Peptide-induced T cell regulation of experimental autoimmune encephalomyelitis: a role for IL-10

Christoph Burkhart, George Y. Liu 1, Stephen M. Anderton, Barbara Metzler and David C. Wraith

Department of Pathology and Microbiology, University of Bristol, Bristol BS8 1TD, UK

1Present address: UCSD, School of Medicine, La Jolla, CA 92093-060, USA

Keywords: anergy, apoptosis, immune deviation, inhalation tolerance

Abstract

Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T cell-mediated, inflammatory disease with similarities to multiple sclerosis in humans. Intranasal (i.n.) administration of a myelin basic protein (MBP)-derived peptide can protect susceptible mice from EAE. The mechanisms underlying this phenomenon, however, remain unclear. To analyze the phenotypic and functional changes taking place during the induction of tolerance by peptide inhalation, we have studied the fate of CD4+ T cells after i.n. peptide application using transgenic mice expressing a TCR specific for the N-terminal peptide (Ac1–9) of MBP. Peripheral T cell death was variably observed in TCR transgenic mice after a single i.n. administration of antigenic peptide but was transient and incomplete. Transgenic spleen cells and cervical lymph node cells responded with a cytokine burst to peptide inhalation and hyperproliferation when re-stimulated in vitro. Transfer experiments demonstrated that the duration of peptide administration required to induce tolerance depended on the precursor frequency of T cells in recipient animals. The stringency of i.n. peptide treatment was increased so as to test the efficacy of tolerance induction both in vitro and in vivo in the presence of high precursor frequencies of antigen-specific T cells. Multiple i.n. doses of peptide completely protected TCR transgenic mice from EAE induced with myelin. Such repeated peptide administration resulted in down-regulation of the capacity of antigen-specific CD4+ T cells to proliferate or to produce IL-2, IFN-γ and IL-4 but increased the production of IL-10. The role of IL-10 in suppression of EAE in vivo was demonstrated by neutralization of IL-10. This completely restored susceptibility to EAE in mice previously protected by i.n. peptide. Considering the immunosuppressive properties of IL-10, T cells which are resistant to apoptosis might act as regulatory cells and mediate bystander suppression.

Introduction

Peptide-specific down-regulation of T cell responses may represent a powerful tool to intervene in T cell-mediated diseases or graft rejection. The non-invasive, intranasal (i.n.) route of peptide administration has proven to be particularly effective and has been demonstrated to abolish responses to allergens in mice (1), and prevent the induction of a variety of experimental autoimmune diseases such as experimental autoimmune uveitis (2), experimental autoimmune myasthenia gravis (3), arthritis in rats (4–6) and diabetes in the NOD mouse (7). Susceptible mice of the H-2k phenotype can be protected from developing experimental autoimmune encephalomyelitis (EAE) by administration of a soluble form of the acetylated N-terminal peptide (Ac1–9) of myelin basic protein (MBP) containing the immunodominant CD4+ T cell epitope. The i.v. (8,9), i.p. (10) or i.n. (11) routes of peptide administration have all proven effective for prevention of disease. Recent studies have shown that treatment with soluble Ac1–9 peptide inhalation not only prevented the induction of the disease but could be used to treat ongoing disease (12,13). The therapeutic benefit was markedly enhanced by exchanging lysine in position 4 with a tyrosine [Ac1–9(4Y)] leading to a much increased affinity for Aα.
Inhalation tolerance in TCR transgenic mice

Possible mechanisms of peptide-induced peripheral T cell tolerance include partial or complete physical elimination of the reactive T cells either by activation-induced cell death/apoptosis (14–17) or clonal exhaustion (for CD8+ T cells) (18). Alternatively, functional elimination via the induction of anergy (19), TCR/co-receptor down-regulation (20) or immune deviation (21–23) are considered possible. In addition, transforming growth factor-β-secreting CD8+ T cells have been implicated in antigen-specific oral tolerance (24), while super-antigen-induced hyporesponsiveness has been associated with production of IL-10 by CD4+ T cells (25). Which of these mechanisms is operational in inhalation tolerance, however, is unclear.

