ELSEVIER

Journal of Neuroimmunology 91 (1998) 135–146

Journal of Neuroimmunology

Bystander suppression of experimental autoimmune encephalomyelitis by T cell lines and clones of the Th2 type induced by copolymer 1

Rina Aharoni, Dvora Teitelbaum, Michael Sela, Ruth Arnon *

Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Received 11 February 1998; revised 4 June 1998; accepted 23 June 1998

Abstract

The synthetic amino acid copolymer, copolymer 1 (Cop 1) induces T suppressor (Ts) lines/clones, which are confined to the Th2 pathway, cross react with myelin basic protein (MBP), but not with other myelin antigens on the level of Th2 cytokine secretion. Nevertheless, Cop 1 Ts cells inhibited the IL-2 response of a proteolipid protein (PLP) specific line. Furthermore, Cop 1 Ts cells ameliorated EAE induced by two unrelated encephalitogenic epitopes of PLP: p139–151 and p178–191, that produced different forms of disease. This bystander suppression demonstrated by the Cop 1 Ts cells may explain the therapeutic effect of Cop 1 in EAE and MS.

Keywords: Experimental autoimmune encephalomyelitis (EAE); Multiple sclerosis (MS); Copaxone®; Bystander suppression; Th2 cytokines; Regulatory T cells

1. Introduction

The dichotomy of the immune system into counteracting Th1 and Th2 subsets, that secrete distinct sets of cytokines, and as a result, perform distinct effector functions, has been established in both mouse and human (Mosmann and Coffmann, 1989; Abbas et al., 1996; Mosmann and Sad, 1996). Although this concept has been recently blamed as oversimplified (Allen and Maizels, 1997), it is accepted that Th1 cells which produce IL-2, IFN-γ, and TNF, mediate pathological processes in inflammatory autoimmune diseases such as multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) (Merrill et al., 1992; Miller and Karpus, 1994; Liblau et al., 1995; Nicholson and Kuchroo, 1996; Adorini and Sinigaglia, 1997). In contrast, Th2 cells produce IL-4, IL-5, IL-6 and IL-10, and antagonize Th1 cell mediated immunity (Kennedy et al., 1992; Khoury et al., 1992; Van der Veen and Stohlman, 1993; Chen et al., 1994; Miller and Karpus, 1994; Liblau et al., 1995; Adorini and Sinigaglia, 1997). Current therapeutic approaches attempt therefore, to induce deviation from the pathological Th1 to the protective Th2 response (Chen et al., 1994; Brennan and Feldmann, 1996; Nicholson et al., 1997; Rocken et al., 1996; Adorini and Sinigaglia, 1997).

The synthetic random copolymer, Copolymer 1 (Cop 1, Copaxone®, glatiramer acetate), composed of L-Ala, L-Glu, L-Lys and L-Tyr (Teitelbaum et al., 1971, 1973), exerts a marked suppressive and protective effect on EAE in various animal species including primates (Teitelbaum et al., 1971, 1973, 1974, 1997; Lando et al., 1979). Cop 1 ameliorated chronic relapsing EAE (Keith et al., 1979), as well as EAE induced by various encephalitogens, e.g., myelin basic protein (MBP) (Teitelbaum et al., 1973, 1974), proteolipid protein (PLP) peptides (Teitelbaum et al., 1996), and myelin oligodendrocyte glycoprotein (MOG) peptides (Ben-Nun et al., 1996). Cop 1 was also shown to slow the progression of disability and to reduce the relapse rate in MS patients (Johnson et al., 1995, 1998), and it was recently approved as a drug for MS under the trade name of Copaxone®. The mechanism of Cop 1 activity in EAE and MS involves high affinity promiscuous binding to various class II major histocompatibility complex (MHC) molecules (Fridkis-Hareli et al., 1994). This binding leads to both competition with myelin antigens for T cell activa-

* Corresponding author. Tel.: +972 8 9344017; fax: +972 8 9469712; e-mail: liarnon@weizmann.weizmannac.il

0165-5728/98/$ - see front matter
PII: S0165-5728(98)00166-0

tion, and induction of specific suppressor T cells (Teitelbaum et al., 1997).

