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Successful prevention and treatment of autoimmune encephalomyelitis by short-term administration of anti-T-cell receptor αβ antibody

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SUMMARY

To identify an effective immunotherapy for T-cell-mediated autoimmune diseases, prevention and treatment of experimental autoimmune encephalomyelitis (EAE) induced in Lewis rats was attempted by administering a monoclonal antibody (mAb), R73, which is specific for rat T-cell receptor (TcR) αβ. Short-term administration of R73 at relatively low doses before immunization with encephalitogenic antigen, myelin basic protein (MBP), prevented the development of EAE. However, treatment with anti-CD4 and anti-Ia mAb in the same protocol was ineffective. Flow cytometric analysis demonstrated that short-term administration of R73 resulted in transient down-regulation of the TcR molecules, whereas the number of CD2-expressing T cells was well preserved. Furthermore, the response to MBP of T cells isolated from rats that were pretreated with R73 and then immunized with MBP was strongly suppressed. On the other hand, the T-cell response of R73-pretreated rats to a third-party antigen which was immunized at a later period was not inhibited. These findings suggest that *in vivo* administration of a low dose of R73 protects rats from EAE by inducing anergy of MBP-reactive encephalitogenic T cells. Furthermore, R73 treatment which started on day 10 of the immunization (shortly before the day of onset of clinical signs) completely suppressed the induction of EAE and that which started on day 11 (the day of onset) hastened recovery. Since the phenotypes of the TcR Vβ chain of encephalitogenic T cells are not so limited as previously believed, immunotherapy with mAb against the TcRαβ framework may be one of the best methods for treatment of T-cell-mediated autoimmune diseases.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is a T-cell-mediated autoimmune disease inducible by immunization of rats and mice with a brain-derived autoantigen, myelin basic protein (MBP). Following immunization, MBP-reactive T cells increase in number[1] and infiltrate the central nervous system (CNS) along with other inflammatory cells such as macrophages. With regard to the nature of encephalitogenic T cells, considerable progress has been made in elucidation of the phenotype of the T-cell receptor (TcR) Vβ chain. Since the initial studies indicated that encephalitogenic T cells predominantly use the Vβ8.2 gene in both mice and rats,[2–5] some investigators proposed the idea of 'V region disease' for EAE.[6]

Based on these characteristics of EAE-inducing T cells, several immunotherapies have been proposed so far. Among them, prevention and treatment of EAE with anti-TcR Vβ-specific antibodies [2,3] and synthetic peptides corresponding to the sequence of Vβ8.2[7–9] are noteworthy. Since these therapies are highly specific, there is only a small possibility that they would affect unrelated immune reactions. However, these highly specific immunotherapies are not particularly effective,[2] primarily because the Vβ phenotypes of encephalitogenic T cells are not as limited as previously believed. Contrary to the initial understanding, recent studies have revealed that rat encephalitogenic T cells reacting with the 87–99 sequence of the MBP molecule (BP87–99) use Vβ genes other than Vβ8.2 (primarily Vβ6 and Vβ14).[10–12] Furthermore, Su and Sriram recently reported, using the mouse model, that there is no correlation between encephalitogenicity of BP91–103-reactive T cells and the Vβ genes which they use.[13]

Taking these findings into consideration, we decided to employ an antibody with a broad specificity to encompass the heterogeneity of disease-inducing T cells instead of employing a highly specific antibody. In the present study, we examined the effect of a monoclonal antibody (mAb), R73, which is specific for a framework component of TcRαβ[14] and found that therapy with R73 was very effective for both prevention and treatment of EAE. More importantly, when R73 was administered for a short time, only immunological events which were activated

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Abbreviations: BP43–67, the 43–67 sequence of MBP; EAE, experimental autoimmune encephalomyelitis; GPBP, guinea-pig myelin basic protein; MBP, myelin basic protein.

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during the treatment were suppressed, and those which were activated before and after the treatment were not inhibited. Therefore, a highly specific therapy using an antibody with a broad specificity can be achieved if the proper treatment protocol is selected.

