, and thus, could represent an in vitro selection advantage of Vβ 8.2 positive T cells. Indeed, in the primed popliteal lymph nodes draining the site of GpMBP inoculation, the percentage of T cells bearing the Vβ 8.2 segment was examined and found to be only 5–10% (Offner et al., 1993; Tsuchida et al., 1993). In order to examine the dynamics of the predominance of Vβ 8.2 positive T cells in culture, we injected Lewis rats with GpMBP and selected a line by repeated stimulation with GpMBP, using the standard protocol (Ben-Nun et al., 1981). Fig. 1 shows that from the 2nd to the 8th stimulation there was a progressive accumulation of cells expressing Vβ 8.2: from 40% after two stimulations to 90% following the eighth stimulation. This line induced severe EAE upon injection to naive rats. T-cell clones derived from the line could also induce EAE in naive recipients (Mor and Cohen, 1993). However, since the response to whole MBP includes other epitopes, such as the 50–69 and the 87–99 peptides (Offner et al., 1989; Vandenbark et al., 1989; Mor and Cohen, 1993), we decided to examine the Vβ response to immunization with the encephalitogenic peptide of GpMBP, Gp71–87.

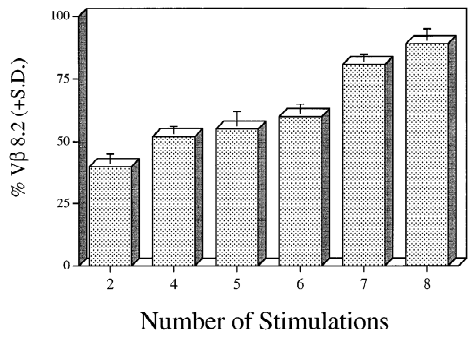

Fig. 1. Percentage of cells expressing the Vβ 8.2 as a function of the stimulation cycle of a GpMBP reactive T-cell line. Samples of cells were obtained at the end of antigen-specific stimulation, washed in FACS buffer (PBS with 1% fetal calf serum and 0.02% sodium azide) and incubated in 4°C for 30 min with FITC-conjugated monoclonal anti Vβ 8.2. The percentage of cells expressing Vβ 8.2 was calculated from the histogram.

3.2. GpMBP encephalitogenic peptide induces a Vβ 8.2 response

We immunized groups of Lewis rats with Gp71–87 in the footpads and obtained draining popoliteal lymph node cells on day 10 post-injection. The cells were stimulated repeatedly with the immunizing peptide. Following three repeated in vitro stimulations, the line was examined by FACS for the expression of the Vβ 8.2 TCR. Fig. 2 shows the results of the analysis, before, during and after the fourth stimulation.

Prior to the activation with Gp71–86 (Pre-Stimulation), 24% of the cells were Vβ 8.2 positive. At 2 and 3 days of stimulation, over 80% of cells expressed the Vβ 8.2 marker. Upon transfer of the cells to the propagation medium (Post-Stimulation), the percentage of cells expressing Vβ 8.2 fell to less than 35%. Thus, stimulation was accompanied by a marked enrichment of the Vβ 8.2 population followed by a decrease in the post-stimulation phase (Fig. 2). A similar experiment performed with a T-cell clone to MBP (A1 clone expressing Vβ 8.2) revealed the percentage of Vβ 8.2 at peak stimulation to be 99%, on day 2 of rest 99%, and after 5 days of rest 92%. Thus, the major part of the Vβ 8.2 reduction in the rest phase (over 50% reduction) was due to cell loss.

3.3. Different forms of stimulation augment Vβ 8.2

To investigate whether different forms of stimulation might influence Vβ 8.2 enrichment differently, we compared the effect on the Vβ 8.2 increase of 5 different modes of line activation (Fig. 3).