We have previously demonstrated that the unresponsiveness to EAE induced by Cop 1 is regulated by T suppressor (Ts) cells, since it could be adoptively transferred to normal recipients (Lando et al., 1979). Cop 1 specific Ts cell lines and Ts hybridomas were established from spleens of mice that had been rendered unresponsive to EAE by Cop 1 (Aharoni et al., 1993). These Cop 1 induced T cells, or their supernatants, inhibited in vitro the response of an encephalitogenic line to MBP. Furthermore, they prevented the development of EAE in vivo, indicating that they are regulatory suppressor cells. The Cop 1 specific T cell lines/clones expressed the CD4 + phenotype. Recently, we have shown that Cop 1 induces T cells which are confined to the Th2 pathway (Aharoni et al., 1997). Thus, the T cell lines and clones generated from Cop 1 immunized mice and selected for Cop 1 secreted high amounts of IL-4, IL-6 and IL-10 in response to Cop 1, but not IL-2 or IFN-γ. The bias towards the Th2 phenotype was found in Cop 1 lines/clones originating from spleens of mice that had been rendered unresponsive to EAE by injection of Cop 1 in ICFA 15–35 days earlier, a regimen previously shown to induce antigen specific suppressor cells (Lando et al., 1979; Aharoni et al., 1993, 1997). Furthermore, even cells originating from lymph nodes of mice that had been immunized with Cop 1 in enriched CFA 10 days earlier, a procedure usually used to obtain effector T cell lines (Ben-Nun and Lando, 1983), exhibited this tendency to Th2 diversion (Aharoni et al., 1997).

The Cop 1 induced T cells had never been exposed to the autoantigen MBP. Nevertheless, they responded to MBP by secretion of Th2 immunosuppressive cytokines (Aharoni et al., 1997). Thus, cross reactivity between Cop 1 and MBP on the level of Th2 cytokine secretion was demonstrated. We have previously shown that the cross reactivity of Cop 1 with the natural autoantigen MBP on the level of both B (Teitelbaum et al., 1991), and T cell response (Webb et al., 1973) is correlated with Cop 1 suppressive activity in vivo (Webb et al., 1976). Indeed the Cop 1/MBP specific T cell lines/clones protected mice against the development of EAE induced by whole mouse spinal cord homogenate (MSCH) (Aharoni et al., 1997). However, MSCH contains in addition to MBP other encephalitogenic antigens that were implicated in disease induction (Lees et al., 1991; Mendel et al., 1995; Van Noort et al., 1995). These findings suggested that Cop 1 specific cells can suppress the encephalitogenic processes induced by other antigens and not only MBP. The phenomenon of T cells specific to one antigen which suppress the immunological response induced by another encephalitogen, has been previously described and termed bystander suppression (Chen et al., 1994; Al-Sabbagh et al., 1994). In the case of EAE and MS bystander suppression must be due to propinquity of the antigens within the myelin sheath. Bystander suppression is especially important in the treatment of both MS and EAE because of the antigen/epitope spreading which has been demonstrated for these diseases (McRae et al., 1995).

In the present study we investigated the reactivity of Cop 1 induced T cells with several myelin antigens. The ability of the Cop 1 specific Ts lines/clones to regulate both in vitro and in vivo, processes which are mediated by myelin antigens other than MBP was tested as well. In the following we demonstrate that even though the Cop 1 specific cells were cross reactive only with MBP and not with PLP or with the other myelin antigens, they still inhibited IL-2 response of a PLP specific line, and prevented disease induced by PLP peptides, indicating that Cop 1 induced T cells that mediate bystander suppression.

2. Materials and methods

2.1. Mice

(SJL/J × BALB/c)F1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Female mice, 7–12 weeks old, were used in all experiments.