MATERIALS AND METHODS

Rats

Lewis rats were obtained from Charles River Japan (Kanagawa, Japan). All rats were used at the age of 8–12 weeks.

mAb and reagents

Hybridomas producing mAb against TcR$\alpha\beta$ (R73),[14] RT1.B (OX6)[15] and CD4 (W3/25)[16] were obtained from European Collection of Animal Cell Culture (Salisbury, U.K.). The following mAb: W3/25, OX8 (anti-CD8),[17] R73, OX34 (anti-CD2)[18,19] and OX19 (anti-CD5),[20] were purchased from Serotec (Blackthorn, U.K.). An mAb against rat CD3 molecules (1F4) was kindly provided by Dr T. Masuko, Dept. Hygienic Chem., Pharm. Inst., Tohoku University, Japan. Synthetic peptides corresponding to the 43–67 (FGSDRAAPKRGSGKDSH-HAARTTHY), 68–88 (GSLPQKSQRSQDENPVVHF) and 87–100 (HFFKNIVTPRTPPP) sequences of guinea-pig MBP (GPBP) were custom prepared by Multiple Peptide Systems (San Diego, CA). Keyhole limpet haemocyanin (KLH) was obtained from Calbiochem (Hoechst Japan, Tokyo, Japan). GPBP were prepared as described previously.[21]

EAE induction

EAE was induced in Lewis rats as described previously.[22] Each rat was injected in the hind footpads with an emulsion containing 100 μg GPBP in complete Freund's adjuvant (CFA). Immunized rats were observed daily for clinical signs of EAE, which were graded into four categories (grade 1, floppy tail; grade 2, mild paraparesis; grade 3, severe paraparesis; grade 4, tetraparesis or moribund condition).[23]

Treatment protocol

The hybridoma cells were grown as ascites in BALB/c mice. While ascitic fluid of R73, W3/25 and OX6 was injected intraperitoneally daily at a dose of 500 μg of protein, each batch (the same batches were used throughout the experiment) was pretested on relevant lymphocyte preparations by flow cytometric (FCM) analysis to examine specificity and titre of the antibodies. Five hundred micrograms of ascites usually contains 30 μg of ammonium sulphate-precipitated antibody. The mAb were administered using two regimens. For inhibition of the development of EAE, the mAb were administered for 7 consecutive days starting from day -7 or twice on days -6 and -2 prior to immunization with GPBP. For the treatment of ongoing EAE, R73 was administered for 7 consecutive days starting from days 10 or 11. Immunized animals developed clinical signs of EAE between days 11 and 13.

FCM analysis

Leucocytes in the spleen, popliteal lymph node and blood were collected and stained with mAb, followed by FITC-labelled anti-mouse Ig (Amersham, Amersham, U.K.). For two-colour analysis, cells were incubated with the first unlabelled mAb (OX34, OX19 and 1F4) followed by phycoerythrin (PE)-conjugated anti-mouse IgG (Biomeda, Foster City, CA). To saturate free binding-sites of the secondary antibody, cells were incubated with normal mouse serum. Then, FITC-conjugated R73 in the second step was applied. Ten thousand cells were analysed in each sample by FACScan flow cytometry. Staining with PE-conjugated anti-mouse IgG in the absence of the first antibody showed no increase of the number of positive cells after the mAb treatment.

Table 1. Effect of mAb administration on the development of EAE

		EAE		
mAb	Treatment*	Incidence†	Onset‡	Severity§
R73	×7	1/4	16	0·3±0·5
	×2	1/5	11	0·2±0·4
W3/25	×7	3/3	11·7±0·6	3
	×2	3/3	12	3·3±0·3
OX6	×7	2/3	11	3
	×2	3/3	11·3±0·6	3·2±0·3
—	—	6/6	10·1±4·9	2·6±1·2

* Lewis rats were treated with the indicated mAb in the form of ascitic fluid. A dose of 500 μg ascitic protein containing approximately 30 μg mAb was given daily from day -7 to day -1 ($\times 7$) or on days -6 and -2 ($\times 2$) and EAE was induced by immunization with GPBP in CFA on day 0.