It is crucial to understand the behavior of antigen-specific T cells after i.n. tolerization if this route of administration is to prove both a safe and valuable tool for immunotherapy. The frequency of naive antigen-specific T cells is virtually undetectable in normal untreated individuals (26). We have therefore used transgenic mice (Tg4) expressing a TCR specific for the N-terminal peptide Ac1–9 of MBP (10), either directly or in an adoptive T cell transfer model, to monitor the fate of a clonal population of reactive CD4+ T cells after peptide inhalation. In this report we investigate the effect of i.n. peptide administration on peripheral T cell death, and the influence of single and multiple i.n. peptide doses on the phenotype and function of peptide-specific T cells in vivo. Our findings address the underlying mechanisms of peptide-induced T cell tolerance and have implications for understanding the consequences of peptide immunotherapy.

Methods

Mice

The Tg4 TCR transgenic mouse was described previously (10). It expresses the αβ TCR (Vα4, Vβ8.2) of the Ac1–9-specific T cell hybridoma 1934.4 derived from an encephalitogenic T cell clone (27). Mice were bred and maintained at the School of Medical Sciences, Bristol, and were between 8 and 14 weeks of age when used for experiments. The screening of TCR transgenic mice was performed using two-colour immunofluorescent staining of peripheral blood lymphocytes with anti-CD4 and anti-Vβ8 mAb, as described below. In the Tg4 TCR transgenic mice 95–100% of all CD4+ T cells were Vβ8+.

Peptides

The acetylated N-terminal peptide of murine MBP (Ac1–9, Ac-ASQKRPSQR) and the high MHC-affinity analogue with a tyrosine substituting the wild-type lysine in position 4 [Ac1–9(4Y)] were synthesized using standard Fmoc chemistry on an AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany).

Antigen administration

Soluble peptide antigen was administered either i.p. (in 200 µl PBS) or i.n. (in 25 µl PBS) as described previously (28). Peptide emulsified in complete Freund's adjuvant (CFA) was injected i.p. or s.c. in the hind footpads and at the base of the tail.

T cell proliferation assays

At indicated days after antigen treatment, spleen or draining lymph nodes were harvested. CD4+ T cell numbers were determined and equal numbers of transgenic cells were cultured in serum-free lymphocyte growth medium (Promocell, Heidelberg, Germany) at 4×10^6 cells/well at 37°C in the presence of different concentrations of Ac1–9. After 24 h, cells were pulsed with 0.5 µCi [3H]thymidine for 8 h and the incorporated radioactivity measured on a liquid scintillation β-counter (1450 Microbeta; Wallac, Milton Keynes, UK). For determination of ex vivo proliferation, cells were cultured without antigen for 12–16 h in the presence of 0.5 µCi [3H]thymidine and processed as described above.

Cytokine production assays

Cytokine release by T cells ex vivo was performed using a modification of the cell-based ELISA recently described by Beech et al. (29). In brief, microtiter plates were coated with the cytokine-specific capture antibodies JES6-1A12 (anti-IL-2), 11B11 (anti-IL-4), JES5-2A5 (anti-IL-10) or R4-6A2 (anti-IFN-γ) at 4°C overnight. Then 2.5×10^6 splenocytes or lymph node cells containing equal numbers of transgenic cells were added per well in 200 µl serum-free lymphocyte growth medium. After 24 h, specifically bound cytokines were quantified using biotinylated secondary antibodies E56-H4 (anti-IL-2), BVD6-24G2 (anti-IL-4), SXC-1 (anti-IL-10) and XMG1.2 (anti-IFN-γ) followed by extravidin peroxidase (Sigma, Poole, UK). All cytokines were quantified with standard curves obtained with known amounts of recombinant mouse cytokines. All mAb and recombinant mouse cytokine standards were purchased from Pharmingen (San Diego, CA). Biologically active TGF-β1 was determined using an ELISA system (Promega, Southampton, UK).

