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Specific inhibition of the T-cell response to myelin basic protein by the synthetic copolymer Cop 1

(experimental allergic encephalomyelitis/autoimmunity/T-cell activation/immunotherapy/interleukin 2)

DVORA TEITELBAUM, RINA AHARONI, RUTH ARNON, AND MICHAEL SELA

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

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ABSTRACT **Cop 1 is a synthetic basic random copolymer of L-alanine, L-glutamic acid, L-lysine, and L-tyrosine in a residue molar ratio of 6.0:1.9:4.7:1.0 and with a molecular weight of 21,000 which proved to be effective in specific suppression of experimental allergic encephalomyelitis and has been proposed as a candidate drug against multiple sclerosis. In the present study we further investigated the mechanism of Cop 1 suppressive activity and tested whether Cop 1 could inhibit the specific T-cell response to myelin basic protein (BP). Eight BP-specific T-cell lines and clones with various H-2 restrictions and antigenic specificities were used. The responses of all these lines and clones to BP, as followed by both cell proliferation and interleukin 2 secretion assays, were affected by Cop 1. For one line, a direct cross proliferation with Cop 1 was observed, whereas in the other seven lines and clones, Cop 1 specifically inhibited the responses to BP in a competitive dose-dependent manner. The inhibition of the response to BP is specific to Cop 1, as D-Cop 1 and another random acidic polymer, poly(Tyr,Glu,Ala) (TGA), both of which were previously demonstrated to be ineffective in suppression of experimental allergic encephalomyelitis, did not inhibit the response to BP. Furthermore, Cop 1 specifically inhibited only the response of the T-cell lines and clones to BP. It did not inhibit their response to the mitogen Con A, nor did it inhibit the responses of the purified protein derivative-specific T-cell line and clone. These results suggest that Cop 1 may be effective in suppression of experimental allergic encephalomyelitis, not only because of the selective stimulation of suppressor T cells, as we have previously demonstrated, but also by specific inhibition of BP-specific effector T cells.**

Experimental allergic encephalomyelitis (EAE) is a T-cell-mediated autoimmune disease of the central nervous system which serves as an animal model for human demyelinating diseases, including multiple sclerosis (1). Cop 1 is a synthetic random copolymer of amino acids which proved to be effective in specific suppression of EAE induced in various animal species (2–6). It was also demonstrated to be effective in reducing the number of relapses in early exacerbating-remitting multiple sclerosis (7).

The basis for the biological activity of Cop 1 lies in its immunological cross reactivity with myelin basic protein (BP), which is the autoantigen in EAE. This cross reactivity was well established at the level of T-cell-mediated immunity (8, 9) and at the level of humoral response, by using monoclonal antibodies (6). Furthermore, in mice, Cop 1 was found to induce suppressor cells specific to BP which mediate protection against EAE (5). This may be a possible mechanism for the protective activity of Cop 1. Nonetheless, some of our early studies suggest that additional immunological mechanisms may also be involved in the suppressive activity of Cop 1. In our studies on the cross reactivity between BP and Cop 1 at the cellular level with the lymphocyte transformation assay of *in vivo*-sensitized lymphocytes from guinea pigs and rabbits, we observed in certain systems direct cross stimulation, while in others, a specific cross inhibition was detected (8). Inhibition by Cop 1 of the response to BP was also observed in an *in vitro*-sensitization system, where Cop 1 inhibited the induction of BP-specific cells from normal lymphocytes (10).

The development of *in vitro*-propagating antigen-specific T-cell lines and clones made a major contribution to the elucidation of the mechanisms of the cellular interactions involved in the immune response in general (11) and in EAE in particular (12). In the present study we have utilized this powerful tool to further investigate the ability of Cop 1 to inhibit the specific T-cell response to BP. We report here that Cop 1 inhibited several BP-specific murine T-cell lines and clones with various H-2 restrictions and antigenic specificities. These findings may suggest an additional or alternative mechanism for Cop 1 suppressive activity in EAE.

MATERIALS AND METHODS

Mice. B10.PL mice were obtained from The Jackson Laboratory. BALB/K and (SJL/J × BALB/K)F_1 mice were obtained from Harlan–Olac (Bicester, England). Mice were used at the age of 6–12 weeks.

Antigens. The BP was isolated from spinal cords of rats, mice, and bovines, as described (13).

The synthetic peptides pR1-11, pR5-16, and pR35-47 of rat BP (RBP) were a gift from Lawrence Steinman (Stanford University).

Purified protein derivative (PPD) of tuberculin was obtained from Statens Serum Institute (Copenhagen, Denmark).