All five forms of activation led to an increase of Vβ 8.2 from 34 to over 70% of the population, on day 3 of

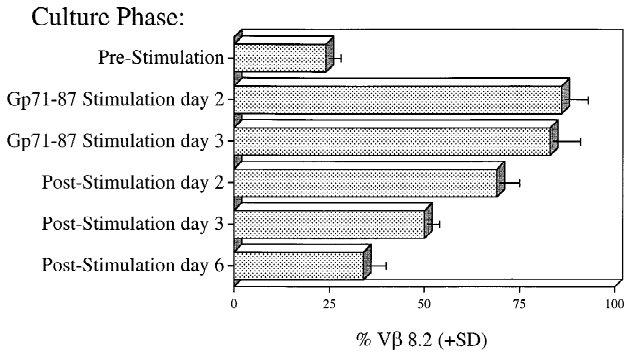

Fig. 2. Percentage of cells expressing Vβ 8.2 as a function of the culture phase in the Gp71–87 reactive T-cell line. The line cells were examined by FACS at the end of 'rest period' in propagation medium (containing T-cell growth factors), 5 days after the 3rd antigen stimulation, designated Pre-Stimulation. Additional samples were tested from cells on the second day of the 4th stimulation designated Gp71–87 Stimulation day 2, and at the third day (designated Gp71–87 Stimulation day 3). The other samples were from propagation medium on the second, third and sixth days (Post-Stimulation day 2, 3 and 6).

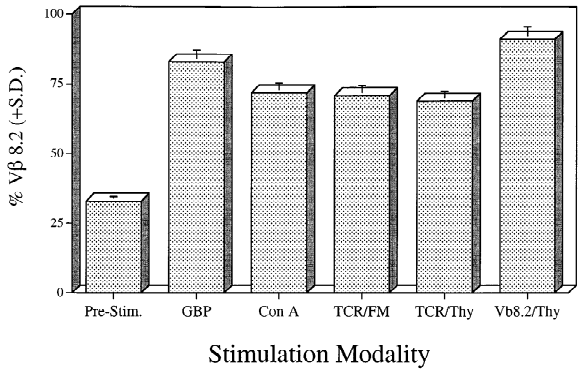

Fig. 3. Percentage Vβ 8.2 expression as a function of the type of T-cell line stimulation. Cells were examined after culture in propagation medium following four stimulations and 5 days in rest (designated Pre-Stim.). Cells were activated with guinea-pig MBP (GBP) or with the mitogen Concanavalin A (Con A), or with plastic-bound anti-T-cell receptor antibodies in full medium (TCR/FM), or anti-TCR with thymocytes (TCR/Thy) or with anti-Vβ 8.2 antibodies with thymocytes (Vb8.2/Thy).

stimulation. The most effective stimuli were plate adherent anti-Vβ 8.2 antibodies with thymocytes and GBP. Similar efficiency was seen with the three other modes of stimulation (Con A, anti-TCR antibodies and IL-2 medium (Reizis et al., 1995) and anti-TCR antibodies and thymocytes). Thus, all modalities of stimulation led to a Vβ 8.2 increase, and anti-Vβ 8.2 antibodies with thymocytes were the most effective in enrichment.

Previous work in both human and mouse T cells, has documented a decrease in TCR expression during stimulation (Itoh et al., 1999; Lanzavecchia et al., 1999). This phenomenon is detectable after 15 min and peaks at 5 h leading to reduced expression of up to 50–70%. However, when using optimal proliferative concentrations of peptides this down regulation is only about 25–50%, and it is not sufficient to render the cells negative by TCR–FACS analysis (especially since the fluorescence intensity of TCR staining is plotted on a logarithmic scale); the residual TCR expression is clearly detectable (Itoh et al., 1999). In our samples, the intensity of TCR staining during activation (measured after 48 or 72 h) was lower than during rest (data not shown), but clearly positive in relation to the control unstained cells. Thus, the increase and decrease in Vβ 8.2 expression is probably due to T-cell population dynamics and not due to TCR up or down regulation resulting from activation–rest cycles.