2.2. Antigens

Copolymer 1 (Cop 1, Copaxone®, glatiramer acetate) is a synthetic random basic polymer, prepared by polymerization of the *N*-carboxyanhydrides of L-alanine, γ-benzyl-L-glutamate, ε,*N*-trifluoroacetyl L-lysine, and L-tyrosine (Teitelbaum et al., 1971) followed by removal of blocking groups. Two Cop 1 batches obtained from Teva Pharmaceutical Industries (Petach Tikva, Israel) were used throughout the study. Batches 02095 and 55495, with average molecular weights of 6000 kDa and 5800 kDa, respectively. Myelin basic protein (MBP) was isolated from spinal cords of mice or rats, as previously described (Hirshfeld et al., 1970). Mouse spinal cord homogenate (MSCH) was prepared by homogenizing four parts of mouse spinal cord and one part of saline. The homogenate was strained through a sieve and lyophilized. Two PLP peptides p139–151 (HSLGKWLGHPDKF) and p178–191 (NTWTTCQSIAFPSK), were synthesized by Merrifield solid-phase method (Merrifield, 1965), using the peptide synthesizer, model 430A of Applied Biosystems, and purified by HPLC. MOG peptide p35–56 (MEVGWYRSPFSRVVHLYRNGKD) was a gift from A. Ben-Nun. αB-crystallin was a gift from J.M. Van Noort. Lysozyme from egg-white was obtained from Sigma (St. Louis, MO).

2.3. T cell lines and clones

Cop 1 specific T cell lines were established from spleens of mice which had been rendered unresponsive to EAE by subcutaneous injection of Cop 1 (5–10 mg/mouse), emul-

sified in incomplete Freund's adjuvant (ICFA, Difco) 15 to 35 days earlier. A control T cell line was established from mice which had been similarly injected with lysozyme (5–10 mg/mouse) in ICFA. MBP specific line was generated from spleens of mice immunized with MBP (200 μg/mouse) in complete Freund's adjuvant (CFA) 10 days earlier. PLP p139–151 specific line was established from spleens of mice immunized with PLP p139–151 (10 μg/mouse) in CFA 10 days earlier. Cells were cultured and selected in vitro using the immunizing antigen (0.1–1 mg/plate), in culture medium (RPMI, 2 mM glutamine, 1 mM sodium pyruvate, non essential amino acids, 5×10^{-5} M 2-mercaptoethanol, 100 μ/ml penicillin, 100 μg/ml streptomycin), supplemented with 1% autologous serum. After 4 days, cells were transferred to culture medium/10% FCS supplemented with 10% supernatant of Con A activated normal spleen cells as T cell growth factor (TCGF). Every 14–21 days, cells were stimulated by exposures to Cop 1 or MBP, presented on syngeneic irradiated (3000 rad) spleen cells (50×10^6/plate) for 3 days, followed by propagation in TCGF medium. Cloning of T cell lines was performed by limiting dilution at 0.3 cells/well in microtiter plate in the presence of Cop 1 (10 μg/well) and irradiated syngeneic spleen cells (5×10^6/well). All the Cop 1 specific T cell lines/clones expressed CD4 + phenotype.

2.4. Proliferation assay

Cells of T lines or clones were tested for their specific proliferative response 10–21 days after antigenic stimulation. T Cells (1.5×10^4) were cultured with 5×10^5 irradiated spleen cells and with the indicated antigens in a final volume of 0.2 ml in 10% FCS culture medium. At the end of 48 h incubation, cultures were pulsed with 1 μCi [^{3}H]-thymidine and harvested 6–12 h later. Results are expressed as mean counts per minute (cpm) thymidine incorporation for triplicate cultures. Standard deviation were under 20% of the mean cpm.

2.5. Cytokine assays

T cells from lines and clones (1×10^6/ml) presented on irradiated spleen cells (5×10^6/ml), were incubated with the indicated antigens in a final volume of 1 ml. Supernatants were collected 24 h later and assayed for cytokine levels. IL-2, IL-4, IL-5, IL-6, IL-10, and IFN-γ were measured using a quantitative sandwich ELISA using pairs of monoclonal antibodies obtained from Pharmingen (San Diego, CA), according to the manufacturer's instructions. The threshold detection for all cytokines was 20–50 pg/ml. Results are expressed in nanograms as mean concentration of duplicate culture supernatants (standard deviations were under 20%), measured in duplicate wells by ELISA (standard deviations under 10%).