The synthetic antigens: Cop 1, composed of L-alanine, L-glutamic acid, L-lysine, and L-tyrosine in a residue molar ratio of 6.0:1.9:4.7:1.0 and with molecular weight of 21,000 (2); D-Cop 1, similar to Cop 1 in composition but composed of D-amino acids (9); and TGA, a random polymer of L-tyrosine, L-glutamic acid, and L-alanine [poly(Tyr,Glu, Ala)] in a residue molar ratio of 2.0:1.0:1.0 (14), were synthesized and characterized as described.

T-Cell Lines and Clones. T-cell lines were derived from lymph node or spleen cells of mice immunized with RBP in complete Freund's adjuvant and selected *in vitro* by using either RBP or PPD as described by Ben-Nun and Lando (15). The cells were propagated in culture with 10% (vol/vol) T-cell growth factor (termed T-cell growth factor/Con A-induced murine spleen cell culture fluid), with exposure to

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Abbreviations: BP, basic protein; BBP, bovine BP; MBP, mouse BP; RBP, rat BP; Cop 1, copolymer 1; EAE, experimental allergic encephalomyelitis; IL-2, interleukin 2; PPD, purified protein derivative; TCR, T-cell receptor; TGA, poly(Tyr,Glu,Ala); MHC, major histocompatibility complex.

antigen presented on syngeneic irradiated spleen cells every 14–18 days.

T-cell clones were established from the T-cell lines by limiting dilution at 0.2–0.3 cell per well in a microtiter plate in the presence of antigen and irradiated spleen cells. The resulting clones were grown thereafter in T-cell growth factor with periodic exposure to the antigen used for the cloning.

T-Cell Proliferation Assay. The proliferative responses of the T-cell lines and clones were determined by incubating 10^4–2.5×10^4 T cells with 5×10^5 irradiated spleen cells and various concentrations of the antigen in a final volume of 0.2 ml in wells of a microtiter plate. After 24–48 hr, the cultures were pulse-labeled with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) and harvested 16 hr later. The mean thymidine incorporation (cpm) was calculated for triplicate cultures. Standard deviations from triplicate cultures were within 10% of the mean value.

Assay of Interleukin-2 (IL-2) Secretion by T-Cell Lines and Clones. Approximately 2.5×10^4 T cells were incubated with antigen and irradiated spleen cells as described for the proliferation assay. Twenty-four hours later 0.05 ml of culture supernatant was collected. The supernatants were tested for IL-2 activity, either immediately or stored at −20°C until assayed. IL-2 activity was measured by the ability of the supernatants to support the growth of IL-2-dependent CTLD cell line. CTLD cells (10^4 cells per well) were cultured with the tested supernatants diluted 1:1 in a final volume of 0.1 ml. After a 24-hr incubation, 1 μCi of [^{3}H]thymidine was added for 5 hr. The cultures were further processed as described above.

Inhibition Studies. Inhibition of the T-cell proliferative activity and IL-2 secretion was studied by adding various concentrations of the tested inhibitors plus the stimulating antigen to the assay system. Inhibition was calculated as: % inhibition = (1 − cpm in the presence of inhibitor/cpm in the absence of inhibitor) × 100.

RESULTS

T-Cell Lines and Clones. Several T-cell lines and clones, listed in Table 1, with various H-2 restrictions and antigenic specificities, were used to study the effect of Cop 1 on the T-cell response to BP. Four clones (Bu-6, -10, -19, and -20) were derived from a line derived from lymph node cells of B10.PL mice immunized with RBP in complete Freund's adjuvant. Although all the clones respond to RBP, they reflect different specificities. Clones Bu-6 and -20 respond also to mouse BP (MBP) and bovine BP (BBP), and they recognize peptide pR1-11, which constitutes the encephalitogenic determinant in H-2^u mice (16). In contrast, clones Bu-10 and -19 did not respond to pR1-11 nor to any of the other H-2^u-linked determinants—i.e., pR5-16 and pR35-47 (16) (data not shown). Clones Bu-10 and -19 differ from each other in their response to BBP. An H-2^u-restricted T-cell clone (PPU-4) specific to PPD was derived from lymph node cells of B10.PL mice immunized with complete Freund's adjuvant alone. This clone is highly specific to PPD and did not respond to BP.