3.4. IL-2 in the post stimulation growth medium induces a decrease in Vβ 8.2 cells

Fig. 2 shows that the percentage of Vβ 8.2 cells falls progressively during culture in propagation medium. One of the most important growth factors in the propagation medium is IL-2. Thus, we designed experiments to examine the role of the IL-2 concentration in the Vβ decline in the propagation medium. We plated cells in propagation medium in various amounts of IL-2 (0, 1, 5, or 25 units/ml), without added T-cell growth factors, and followed them longitudinally for various days after stimulation recording the cell count, the percentage of apoptotic cells and the percentage of cells expressing Vβ 8.2 TCR (Fig. 4). As can be seen from Fig. 4A, cell numbers increased progressively with higher concentrations of IL-2, peaking on day 3, and decreasing on day 6. Without IL-2, as expected, the percentage of dead cells was the highest, and it decreased with the addition of IL-2 to the medium (Fig. 4B). There appeared to be no difference regarding the mount of IL-2 added. The curve of dead cells behaved in a bimodal pattern: a high percentage of dead cells were seen 1 day following stimulation, which could represent activation induced cell death (Wesselborg et al., 1993). This was followed by a decrease in the percentage of dead cells on days 2 and 3 in culture. A second increase in dead cells

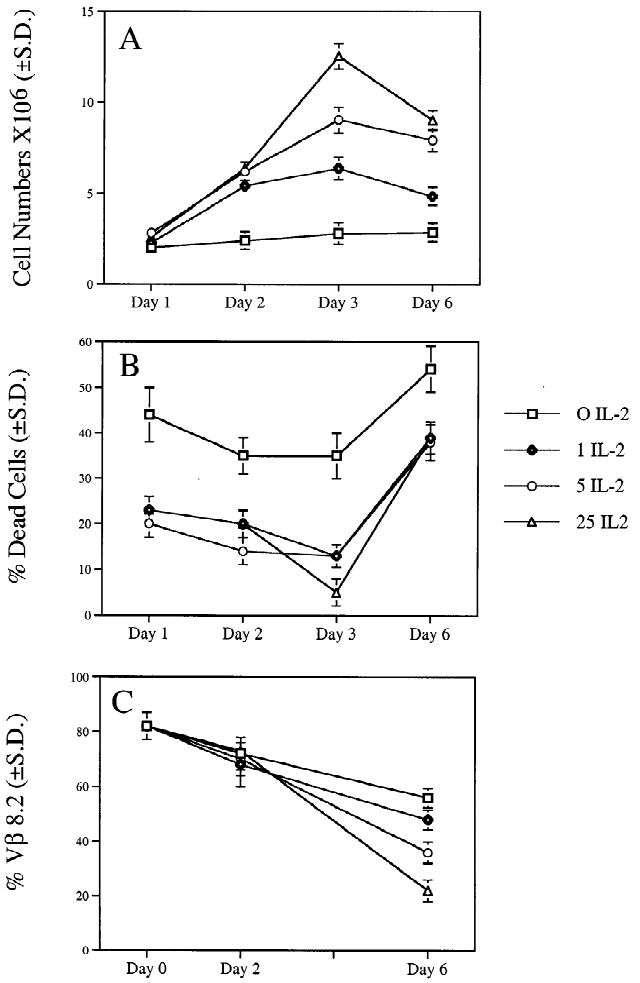

Fig. 4. Effect of IL-2 on Vβ 8.2 expression during the propagation period. Following the 4th stimulation of line Gp71–90, cells were plated in medium containing different IL-2 concentrations (without T-cell growth factors). At four time points, the cells were counted (A), the percentage of dead cells in culture were recorded (B), and the percent expression of Vβ 8.2 was analysed by FACS (C).

appeared at day 6. The second peak could represent a decline in IL-2 receptor expression and thus apoptosis for lack of growth stimulus (Mor and Cohen, 1996).