The quantity of cytokines released by T cells into the culture medium after antigen re-stimulation was assayed by conventional ELISA. For determination of IL-2, supernatant was harvested after 24–48 h. For IFN-γ, IL-4, IL-10 and TGF-β supernatant was collected after 72 h. mAb and mouse cytokine standards as described above were used.

Propidium iodide (PI) staining

Apoptosis was visualized as described by Nicoletti et al. (30). Splenocytes, thymocytes or lymph node cells were fixed in 70% ethanol at 4°C for 1 h, then washed twice with PBS and resuspended at 10^6 cells/ml in PBS. RNase (10 mg/ml in PBS; Sigma, Poole, UK) was added to a final concentration of 20 µg/ml followed by PI (2 mg/ml in PBS; Sigma) solution to a final PI concentration of 2.5 µg/ml. After incubation in the dark at room temperature for 1 h, cells were kept at 4°C in the dark until FACS analysis.

Induction and assessment of EAE

EAE was induced by subcutaneous injection of myelin antigens consisting of 1 mg spinal chord homogenate in CFA containing Mycobacterium tuberculosis H37 RA (4 mg/ml). On the same day and 2 days later, all mice received two i.p. doses of pertussis toxin (200 ng/injection; Porton Products, Salisbury, UK). Mice were scored for symptoms of EAE as follows: 0, no signs; 1, flaccid tail; 2, partial hind limb paralysis.
Inhalation tolerance in TCR transgenic mice

Fig. 1. Apoptosis induced in cervical lymph nodes by multiple peptide inhalation. TCR transgenic mice were either treated with 100 µg Ac1–9(4Y) in PBS i.n. or i.p., or immunized with 100 µg Ac1–9(4Y)/CFA i.p. One day after the last treatment, mice were sacrificed and lymphocytes stained with propidium iodide as described in Methods. Representative data of one out of three mice per group is shown.

and/or impaired righting reflex; 3, full hind limb paralysis; 4, hind limb plus fore limb paralysis; and 5, moribund or dead.

Neutralization of IL-10 in vivo

Cells secreting anti-IL-10 mAb JES5-2A5 were a kind gift from Michel Goldman (Brussels, Belgium). Anti-IL-10 mAb was purified from culture supernatant through a Protein G column. Purified mAb (1 mg/injection) was given i.p. on day –1, 1, 2 and 3 in respect of EAE induction on day 0.

Results

Peptide-specific cell death in central and peripheral lymphoid organs

Physical elimination of reactive cells is considered a possible underlying mechanism for the establishment of T cell unresponsiveness. This has previously been demonstrated in mice transgenic for an ovalbumin (OVA)-specific TCR following oral administration of antigen (15). In some experimental systems i.v. injection of very high doses of Ac1–11 has been shown to cause deletion of peptide-specific peripheral CD4+ TCR transgenic T cells (9,16). To evaluate the effect of peptide inhalation on the peripheral T cell population, TCR transgenic Tg4 mice were treated i.n. or i.p. with a single dose of 100 µg of Ac1–9(4Y) or were given Ac1–9(4Y)/CFA i.p. A significant increase in cell death, as measured by PI staining of DNA, was observed in the cervical lymph nodes (CLN) (Fig. 1) and in the thymus (not shown) 16 h after a single dose of Ac1–9(4Y) peptide either with or without CFA. No measurable increase of hypodiploid cells could, however, be detected in the spleen. Furthermore, cell numbers in all lymphoid organs had recovered to normal levels by 48 h, and there was no discernible lasting effect on the ratio of CD4+ and CD8+ T cells after a single dose of peptide i.n.

Hyperproliferation of peptide-specific CD4+ T cells after single peptide inhalation

Next, we addressed the question whether the observed deletion of CD4+CD8+ T cells in the thymus and the cell death in the periphery after i.n. peptide application was accompanied by peripheral T cell activation and proliferation. To this end, the ex vivo proliferation of splenocytes from TCR transgenic mice was measured after Ac1–9(4Y) inhalation. As shown in Fig. 2(A), Ac1–9(4Y) i.n. led to proliferation of splenocytes ex vivo 18 h post i.n. treatment suggesting priming of peptide-specific T cells. This proliferation was transient and declined to the level of PBS-treated controls after ~6 days. Ex vivo T cell activation correlated with up-regulation of CD69 and IL-2R, starting 2–3 h after peptide application, and reaching a maximum between 8 and 12 h. Cell surface expression of these markers returned to normal levels by 72 h. TCR down-regulation was found to be maximal between 12 and 16 h, and reached normal levels by 24 h (data not shown).