† Number of rats with clinical EAE per total number of rats tested.

‡ Days of onset represent the average scores only for the animals which developed clinical signs.

§ Mean values of maximal clinical score ± SD are shown.

Proliferation assay

LNC (2×10^5 cells/well) were cultured with GPBP, BP43–67, BP68–88 or BP87–100 (0·54 μM–5·4 μM). KLH was used at doses ranging from 0·1 μg to 100 μg. The microwell cultures were incubated for 3 days, the last 18 hr in the presence of 0·5 μCi [^{3}H]thymidine (Amersham). The cells were harvested on glass-fibre filters, and the label uptake was determined using standard liquid scintillation techniques.

RESULTS

Effect of *in vivo* treatment with TcR$\alpha\beta$-specific mAb (R73) on EAE development

We initially tried to suppress the development of EAE using mAb against molecules associated with antigen-recognition by T cells, such as the TcR, CD4 and Ia molecules. R73 (anti-TcR$\alpha\beta$), W3/25 (anti-CD4) and OX6 (anti-Ia frame work) were administered to Lewis rats for 7 consecutive days starting from day -7 or twice on days -6 and -2 and then challenged with GPBP on day 0. All the mAb are mouse IgG1. As shown in Table 1, R73 administered according to either schedule prevented the induction of EAE almost completely. The clinical course of EAE in rats treated with R73 and in untreated controls is shown in Fig. 1. However, administration of anti-CD4 or anti-Ia antibody, both of which are reported to be effective for prevention of EAE,[24–28] did not suppress the development of

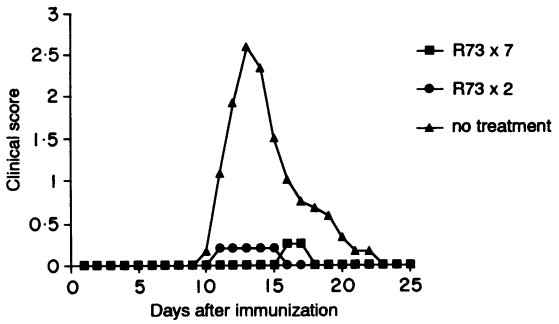

Figure 1. Clinical course of EAE in rats pretreated with R73 for 7 (■) or 2 (●) days and in untreated controls (▲). Each symbol indicates a mean clinical score of each group on the indicated day. The groups shown here are the same as those in Table 1.

EAE by the treatment protocols employed here (Table 1). Some rats treated with the mAb were examined histologically between days 10 and 15 when untreated control rats showed severe EAE, and the rest were examined between days 21 and 25. All the untreated rats had histological EAE to some extent at these time-points.[29] On the other hand, inflammatory foci were not present in the spinal cord of rats that had been treated with R73 and had no clinical signs of EAE after the challenge (data not shown).