Interestingly, the percentage of Vβ 8.2 was inversely related to the IL-2 concentration, with a larger decrease in Vβ 8.2 in the population as more IL-2 was added to the medium (Fig. 4C). Thus, the decrease in percentage of Vβ 8.2 in the post-stimulation period could be related to a propriocidal effect of IL-2 (Lenardo, 1991; Boehme and Lenardo, 1993). Recent work has demonstrated that IL-2 leads to the induction of effector molecules involved in apoptosis, and the effect was not seen in IL-2 receptor alpha knockout mice (Zheng et al., 1998). Thus, the Vβ 8.2 cells had an advantage during stimulation, but after stimulation, the same cells were more susceptible to apoptosis apparently induced by IL-2 in the propagation medium.

3.5. Comparing the Gp71–87 and Rat70–86 peptides: a single amino acid determines Vβ 8.2 selection

Immunization of rats individually with either GpMBP or Rat MBP resulted in lines that were markedly different in Vβ 8.2 composition after 6–8 stimulations: 70–90% for GpMBP and 25% for rat MBP. To test the effect of the immunogenic peptides on the process of Vβ 8.2 selection, we immunized five Lewis rats with the Gp71–87 peptide in one footpad, and with Rat70–86 in parallel in the other footpad. We immunized with both peptides simultaneously to minimize the possibility that different rats might have different T-cell repertoires, and thus the difference in T-cell responses would not be attributable to the differences in the immunizing peptide. We obtained the draining popliteal lymph nodes injected with Gp71–87 or Rat70–86 and expanded them in vitro to short-term T-cell lines, using the immunizing peptide. After four stimulations, cells from each line were tested in proliferation assays. Fig. 5 shows the specificity of the lines. The Gp71–87 line, similar to GpMBP T-cell lines, showed proliferation to Gp71–87 and to GpMBP, with lower but significant proliferation to Rat70–86. In contrast, the Rat70–86 line, raised using an identical protocol from the same rats, had a much higher background proliferation response to thymic APCs in the absence of added antigen. Similar elevated background proliferation was seen in T cell lines to rat MBP (data not shown). There was a small but significant response to Rat70–86 and no response to Gp71–87 or GpMBP above background. The reason for the elevated background proliferation might be the endogenous expression of MBP in thymic cells, a phenomenon documented by Fritz (Fritz and Zhao, 1996) and others (Kojima et al., 1994; Mor et al., 1998).

To examine the effects of the two peptides on Vβ 8.2 selection, we tested both lines by FACS after the third and the forth stimulations in vitro (Fig. 6). Only the Gp71–87 line demonstrated enrichment of T cells of the Vβ 8.2 family, similar to the enrichment observed with whole GpMBP. Thus, the classical Vβ 8.2 could be induced using heterologous GpMBP and its encephalitogenic peptide but not with Rat70–86 which differs from Gp71–87 only in a single amino acid substitution: serine in place of threonine at position 78.

3.6. TCR sequence analysis of Gp71–87 and Rat70–86 lines

The TCR motif of encephalitogenic anti-MBP T cell lines has been characterized by short VDJ sequences containing AS or DS (Gold et al., 1991; Buenafe et al., 1994; Lannes-Viera et al., 1995; Kim et al., 1998; Wilson et al., 1998). To examine the CDR3 motifs in our cultures, we first analyzed a panel of Gp71–87-reactive T cell lines and clones. Table 1 shows the results of this analysis. Clones designated C and A were derived from the cloning of line BP10 (Mor and Cohen, 1993), N lines were derived from naive rats, and B lines from rats 40 days post induction of encephalomyelitis. These lines were initiated using a first stimulation with Con A, followed by seeding at 500 cells per well and repeated stimulation with GpMBP