Transgenic splenocytes or CLN cells proved to be hyper-responsive to re-stimulation with Ac1–9 24 h or 72 h after i.n. Ac1–9(4Y) administration (Fig. 2B). Although the proliferative response at day 3 was reduced compared to day 1 no apparent state of hyporesponsiveness had been reached. Hyper-responsiveness decreased to the level of PBS-treated controls but not below after 6 days.
Inhalation tolerance in TCR transgenic mice

Fig. 2. Hyper-responsiveness of peripheral T cells to Ac1–9 after i.n. treatment with Ac1–9(4Y). (A) Transgenic mice received a single i.n. dose of 100 µg Ac1–9(4Y) at indicated time points. Splenocytes were then cultured without antigen in the presence of 1 µCi [3H] thymidine for 12 h and proliferation was determined by incorporation of radioactivity. (B) Transgenic mice were treated i.n. with a single dose of 100 µg Ac1–9(4Y) or PBS. Three days (squares) or 1 day (diamonds) after peptide treatment or 1 day after PBS treatment (triangles), mice were sacrificed and the cervical lymph node cells and splenocytes were cultured with varying concentrations of Ac1–9 for 24 h. Proliferation was determined by [3H]thymidine incorporation over an additional 8 h. Data represent mean values of two mice and show one representative out of three experiments performed.

Cytokine burst by TCR transgenic splenocytes and lymph node cells after single i.n. peptide administration

In our previous experiments transient proliferation and changes in cell surface phenotype after i.n. peptide application had indicated priming of CD4+ T cells as an integral part of tolerance induction. Cytokine production in peptide-treated TCR transgenic mice was measured ex vivo with a sensitive cell-based ELISA so as to further characterize the early events that lead to unresponsiveness after peptide inhalation. This assay incorporates the cytokine producing cells into the ELISA system and thereby combines the conventional ELISA method with the sensitivity of an ELISPOT assay (29). As shown in Fig. 3, both local draining lymph node cells and splenocytes produced T helper 1 and T helper 2 cytokines transiently in response to Ac1–9(4Y) inhalation. However, whereas IFN-γ production had ceased in the lymph node 3 days after treatment, it was maximal in the spleen at this time point (Fig. 3A), suggesting a shift of the responding cells from draining lymph nodes to the spleen. Production of IL-4 was consistently higher in the spleen than in the cervical lymph nodes, while IL-2 production was more pronounced in the draining lymph nodes. A transient production of IL-10 and TGF-β was detected in the spleen (Fig. 3B), and to a lesser extent in the cervical lymph nodes. Similar cytokine profiles were observed with splenocytes from mice immunized i.p. with Ac1–9(4Y)/CFA.

Partial unresponsiveness of adoptively transferred TCR transgenic T cells after i.n. peptide treatment

There is no a priori reason why Ac1–9-specific transgenic T cells should behave differently to T cells with the same specificity in normal mice in terms of activation requirements, susceptibility to apoptosis or tolerance induction. We reasoned, therefore, that the lack of tolerance after a single i.n. peptide administration would be due to the abnormally high frequency of these cells in Tg4 mice.