In vivo and *in vitro* effects of R73 on TcRαβ-expressing cells

We next examined the *in vivo* effects of R73 on TcRαβ-expressing cells. R73 was administered twice at 4-day intervals to naive rats and the day of the second R73 injection was designated as day 0. T cells expressing the TcRαβ, CD2, CD3 and CD5 antigens in the lymph node, spleen and blood were examined on days 1, 3, 5 and 7 by FCM analysis. The number of cells in each preparation did not substantially decrease. Figure 2 depicts two-colour analysis for CD2 and TcRαβ. Under normal conditions, virtually all the $CD2^+$ cells expressed TcRαβ (Fig. 2a). However, T cells expressing TcRαβ decreased on days 1 and 3 and returned rapidly towards normal levels thereafter. The number of bright $CD2^+R73^+$ T cells on day 5 (Fig. 2d) became about twofold higher than that on days 1 and 3 (Fig. 2b and 2c, respectively). In sharp contrast to the down-regulation of the TcRαβ molecules, the number of CD2-expressing cells was relatively preserved. These findings indicate that the decrease of R73 bright positive cells was mainly due to immunomodulation of the TcR molecules. Double staining with R73 and CD2, 3 or 5 is summarized in Fig. 3. On day 3, $CD2^+$ cells in the lymph node (Fig. 3a, circle) accounted for about 70% of the total cells, which was almost the same as in the untreated controls after a slight increase on day 1. The percentages of $CD2^+$ cells decreased gradually thereafter. In sharp contrast, $TcR\alpha\beta^+$ cells decreased drastically from about 70% on day 1 to 15% on day 3 and recovered rapidly to a level slightly lower than that in the controls (Fig. 3a, square). $CD3^+$ cells (Fig. 3a, triangle) and $CD5^+$ cells (Fig. 3a, diamond) were intermediate between $CD2^+$ and $TcR\alpha\beta^+$ cells. Splenic (Fig. 3b) and blood (Fig. 3c) T cells showed essentially the same pattern. Some minor differences were that splenic $CD2^+$ cells increased transiently on day 3 and that $CD3^+$ cells in the blood (Fig. 3c, triangle) decreased to about 3% on day 5. These findings indicate that the transient decrease of $R73^+$, $CD3^+$ or $CD5^+$ cells in the lymphoid organs on day 3 was the result of immunomodulation of these molecules by R73 treatment, because $CD2^+$ cells were relatively preserved during this period (Fig. 3). However, a very small number of T cells treated with R73 may have been gradually depleted, as evidenced by the gradual decrease of $CD2^+$ cells by day 7 (Fig. 3).

Proliferative responses to GPBP and a third-party antigen of T cells recovered from rats protected from EAE by treatment with R73

Reactivities of T cells taken from rats protected from EAE by R73 pretreatment and from rats with full-blown EAE were measured using GPBP (Fig. 4) and synthetic peptides corresponding to the 43–100 sequences (not shown). Representative results are shown in Fig. 4. T cells from an EAE-protected rat only marginally responded to the antigens used. A small response to GPBP was observed only when T cells were cultured with a low concentration of the antigen (Fig. 4a). In contrast, T cells from an untreated rat responded markedly to GPBP at all the concentrations tested (Fig. 4b).

In the other experiment (Fig. 5), rats were pretreated with R73 or saline on days −6 and −2, and challenged with GPBP in CFA on day 0. Over the subsequent 3 weeks, rats were either protected from, or developed and recovered from, EAE. These rats were then immunized with KLH in CFA. Ten days later, the proliferative responses of T cells from the regional lymph node to KLH were measured. As shown in Fig. 5, T cells from rats which had been protected from EAE by R73 pretreatment (Fig. 5a), or rats which had recovered from EAE without R73 pretreatment (Fig. 5b), responded strongly to KLH. The magnitude of the responses was almost the same as that in control rats which had been immunized with KLH alone (Fig. 5c). These findings indicate that pulse treatment with R73 is only effective for a short time after administration and does not modulate the immune response to a third-party antigen.

Protective effect of R73 administration is short-lived

We examined whether the suppressive effect of R73 on antigen-driven T-cell activation is long lasting or short-lived. To do this, rats were treated twice with R73 and challenged with GPBP at various times before and after the R73 treatment (Table 2). Rats treated with R73 16 and 14 days before the immunization (group A) developed severe EAE. R73 treatment at 11 and 9 days before immunization with GPBP reduced the clinical severity; however, all the rats developed EAE (group B). R73 treatment on days −6 and −2, which was the routine protocol employed in the present study (group C), was the most effective protocol among those tested. R73 treatment immediately before and after the immunization was partially protected rats from EAE (group D). Finally, R73 treatment after the immunization was ineffective (group E). These findings indicate that effective R73 treatment is achieved only when the mAb is given just prior to immunization with encephalitogenic antigen and further suggest that the effect of R73 is short-lived.