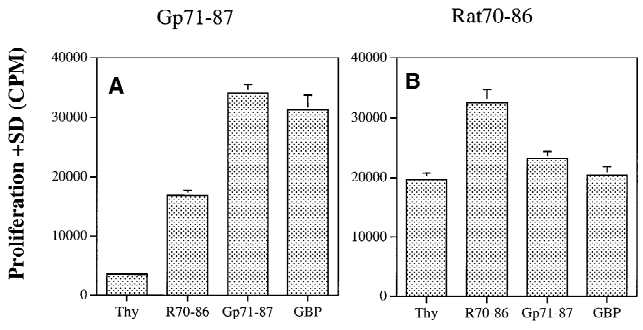

Fig. 5. Comparison of the specificities of Gp71–90 to rat71–90 T cell lines. After four cycles of peptide stimulation, the cells were tested in T-cell proliferation assays to peptides Rat 70–86, Gp71–87, GpMBP or thymocytes without added antigen (Thy).

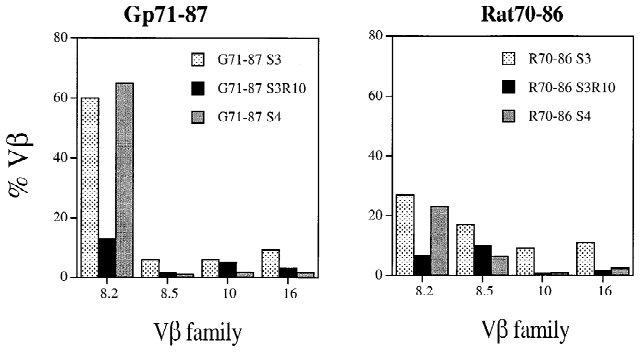

Fig. 6. Comparison of Vβ 8.2 usage in Gp71–90 and in rat71–90 T-cell lines. Vβ expression was examined by FACS, after the third stimulation (designated S3) after 10 days in propagation medium (designated S3R10) and after the 4th stimulation (S4).

Table 1
CDR3 sequences of T cells responding to Gp71–87

Clone/line	Vβ	J	CDR3 sequence													CDR3 length
C4	8.2	1.3	C	A	S	S	N	S	G	N	Y	L	Y			11
C19	8.2	2.6	C	A	S	S	D	S	S	Y	E	Q	Y			11
C28	8.2	1.3	C	A	S	S	A	S	G	N	V	L	Y			11
C37w	3.3	1.3	C	A	S	R	H	S	G	N	Y	L	Y			11
C37w	8.2	2.6	C	A	S	S	D	T	S	Y	E	Q	Y			11
C43	8.2	2.4	C	A	S	S	A	S	Q	N	T	L	F			11
C46	8.2	2.3	C	A	S	G	D	S	S	T	D	K	I	Y		12
C52w	8.2	1.3	C	A	S	S	A	S	G	N	Y	L	Y			11
C76w	8.2	2.6	C	A	S	S	P	S	W	G	E	Q	Y			11
N2	8.2	1.3	C	A	S	S	D	S	G	N	V	L	Y			11
N18	2	2.5	C	S	T	R	D	W	G	G	R	E	T	Q	Y	13
N18	17	1.2	C	A	S	S	I	G	Q	G	N	D	Y	T		12
B3	3.3	2.4	C	A	S	S	H	D	W	G	Q	N	T	L	F	13
B3	8.2	2.4	C	A	S	S	D	S	G	N	T	L	F			11
A6	8.2	2.3	C	A	S	G	D	S	S	T	D	K	I	Y		12
A8	8.2	2.4	C	A	S	R	D	S	Q	N	T	L	F			11

(Mor and Cohen, 1993). In accord with the literature, the length of the segment of the CDR3 in Vβ 8.2 T cells was short: 11 ± 0.37 amino acids. As found in other studies (Gold et al., 1991; Buenafe et al., 1994), most T cells manifested the AS or DS motifs in their CDR3 sequences.