Fig. 3. Ex vivo cytokine production of cervical lymph node cells (A, top row) and splenocytes (A, bottom row, and B) after i.n. treatment with Ac1–9(4Y). Transgenic mice were treated with one dose of 100 µg Ac1–9(4Y) in PBS or with PBS only at indicated time points. On day 0, mice were sacrificed and lymphocytes were cultured in the absence of antigen in anti-cytokine mAb-coated microtiter plates as described in Methods. After 24 h, cells were washed off, and plate-bound IFN-γ, IL-2, IL-4, IL-10 and TGF-β were quantified using recombinant cytokine standards. Data of individual mice (A) or pooled from two mice (B) are presented and show one representative out of two experiments performed.
Fig. 4. TCR transgenic splenocytes were adoptively transferred into non-transgenic sex-matched littermates (3x10^7 cells/mouse). Eleven days after transfer, mice were immunized with 100 µg Ac1-9(4Y)/CFA s.c. At indicated time points before peptide/CFA challenge, groups of two mice were given one or two doses of 100 µg soluble Ac1-9(4Y) i.n. or one dose of PBS i.n. only. Control animals were i.n. treated with PBS followed by immunization with PBS/CFA. Eight days after challenge, spleen and draining lymph nodes were excised, and pooled cells within the group re-stimulated in vitro with Ac1-9. Proliferative responses by splenocytes and lymph node cells (A) and cytokine release into the culture supernatant by spleen cells (B) were evaluated as described in Methods.

In order to test whether it would be possible to induce unresponsiveness in the presence of fewer antigen-specific T cells, we adoptively transferred 3x10^7 TCR transgenic splenocytes into non-transgenic, sex-matched littermates. The recipient mice were then treated either once or twice with soluble Ac1-9(4Y) peptide i.n. followed by immunization with Ac1-9(4Y) s.c. in CFA. Control mice received PBS i.n. before Ac1-9(4Y)/CFA challenge or PBS i.n. followed by PBS/CFA s.c. Eight days after the challenge immunization, spleen and draining popliteal and inguinal lymph nodes were harvested, and their proliferation and cytokine production in response to Ac1-9 was determined. Equal numbers of transgenic CD4+ T cells were analyzed in vitro, in order to ensure that the resulting responses were due to qualitative and not quantitative differences of the antigen-specific cells. As Fig. 4 shows, one i.n. administration had no or very little effect on proliferation or IFN-γ production but reduced IL-2 and IL-4 production to background levels. Two doses of peptide i.n., however, clearly reduced proliferation as well as IFN-γ secretion, and abrogated IL-2 and IL-4 production. This result demonstrates that the precursor frequency influences the outcome of soluble peptide treatment since one dose of peptide is sufficient to totally abrogate proliferation and IFN-γ production in normal mice (11,28).
Unresponsiveness induced in transgenic peripheral T cells after repetitive peptide administration

Tolerance induction in TCR transgenic mice has been difficult to achieve due to the high frequency of reactive peripheral T cells (32,33). Having observed a partial tolerizing effect on transferred transgenic T cells by antigenic peptide inhalation, we tested the efficiency of the i.n. route in tolerance induction, even in the presence of a high frequency of responding CD4+ T cells. Tg4 TCR transgenic mice were given either a single dose or five or 10 successive doses of 100 µg soluble Ac1–9(4Y) at intervals of 3–4 days and equal numbers of transgenic CD4+ T cells were compared for their proliferative response to Ac1–9. As shown in Fig. 5 almost complete unresponsiveness could be induced in our model after repeated inhalation of soluble Ac1–9(4Y). Thymic and peripheral deletion gradually decreased over the course of chronic peptide administration to 50–25% of the level in single peptide-treated mice. Additionally, both the total number of CD4+ cells in the periphery as well as the ratio of CD4+ and CD8+ cells remained fairly constant over the time of treatment. Therefore, this unresponsiveness was unlikely to be due to deletion of transgenic T cells alone. Addition of exogenous IL-2 (20 U/ml) to the cell culture had no effect on the observed state of tolerance (data not shown). No adverse side effects were observed in i.n. treated animals.

Protection of TCR transgenic mice from EAE by multiple inhalation of antigenic peptide

The efficacy of i.n. peptide administration in vivo in the presence of high frequencies of antigen-specific precursor T cells was tested. Multiple i.n. doses of Ac1–9(4Y) completely protected TCR transgenic mice from EAE induced with myelin (Table 1). None of the peptide-treated mice showed signs of disease while all untreated or PBS-treated animals developed EAE with a mean maximal score of 3.6 ± 1.9 and 3.4 ± 0.8 respectively.