IL-2 secretion was measured also using indicator cells by evaluating the ability of culture supernatants to support the proliferation of the IL-2 dependent CTLD line. The tested supernatants were incubated with the indicator cells (1×10^4/well) at a 1:1 dilution to a final volume of 0.1 ml for 48 h and then labeled with 1 μCi thymidine for 16 h. Results are expressed as mean cpm thymidine incorporation for triplicate cultures, and the standard deviations were less then 20%.

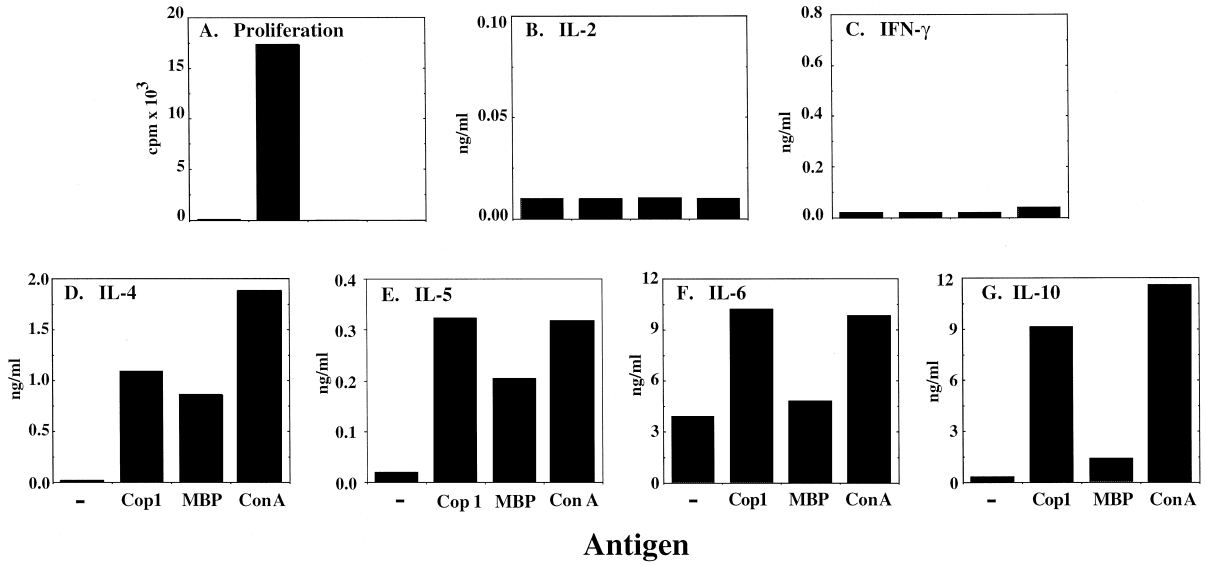

Fig. 1. Proliferation and cytokine secretion profile of Cop 1 specific clone S-1-4. The response to medium, Cop 1 (50 μg/ml), MBP (100 μg/ml) and ConA (5 μg/ml) was tested by proliferation and cytokine secretion. Proliferation was measured by thymidine incorporation for triplicate cultures. Cytokine concentration was measured by quantitative ELISA in duplicate wells for each one of duplicate culture supernatants. Standard deviations were under 20% of the mean. Results represent one of four independent experiments.