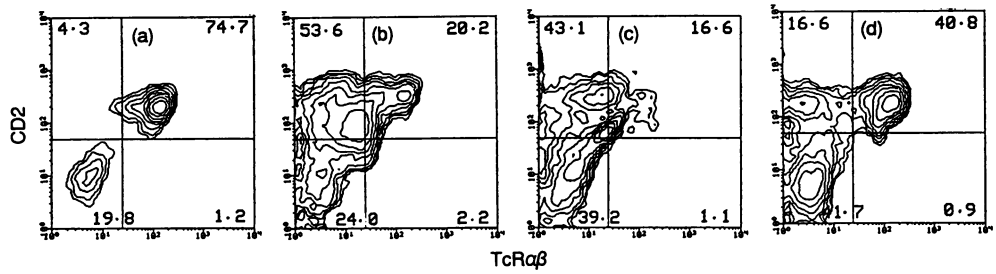

Figure 2. Two-colour FCM analysis of T cells in the lymph node for CD2 and TcR$\alpha\beta$ after *in vivo* administration of R73. R73 was given twice with a 4-day interval, and the day of the second injection was designated as day 0. On days 1, 3 and 5, the lymph node, spleen and blood were taken and processed for FCM analysis to evaluate the percentages of TcR$\alpha\beta^+$, CD2$^+$, CD3$^+$ and CD5$^+$ cells. Representative results regarding two-colour analysis for TcR$\alpha\beta$ and CD2 on days 1, 3 and 5 (b, c and d, respectively) are shown. Figure 2a is a profile of untreated control rats.

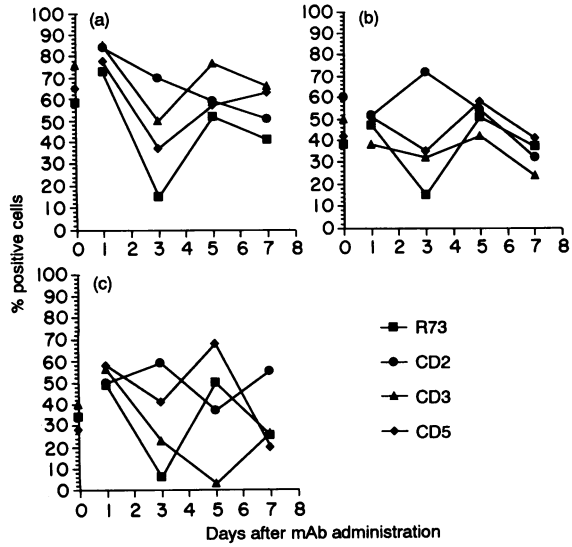

Figure 3. Time profile of R73$^+$, CD2$^+$, CD3$^+$ and CD5$^+$ cells in the lymph node (a), spleen (b) and blood (c) after treatment with R73 (for detail, see the legend for Fig. 2). From the results obtained by FCM analysis including the data shown in Fig. 2, R73$^+$, CD2$^+$, CD3$^+$ and CD5$^+$ cells were separately calculated and plotted. Each symbol represents the mean value of two to three experiments. The symbols on the *y*-axis of the figures (day 0) are from results of untreated control rats.

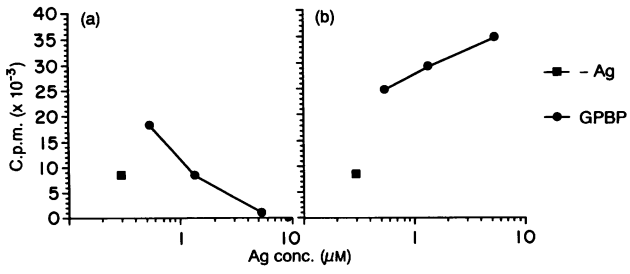

Figure 4. Proliferative responses of lymph node cells (2×10^5 cells/well) to GPBP (●) at the indicated concentrations. Lymph node cells were taken from a R73-treated/GPBP-immunized rat which was protected from EAE (a) and from an untreated/GPBP-immunized rat with 'full-blown' EAE (b). The closed square indicates c.p.m. in the absence of antigen (Ag). This assay was repeated three times and the representative results are shown.