Altered cytokine profiles after multiple i.n. peptide administration

In order to clarify the functional changes induced by repetitive i.n. administration of antigenic peptide and to test the possibility of a Th1 to Th2 immune deviation, the cytokine production of transgenic splenocytes in response to antigen in vitro after multiple peptide inhalation was evaluated. Five and 10 consecutive i.n. treatments resulted in down-regulation of the Th1 cytokines IL-2 and IFN-γ, and the Th2 cytokine IL-4. On the other hand, multiple peptide inhalation led to an increase in production of the immunosuppressive cytokine IL-10 (Fig. 6). In vitro IL-10 production correlated positively with peptide concentration and was strictly dependent on previous antigenic challenge of specific T cells in vivo. Antigen-specific down-regulation of IFN-γ and IL-2, and up-regulation of IL-10 was also observed in CLN. Production of IL-5 and TGF-β in the spleen following a single i.n. peptide administration was abolished by repetitive i.n. peptide treatment (data not shown).

Abrogation of peptide-induced protection from EAE by anti-IL-10 treatment

In order to test the dependence of peptide-induced T cell tolerance on IL-10 in vivo, i.n. tolerized Tg4 mice were given four 1 mg doses of anti-IL-10 mAb JESS-2 AS around the time point of disease induction with myelin. Neutralization of IL-10 in vivo restored the susceptibility to EAE induction in i.n. tolerized mice (Fig. 7). While none of the peptide-tolerized control mice showed any sign of disease, all tolerized and antibody-treated animals developed severe EAE. The disease onset was delayed compared to untreated mice (15.3 ± 1.1 versus 9.0 ± 0.7 days) and signs of disease were more

Table 1. Nasal pretreatment of TCR transgenic mice with Ac1–9(4Y) prevents spinal chord-induced EAE

<table>
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<tr>
<th>Treatment</th>
<th>Incidence of disease</th>
<th>Fatality</th>
<th>Mean day of onset (± SD)</th>
<th>Mean maximal grade (± SD)</th>
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</thead>
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<tr>
<td>No</td>
<td>5/5</td>
<td>4/5</td>
<td>7.8 (± 1.0)</td>
<td>3.6 (± 1.9)</td>
</tr>
<tr>
<td>PBS i.n. 5×</td>
<td>5/5</td>
<td>1/5</td>
<td>9.0 (± 0.7)</td>
<td>3.4 (± 0.8)</td>
</tr>
<tr>
<td>4Y i.n. 5×</td>
<td>0/4</td>
<td>0/4</td>
<td>–</td>
<td>0</td>
</tr>
<tr>
<td>4Y i.n. 14×</td>
<td>0/5</td>
<td>0/5</td>
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Groups of four to five Tg4 mice were treated either with five or 14 consecutive doses of 100 µg soluble Ac1–9(4Y) i.n. at intervals of 3–4 days. Control mice were treated either with five consecutive applications of PBS i.n. at intervals of 3–4 days or were left untreated. One day after the last treatment, EAE was induced and disease monitored as described in Methods.
Inhalation tolerance in TCR transgenic mice

**Fig. 6.** Cytokine production of transgenic T cells to Ac1–9 after multiple i.n. peptide treatment in vivo. Groups of Tg4 mice were administered a single i.n. dose (▲) of 100 µg soluble Ac1–9(4Y) or five (●) or 10 (■) successive i.n. doses at intervals of 3–4 days. Control mice (○) were left untreated. Supernatant was collected after 24 h for IL-2 detection, and after 72 h for IL-4, IL-10 and IFN-γ detection. Cytokine production was quantified by a sandwich ELISA. Results are from pooled splenocytes from three mice per group.