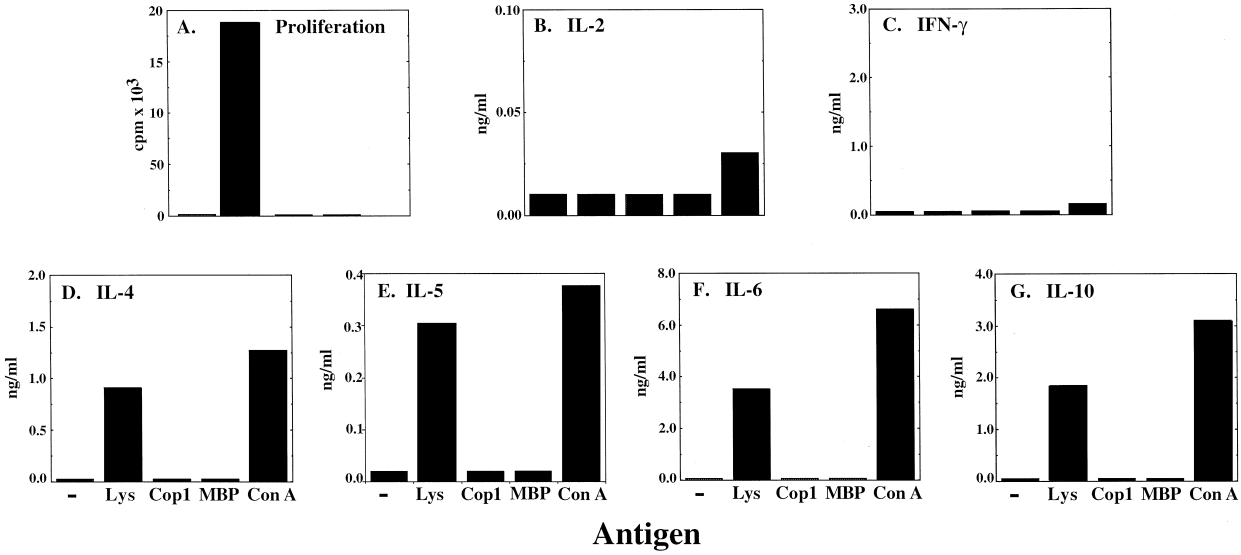

Fig. 2. Proliferation and cytokine secretion profile of lysozyme specific line Lys-1. The response to medium, lysozyme (100 μg/ml), Cop 1 (50 μg/ml), MBP (100 μg/ml) and ConA (5 μg/ml) was measured by proliferation and cytokine secretion as described in Fig. 1. Standard deviations were under 20% of the mean. Results represent one of two independent experiments.

2.6. Induction of CR-EAE by PLP peptides

(SJL/J × BALB/c)F1 7–8 weeks old female mice were injected subcutaneously in the flank with 200 μg/mouse PLP peptide 139–151 or 178–191 emulsified in a 1:1 ratio in CFA supplemented with 3 mg/ml micobacterium tuberculosis H37Ra. Pertussis toxin (0.25 ml, 250 ng, Sigma) was injected i.v. immediately thereafter and 48 h later. Mice were examined daily from day 10 post induction for signs of EAE, and assessed for clinical severity from 0 to 5 as follows: 0: healthy, 1: flaccid tail, 2: hind limbs paralyzed, 3: hind and fore limbs paralyzed, 4: total paralysis, 5: moribund.

2.7. Inhibition of CR-EAE by Ts lines and clones

T cells of lines and clones (0.5×10^6/ml) were incubated with their specific antigen (50–100 μg/ml) and with irradiated antigen presenting cells (5×10^6/ml) for 3 days. Then cells were washed and injected intravenously