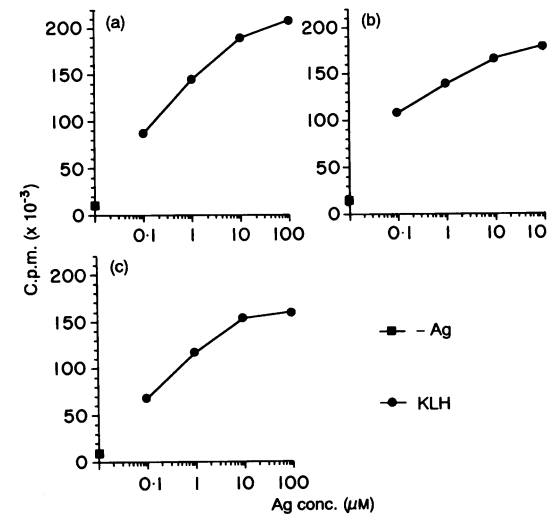

Figure 5. Proliferative responses of T cells to KLH. Lewis rats ($n=3$) were treated with R73 on days -6 and -2 (a) or left untreated (b) and challenged with GPBP on day 0. On day 24, R73-treated and EAE-free rats (a) and R73-untreated rats which had developed, and subsequently recovered from EAE (b) were immunized with KLH along with naive rats (c). Proliferative responses of lymph node cells were measured 10 days after KLH immunization.

Effect of R73 administration on ongoing EAE

Finally, we tested whether or not *in vivo* administration of R73 was effective against established EAE. Rats were immunized with GPBP and treated with R73 for 7 days starting either from 11 (Fig. 6a) or 10 (Fig. 6b) days after GPBP immunization. In an experiment shown in Fig. 6a, R73 were given when rats already showed mild EAE. Clinical signs of EAE of both control rats and R73-treated rats proceeded at the same rate and reached a maximal level on day 13. However, while rats treated with R73 had started to recover on day 14, untreated control rats still exhibited 'full-blown' EAE. Furthermore, the R73-treated rats became asymptomatic by day 16, 3 days earlier than the control animals. In another experiment, R73 treatment was initiated from day 10 (Fig. 6b) following GPBP immunization. In this case, induction of EAE was completely eliminated. Evidently, R73 administered before the onset of EAE suppressed induction of the disease and mAb given shortly after the onset hastened recovery.

Table 2. Effect of R73 administered at various time-points on EAE induction*

Group	Treatment	EAE†		
		Incidence	Onset	Severity
A	−16,−14	3/3	11·7±1·5	3·3±1·2
B	−11,−9	3/3	15	1
C	−6,−2	1/5	11	0·2±0·4
D	−2,+2	2/3	13·5±0·7	1·8±1·6
E	+2,+6	3/3	12·3±0·6	3

* R73 was administered to Lewis rats on the indicated days before (−) and/or after (+) the immunization with GPBP. The rats were observed daily for clinical signs of EAE.

† See footnotes of Table 1.

‡ Data shown in group C are the same as those in Table 1.

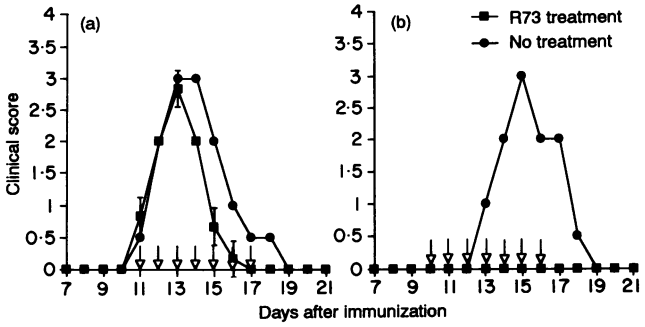

Figure 6. Treatment of established EAE with R73. Rats were immunized with GPBP on day 0 and treated with R73 for 7 consecutive days (indicated by arrows) starting on day 11 (a) or day 10 (b). Closed squares indicate the mean values of clinical score of the treated group ($n=5$)