**Discussion**

In order to analyze the phenotypic and functional changes taking place during the induction of tolerance by peptide inhalation we have studied the fate of CD4⁺ T cells after i.n. peptide application using a TCR transgenic mouse model. A high degree of apoptosis was observed in thymus and cervical lymph nodes but not in spleen, possibly due to the low number of dying cells in a given time window and the sensitivity of the PI staining. Apoptosis has been previously documented after in vivo encounter with soluble peptide (9,14) and was dependent on the affinity of the peptide for MHC (10,17). Deletion of CD4⁺ T cells in the periphery, however, is never complete and leads usually to a small residual population of T cells. Clonal exhaustion as a sole mechanism for antigen-specific unresponsiveness has so far been demonstrated only for CD8⁺ T cells (18). Its role in the induction and maintenance of immunological unresponsiveness therefore remains controversial. In our studies, cell death was as evident in peptide/CFA-treated as in soluble peptide-treated mice, arguing against a mandatory requirement for apoptosis in the establishment of an unresponsive state.

T cells from Tg4 TCR transgenic mice did not show signs of unresponsiveness when a single peptide dose was applied i.n. When defined numbers of transgenic cells were adoptively transferred into non-transgenic hosts, however, a single i.n. Ac1–9(4Y) administration reduced IL-2 and IL-4 secretion in vitro after a challenge immunization with Ac1–9(4Y)/CFA. These results suggest that unresponsiveness resulting from peptide inhalation is inversely proportional to the frequency of Ac1–9-specific T cells.

Our results are in agreement with those obtained by the group of Jenkins (14,36,37) using an adoptive transfer system where transferred OVA-specific TCR transgenic CD4⁺ T cells were followed in non-transgenic recipients with a clonotypic antibody. They found that the presence of inflammation or adjuvants during T cell activation led to an accumulation of proliferating cells, whereas in the absence of inflammation the majority of the activated antigen-specific T cells disappeared 30 days after exposure to soluble peptide. In a similar system, Degerman et al. (38) interpreted the persistence of transferred cells 10 days after soluble peptide administration as indicating the absence of peripheral deletion. It has to be noted, however, that the detection of the specific T cell population with a clonotypic antibody does not reveal the dynamics of concomitant proliferation and apoptosis, but rather shows the net result of proliferation, migration and cell death.

Other investigators have shown that a significant deletion of peripheral antigen-specific T cells could be detected after repeated antigen injection or feeding (33,39). Chronic antigen administration could even induce tolerance in TCR transgenic mice with a high frequency of reactive T cells (32, 33). Tolerance induction by chronic peptide administration could be explained by the preferential deletion of T cells with a high-affinity TCR, whereas T cells with low-affinity receptors would escape deletion [similar to escape of autoreactive T cells from negative selection in the thymus (11)] or become anergic. Interestingly, however, multiple peptide inhalation had no effect on the overall number of peripheral T cells in our model.

**Fig. 7.** Anti-IL-10 mAb restores susceptibility to EAE induction in TCR transgenic mice tolerized with antigenic peptide. Groups of three to five Tg4 mice were treated either with eight consecutive doses of 100 µg soluble Ac1–9(4Y) i.n. at intervals of 3–4 days (full symbols) or were left untreated (○). On day 0, one day after the last treatment, EAE was induced as described for Table 1. IL-10 was neutralized by four i.p. injections of 1 mg purified mAb JES5–2A5 in PBS on day –1, 1, 2 and 3 (▲). EAE symptoms were monitored as described for Table 1.

severe (mortality: 2/3 versus 1/5; mean maximal score: 4.3 ± 1.1 versus 3.4 ± 0.8).
A cell-based ELISA system was used to detect cytokines produced by activated peptide-specific T cells ex vivo. This method avoids further manipulation with antigen in vitro and limits the problem of cytokine loss due to consumption by other cells. Upon a single i.n. peptide administration, peptide-specific T cells produced a burst of cytokines of both the Th1 and Th2 type. Cytokine production in draining lymph nodes and spleen was maximal 24 h after i.n. treatment, with the exception of IFN-γ which reached a maximum level in the spleen at day 3. Hoyne et al. had previously reported transient cytokine production after i.n. administration of allergen-derived peptide. In their studies IFN-γ and IL-2 production in spleen and CLN reached a maximum 4 days after peptide inhalation (1,40). Their experiments differ from ours in that peptide was given i.n. 3 times on consecutive days and lymphocytes were re-stimulated with antigen in vitro. They provide, therefore, little information on the initial cytokine profile and kinetics after peptide inhalation.