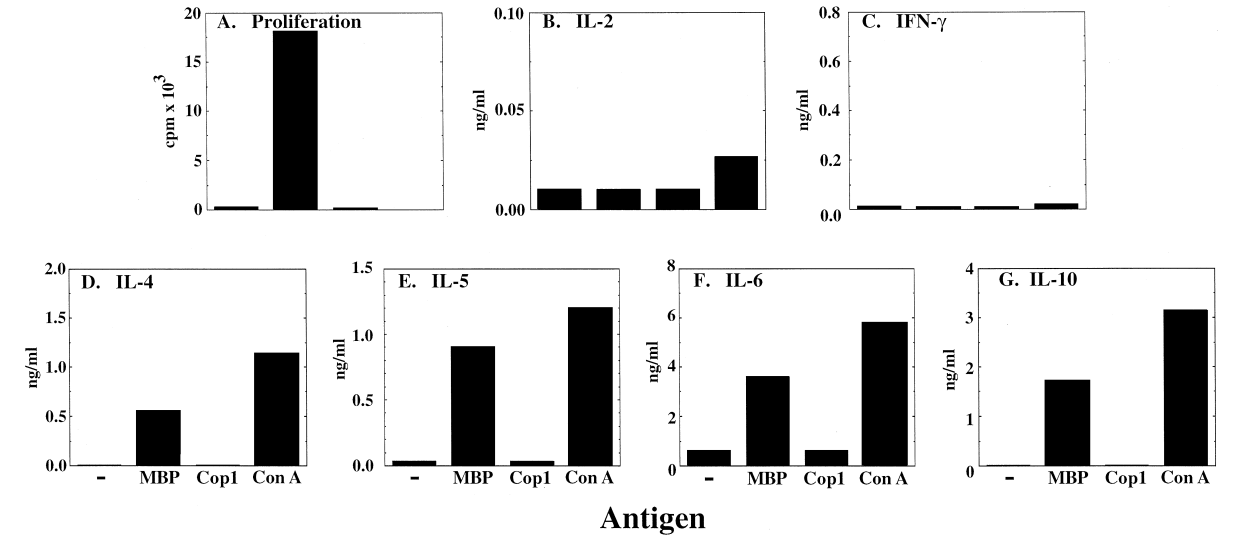

Fig. 3. Proliferation and cytokine secretion profile of MBP specific line. The response to medium, MBP (100 μg/ml), Cop 1 (50 μg/ml), and ConA (5 μg/ml) was measured by proliferation and cytokine secretion as described in Fig. 1. Standard deviations were under 20% of the mean. Results represent one of two independent experiments.

$(10 \times 10^6$/mouse), to (SJL/J × BALB/c)F1 mice, followed by disease induction.

2.8. Statistical analyses

Mice treated with T cell lines/clones before EAE induction were compared to control mice induced with disease without any treatment. Disease incidence was analyzed by Fisher's exact test. Maximal score and day of onset were analyzed by Wilcoxon Rank Sums test (Mann–Whitney test). Combination statistical test was performed by combining several independent tests on the same hypothesis (Winer, 1971).