To test whether the TCR CDR3 motifs might be present in other MBP reactive T cells, we examined a panel of T-cell lines and clones that responded to the 50–69 peptide of GpMBP. These T cells are not reactive to rat MBP and are not pathogenic (Mor and Cohen, 1993). These cells showed a more diverse length of the CDR3 region, and a specific motif could not be seen in the TCR of these T cells (Table 2). With this background information, we sequenced the TCR from the peptide-induced T-cell lines after five cycles of in vitro stimulation. We isolated cDNA from both lines, performed PCR for Vβ 8.2, and cloned the PCR product into Phagescript SK(+) cloning kit and the PCR-Script SK(+) plasmid. The sequences of the CDR3 of these PCR clones are shown in Table 3. We found that the Gp71–87 line, similar to the previous GpMBP lines and clones, showed a predominance of Vβ 8.2 cells, and the sequences showed the short CDR3 segment with the DS motif (Table 3). In contrast, the Rat70–86 line was much more heterogenous in terms of its Vβ composition, and the CDR3 sequences were of varying length. Moreover, the DS motif was seen in only 4 of 31 sequenced PCR clones. The Rat70–86 peptide did not lead to the expected Vβ 8.2 enrichment and the sequences of T cells were only infrequently of the previously described DS or AS motifs.

4. Discussion

The issue of T-cell repertoire selection to MBP in EAE has been extensively studied. The generally accepted paradigm is that in Lewis rats, upon immunization with GpMBP, the pathogenic T cells are directed to peptide 71–87, use the Vβ 8.2 TCR element and have short CDR3 motifs that contain DS or AS. Some authors, however, have reported that the T-cell repertoire to MBP in the Lewis rat is much more diverse (Sun et al., 1993a,1994a)

Table 2
CDR3 sequences of T cells responding to Gp50–69

Clone/line	Vβ	J	CDR3 sequence													CDR3 length
B9	8.2	1.3	C	A	S	G	G	S	G	N	V					9
N8/2	8.2	1.4	C	A	S	S	D	S	G	Y	E	R				10
N8/10	10	1.1	C	A	S	S	P	D	R	G	D	T	E			11
A14	6	1.1	C	A	S	S	L	D	R	N	T	E				10
N16	8.2	1.4	C	A	S	R	E	G	A	F	S	N	E	R		12
B32	9	2.5	C	A	S	S	R	S	G	L	G	Q	E	T		12
N15	3.3	2.3	C	A	S	I	L	M	G	G	Y	T	T	D	K	13
N16	3.3	2.3	C	A	S	I	L	M	G	G	Y	T	T	D	K	13
A7	6	2.6	C	A	S	S	L	P	G	Q	F	S	Y	E		12
A13	6	2.6	C	A	S	S	L	P	G	Q	F	S	Y	E		12
C62	8.3	2.1	C	S	S	N	E	G	W	G	K	A	E			11
	8.1	2.1	C	A	S	S	D	N	W	G	R	Y	A	E		12