Administration of Ac1–9(4Y) i.n. led to hyperactivation of specific CD4+ T cells which could be prevented by a single i.n. pretreatment with peptide. Pretreatment resulted in an abrogation of IL-2 and IFN-γ production after subsequent i.n. challenge in vivo. Down-regulation of T,1 cytokines was not associated with an increase of Th2 cytokines such as IL-4 and IL-5. This is in contrast to findings by Pearson et al., where a TCR transgenic mouse carrying a receptor originating from the same T cell hybridoma as Tg4 responded in a shift from Th1 to Th2 cytokine production after the in vivo administration of 2.4 mg Ac1–11(4Y) (16). Since there is so far no evidence that mice on the PL/J background would be able to inhibit the onset of EAE and to treat animals therapeutically after onset of neurological signs of disease (47). We have shown previously that treatment with Ac1–9 blocked disease induced with whole myelin which contains a heterogeneous mixture of potential autoantigens (12). Furthermore, inhalation of soluble peptide containing a single H-2k-restricted epitope of proteolipid protein (PLP) suppressed disease induced with whole myelin which contains a heterogeneous mixture of potential autoantigens (12). Therefore, even high numbers of potentially reactive cells can be rendered unresponsive by the effects of neutralization of IL-10 in vivo. Peptide-mediated protection from EAE was abolished after treatment of tolerized mice with an anti-IL-10 mAb.

IL-10 has been demonstrated by several studies to exert inhibitory effects on MHC class II-dependent antigen presentation (42) with consequences for Th1 cytokine production (43) and T cell proliferation (44). More recently, IL-10 has been associated with tolerance induction. One report has documented a role for IL-10 in the induction of long-lasting anergy in human CD4+ T cells (45) and Sundstedt et al. have described a situation where superantigen-induced unresponsiveness was correlated with the down-regulation of IL-4 and the up-regulation of IL-10 (25). CD4+ T cells producing high levels of IL-10 but low levels of IL-2 and IL-4 have the capacity to prevent colitis induced in SCID mice by pathogenic CD4+CD45RBhigh cells (46). Moreover, transfected T cell clones expressing antigen-inducible IL-10 were able both to inhibit the onset of EAE and to treat animals therapeutically after onset of neurological signs of disease (47). We have shown previously that treatment with Ac1–9 blocked disease induced with whole myelin which contains a heterogeneous mixture of potential autoantigens (12). Furthermore, inhalation of soluble peptide containing a single H-2k-restricted epitope of proteolipid protein (PLP) suppressed both PLP- and MBP-induced disease in the H-2k/k model of EAE (48). These observations suggest that, in addition to an antigen-specific component, inhalation tolerance induces bystander regulation of responses to epitopes within a tissue. This bystander suppression could not be explained by a wholesale shift to Th2 cytokines such as IL-4 and IL-5 in the H-2k/k model. So far, however, we have not been able to detect significant amounts of IL-10 from tolerant versus immune lymph nodes in H-2k/k mice (48). This may be explained by the low frequency of regulatory T cells generated by the different tolerizing regime in normal mice. Efforts are under way to investigate the role of IL-10 in peptide-induced tolerance in non-transgenic mice. Nevertheless, the increased production of IL-10 in TCR transgenic mice after i.n. peptide administration strongly suggests the persistence of a cell population which escapes apoptosis and mediates immune regulation through production of this cytokine.

**Acknowledgements**

We thank Beata Burkhart and Pauline A. Lowrey for excellent technical assistance. This work was supported by grants from the Wellcome Trust. C. B. was a recipient of a fellowship from Deutsche Forschungsgemeinschaft Bu 995/1-1. B. M. was supported by a grant from the Multiple Sclerosis Society of Great Britain and Northern Ireland.

**Abbreviations**

- OVA: ovalbumin
- MBP: myelin basic protein
- PI: propidium iodide
- PLP: proteolipid protein
- TGF: transforming growth factor
References