DISCUSSION

By molecular biological approaches, considerable progress has been made in characterizing T cells responsible for the induction of EAE. Encephalitogenic T cells were initially believed to use exclusively the Vβ8.2 of TcR genes.[2-5] Recently, however, it has been revealed that encephalitogenic T cells which are especially reactive with the C-terminal sequence of the immunodominant epitope for Lewis rats use TcR other than Vβ8.2. Gold *et al.*[10,11] and Sun *et al.*[12] independently reported that rat BP87–99-reactive T cells predominantly use Vβ6, whereas Su and Sriram[13] found no predominant use of TcR Vβ genes by mouse encephalitogenic T-cell clones. This heterogeneity of TcR gene usage would cause anti-Vβ8.2 mAb to be only partially effective as a therapy for EAE.[2] In the present study, we attempted to prevent and treat EAE using an mAb against a TcRαβ framework instead of a highly specific mAb against one of the Vβ subtypes. We believed that by administering *in vivo* anti-TcRαβ antibody at a low dose for a short duration, only autoimmune processes which occurred during the treatment would be suppressed without general unwanted immunomodulation. Short-term administration of anti-TcRαβ mAb was shown to suppress EAE almost completely. Although treatment with anti-CD4 and anti-Ia mAb has been reported to suppress the development of EAE,[24-28] it was ineffective. In order to compare the effect of anti-CD4 and anti-Ia antibodies to that of R73, lower doses of mAb were administered for a short duration in the present study than those reported before. This is probably attributable to the outcome.

We were interested in the mechanism by which R73 administered according to the present protocol suppressed the development of EAE, because antibody therapy against molecules associated with antigen recognition by T cells generally causes two types of immunological changes, i.e. anergy and deletion of relevant T cells. In the present study, we have tried to elucidate the mechanism by FCM analysis and by examining antigen reactivity of T cells isolated from R73-treated/EAE-protected rats. FCM analysis revealed marked but transient down-regulation of TcRαβ and relative preservation of CD2 (Figs 2 and 3). These findings suggest that short-term administration of R73 mainly down-regulates the expression of the TcR-CD3 complex and that depletion of T cells is minimal. Decrease in number of $CD5^+$ cells on day 3 of R73 treatment (Fig. 3) does not always indicate T-cell depletion, because unlike CD2,[18,19] the CD5 molecules on T cells often disappear after various stimulation.[30] Furthermore, the response to MBP, which was immunized shortly after R73 administration, of T cells taken from EAE-protected rats was greatly suppressed (Fig. 4), but that to a third-party antigen was not inhibited (Fig. 5). Taken together, it can be said that protection of rats from EAE development by R73 treatment results from clonal anergy of MBP-reactive T cells which are activated shortly after mAb administration. Although the immunosuppressive effects due to tolerance induction by anti-CD2 and anti-CD4 mAb are well known,[31-33] we have been able to demonstrate that anti-TcRαβ mAb has the same immunological function.

We finally examined the effect of R73 administration on established EAE. It was revealed that the treatment started just before the day of onset of clinical signs completely inhibited the development of EAE and that the administration started on the day of onset hastened recovery. R73 treatment started after the disease onset did not reduce severity of maximum clinical signs. This is probably because, in contrast to arthritis,[33-35] the period between the days of onset and of the plateau phase is so short that rats develop full-blown EAE before the mAb can down-regulate the function of relevant T cells.

In summary, we have succeeded in preventing the development of EAE by administering anti-TcRαβ mAb at a low dose for a short duration. This treatment induced a decrease of TcRαβ-expressing cells mainly by immunomodulation of this molecule. Since the effect of R73 pulse treatment was short-lived, clonal anergy may have been induced in T cells which were activated during the treatment. If the appropriate treatment protocol is selected, immunotherapy with an antibody against the framework molecule of TcR is very effective, has minimal side-effects, and is potentially applicable to the treatment of human autoimmune diseases.

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