Table 3
CDR3 sequences of Rat70–86 and Gp71–87 lines

Line	#[a]	Vβ	V[b]	nDn	J	J#
Gp71–87	1	8.2	tgtgccagcagt	gacagtggc	aacacc	2.4
			CASS	DSG	NT	
	1	8.2	tgtgccagcagt	gactctt	ctacagacaag	2.3
			CASS	DSS	TDK	
	1	8.2	tgtgccagca	ccgacag	ttctggaaatgtg	1.3
			CAS	TDS	SGNV	
	2	8.2	tgtgccagcagt	gacag	ctcctatgag	2.6
			CASS	DS	SYE	
	1	8.2	tgtgccagcagt	gattcccgactc	gaaaga	1.4
			CASS	DSRL	ER	
Rat70–86	7	8.2	tgtgccagcag	gacctcag	aaaacacc	2.4
			CAS	RTSE	NT	
	1	8.2	tgtgccagcag	cttgggggggag	ctcctatgag	2.6
			CAS	SLGGS	SYE	
	2	8.2	tgtgccagcagt	tct	agtcaaaacacc	2.4
			CASS	S	SQNT	
	4	8.2	tgtgccagcag	cgacag	ctcctatgag	2.6
			CAS	SDS	SYE	
	2	4	tgtgccagtag	agaggga	actacagacaag	2.3
			CAS	REG	TTDK	
	1	4	tgtgccagtagc	ccaacgacag	atgag	2.6
			CASS	PTTD	E	
	7	4	tgtgccagtagc	caccccggcggggcgg	actatgac	1.2
			CASS	HPGGAD	YD	
	6	10	tgtgccagcagc	tccat	tacaggcaag	2.3
			CASS	SI	TDK	
	1	10	tgtgccagcagc	ccgacagggac	caccgggcag	2.2
			CASS	PTGT	TGQ	

[a] Number of times a sequence was seen in PCR cloning.
[b] DNA sequence given in upper row and amino acid sequence in the second row.

and includes many non-Vβ 8.2 cells. The same authors generated T-cell lines from the spinal cord and found them to be heterogenic in terms of TCR diversity (Sun et al., 1994b). Our present study indicates that the majority of T-cells developing in vitro were of the Vβ 8.2, but that some cell lines were of other Vβ families and had non-conserved CDR3 motifs (Table 1). A possible explanation for the high level of diversity found by Sun et al., is the use of non-conventional APCs that may select for diverse T cells (Sun et al., 1993a). Others have found that modification of the MBP molecule has a diversifying effect on the T cell repertoire (Cao et al., 1998).

Novel findings in the present work are that the predominance of Vβ 8.2 occurs in culture in a stepwise process; T-cells expressing Vβ 8.2 having a clear survival advantage during various forms of stimulation, followed by increased apoptosis after exposing the cells to IL-2 containing medium (Figs. 2–4). Attempts to deplete Vβ 8.2 cells by magnetic beads and expansion of the non-Vβ 8.2 cells were not successful, as the Vβ 8.2 TCR regained dominance in culture after a few cycles of stimulation (data not shown). Previous studies have analyzed long-term cultured T cell clones that were composed of cells expressing Vβ 8.2 (Chluba et al., 1989; Gold et al., 1991), raising the possibility that the observed homogeneity is the result of a survival advantage of Vβ 8.2 cells over other Vβ families in culture. Our work shows that the Vβ 8.2 dominance is a relatively early phenomenon occurring after 4–5 cycles of stimulation. The nature of the non-Vβ 8.2 cells in the MBP reactive culture in the early stages of the line selection is not clear; these cells could represent bystander cells carried in culture due to non-antigen specific stimulation (cytokines), regulatory cells (Huang and Sriram, 1989) or MBP reactive cells that are of other Vβ families (Sun et al., 1992). As the kinetics of Vβ 8.2 accumulation was similar for GpMBP and its peptide Gp71–87, it is unlikely that the non-Vβ 8.2 cells are the result of expansion of cells reacting to other MBP epitopes. However, in some of our GpMBP reactive lines, a significant proportion of cells respond to peptide 50–69, and in these lines the proportion of Vβ 8.2 is lower and does not increase upon successive cycles of stimulation (Table 2).

Comparison of the encephaloitogenic peptides of guinea-pig and rat origin, which differ in only a single amino acid, revealed a striking effect on T-cell repertoire selection (Figs. 5–6 and Table 3). A similar broad TCR usage by T cells reactive to rat encephalitogenic peptide was reported by Johnson et al. (1997). The crucial importance of the serine at position 78 was noted previously in a study that used FACS analysis and double staining

of primed LN cells with anti-Vβ 8.2 plus anti IL-2 receptor alpha chain (Weissert et al., 1998). How can this difference between the two peptides be explained?