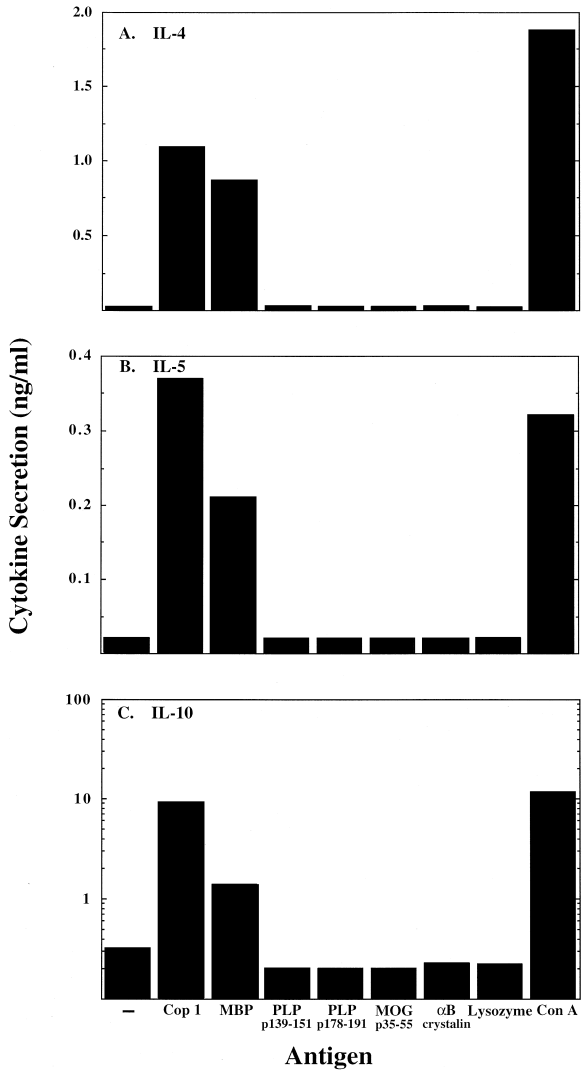

Fig. 4. Specificity of Cop 1 induced S-1-4 clone. IL-4 (A), IL-5 (B), and IL-10 (C), secretion in response to medium, Cop 1 (50 μg/ml), MBP (100 μg/ml), the following peptides (10 μg/ml): PLP 139–151, PLP 178–191 and MOG 35–56, αB-crystallin (100 μg/ml), lysozyme (100 μg/ml), and ConA (5 μg/ml) was measured by quantitative ELISA. Results are expressed as mean concentration of duplicate culture supernatants, measured in duplicate wells. Standard deviations were under 20% of the mean. Results represent one of two independent experiments.

Table 1
Specificity of IL-4 secretion by F1-Ts-Cop 1 lines/clones

Ts-cell type	–	Cop 1	MBP	PLP p139–151	PLP p178–191	ConA
S-1-4	< 0.02	1.08	0.85	< 0.02	< 0.02	1.87
S-22-1	< 0.02	0.82	0.43	< 0.02	< 0.02	1.85
S-3	< 0.02	1.70	0.35	< 0.02	< 0.02	1.78
S-4	< 0.02	1.85	0.31	< 0.02	< 0.02	1.98

IL-4 secretion in response to medium, Cop 1 (50 μg/ml), MBP (100 μg/ml), PLP p139–151 (10 μg/ml), PLP p178–191 (10 μg/ml), and to ConA (5 μg/ml) was measured by quantitative ELISA.
Results are expressed in ng/ml as mean concentration of duplicate culture supernatants, measured in duplicate wells.
Standard deviations were under 20% of the mean.

3. Results

3.1. Specificity of Cop 1 induced Ts cells

We have previously demonstrated that Cop 1 specific T cell lines and clones, generated from spleens of mice that

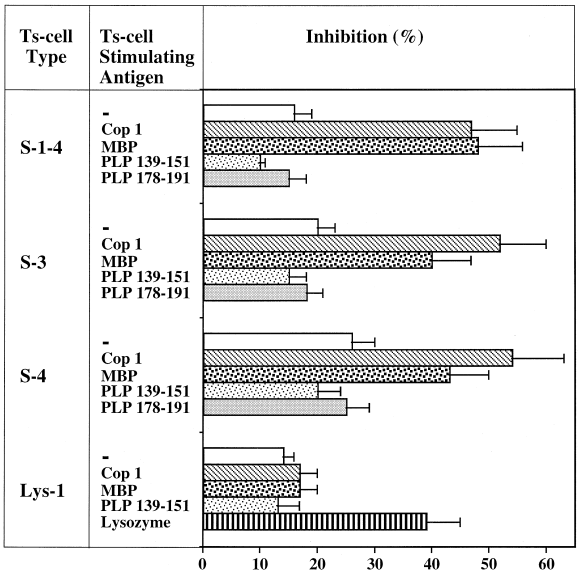

Fig. 5. Inhibition of IL-2 secretion of PLP p139–151 specific clone by supernatants of Ts lines and clones. Ts cells (1×10^6/ml) were stimulated with the indicated antigens (Cop 1 50 μg/ml, MBP 100 μg/ml, PLP peptides 139–151 and 178–191 10 μg/ml). After 24 h, 50 μl of culture supernatants were transferred to a PLP specific T cell clone stimulated with PLP p139–151 (1 μg/ml). The IL-2 secretion of the PLP specific clone was measured after 24 h using the CTLD IL-2 dependent indicator line, as described in Section 2. Results are expressed as percent inhibition from the uninhibited response of the PLP clone to PLP p139–151—10,311 cpm. Background response of the PLP clone without antigen was 333 cpm. Standard deviations of thymidine incorporation for triplicate cultures were less then 20%.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.