In our analysis of the peptide binding specificity of the class II IA molecules in the Lewis rat RT1Bl., we identified a motif that consisted of a nonamer peptide with 4 anchor positions: P1, P4, P6 and P9 (Reizis et al., 1996). Of these positions P4 and P9 were particularly important for binding. According to this motif, the serine/threonine in position 78 is located at anchor position P6, which was found to be a shallow pocket that does not contribute much to the strength of binding. Thr is larger than Ser and is probably less favorable at this position. So it is conceivable that the seemingly conservative substitution might have a large effect on TCR recognition. First, the P6 fits into a small and shallow pocket close to the surface, and might contact the TCR directly, especially as the neighboring P5 is a major TCR contact. Another possibility is that a poor fit of the Thr at pocket 6 might induce conformational changes in the peptide backbone, and other residues might be slightly displaced — indeed the whole TCR contact surface could be modified by this Ser/Thr substitution. In the case of PLP, substitution of major TCR contact residues on the encephalitogenic peptide yielded TCR antagonists with a therapeutic effect on EAE (Das et al., 1997; Legge et al., 1997; Nicholson et al., 1997).

With regard to the mechanism of lack of Vβ 8.2 dominance upon immunization with Rat MBP or Rat70–86, the finding of self MBP expression by immune cells may be relevant. Demonstration of antigen-specific proliferation by a T-cell population involves the evaluation of the excess proliferation as compared to the 'background proliferation' of line cells and APCs without added antigen. Since thymic APCs may be expressing self MBP from endogenous sources, the elevated background proliferation in this case could be related to self MBP epitopes presented in the thymus. Work performed by Fritz and colleagues in mice (Fritz and Zhao, 1996) documented such proliferation in SJL mice. Indeed, we presented evidence for the expression of a panel of auto-antigens by cells of the immune system (Mor et al., 1998). This 'ectopic' expression of MBP is apparently not sufficient to induce total clonal deletion as pathogenic MBP reactive T cell clones can be recovered from the rat thymus (Lannes-Viera et al., 1995; Wilson et al., 1998) and from the periphery (Mor and Cohen, 1993). The expression of self-antigens in the thymus was reported for insulin (Jolicoeur et al., 1994; Pugliese et al., 1997) acetyl-choline receptor (Wheatley et al., 1992) and other disease-related self antigens (Mor et al., 1998). On theoretical grounds, the relative inefficiency of RBP to induce EAE [to cause similar level of paralysis one needs 10 fold more RBP than GpMBP (Happ and Heber Katz, 1988)] may be related to the thymic expression of the self-antigen; thus, the weaker pathogenic potential and the weaker ability to recruit the Vβ 8.2 disease-mediating T cells by Rat70–86 may be related to the occupancy by self 70–86 in the thymus that results in major deletion of these cells from the mature repertoire.

Other possibilities, however, are conceivable. It is generally accepted that T cells maturing in the thymus are positively selected for low or moderate affinity to self peptides presented by self MHC class II molecules (Siegel et al., 1990; Liu et al., 1995, 1998; Alam et al., 1996). Foreign T-cell epitopes are thus higher affinity peptides for TCRs selected on self; foreign T-cell epitopes can be regarded as altered self-peptides (Nicholson et al., 1995). Now it is likely that Gp71–87 T cells are cross reactive to R71–86; our Gp71–87 line reacted to R71–86 in vitro (Fig. 5) and the line could cause EAE, so it must be able to recognize rat BP. Therefore, it is possible that T cells selected endogenously on Rat70–86 for low affinity could have a higher affinity for Gp71–87. Hence, Gp71–87 is much more effective than Rat70–86 in activating the T cells into expression and effector potential. Gp71–87 thus may be a pathogenically altered self-peptide. Recent work on human CD8 clones reactive to MBP showed that altered peptides can act as super-agonists if TCR contact residues are modified (Dressel et al., 1997).

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