Three T-cell lines specific to RBP were derived from lymph node cells (B-SK-LNI line) and spleen cells (B-SK-SpI and B-SK-SpII lines) of another EAE-sensitive mouse strain—(SJL/J × BALB/K)F$_1$. These lines manifest difference in their fine specificities as reflected in the pattern of response to RBP and MBP (Table 1). All the three lines responded to BP when presented on syngeneic F$_1$ spleen cells or both parental spleen cells (data not shown). Another line PP-SK-Sp was developed form spleen cells of the same mice immunized with RBP in complete Freund's adjuvant, but selected *in vitro* with PPD. This line after several *in vitro* cycles, was highly reactive with PPD, while completely losing the ability to recognize RBP, the immunizing antigen.

A third source of RBP-specific lines was the BALB/K strain of mice which is EAE-resistant under normal conditions but can be converted to EAE sensitivity after treatment with a low dose of cyclophosphamide (17). The line developed from spleen cells of these RBP-immunized mice, denoted B-K-Sp, is reactive only with RBP (Table 1).

Inhibition of BP-Specific H-2^u-Restricted T-Cell Clones by Cop 1. The specific response of the T-cell clones was followed either by their proliferative activity or IL-2 secretion. None of these clones could be directly stimulated by Cop 1. The effect of Cop 1 on the response of the four BP-specific T-cell clones of H-2^u origin was, therefore, evaluated by their capacity to inhibit the response to BP. The T-cell clones were incubated with various concentrations of BP and Cop 1.

The results with a representative clone, Bu-6, are shown in Fig. 1. Cop 1 inhibited RBP activation of clone Bu-6 in a competitive dose-dependent manner. As can be seen (Fig. 1*A*), 50% inhibition of the proliferative response to RBP could be obtained by using an ≈2-fold molar excess of Cop 1, when tested in the linear phase of the dose–response curve of RBP, and up to 96% inhibition was obtained by using a 10-fold molar excess of Cop 1. The response of this clone to RBP, as followed by IL-2 secretion, could also be inhibited with Cop 1, with a similar efficiency and profile as in the proliferation assay (Fig. 1*B*). The inhibition of the response to BP was specific to Cop 1, as D-Cop 1, and another random polymer TGA, both of which were shown (2, 9) to be ineffective in EAE suppression, did not inhibit the response to BP (Fig. 1*A*). Furthermore, Cop 1 specifically inhibited only the responses to RBP and MBP of the T-cell clone and did not inhibit the response to the mitogen Con A (Fig. 1*C*).

Table 1. Specificity of T-cell lines and clones

Strain of origin	Line/clone	[^{3}H]Thymidine incorporation response, cpm					
		—	RBP	MBP	BBP	pR1-11	PPD
B10.PL (H-2^u)	Bu-6	302	88,839	93,268	58,355	97,138	405
	Bu-10	393	113,071	2,063	656	2,262	345
	Bu-19	101	109,308	1,404	55,034	313	207
	Bu-20	451	156,082	168,730	152,327	182,009	501
	PPu-4	1272	1,502	NT	NT	NT	63,053
(SJL/J × BALB/K)F$_1$ (H-2^{s,k})	B-SK-LN I	850	40,767	24,547	NT	NT	950
	B-SK-Sp I	133	11,156	187	NT	NT	203
	B-SK-Sp II	661	11,867	8,878	NT	NT	509
	PP-SK-Sp	2159	2,791	NT	NT	NT	61,524
BALB/K (H-2^k)	B-K-Sp	242	46,199	659	NT	NT	332

RBP, MBP, and BBP were used at concentration of 10 μg per well, pR1-11 was at 1 μg per well, and PPD was at 2.5 μg per well. These antigen concentrations gave maximal responses in all the clones. NT, not tested.

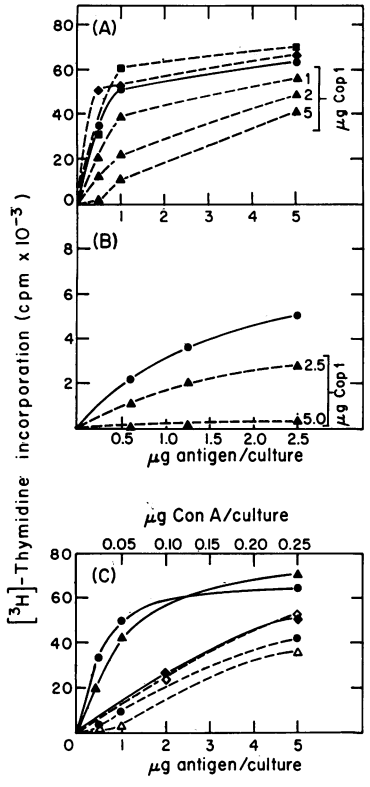

FIG. 1. Inhibition of Bu-6 clone activation by Cop 1. (*A*) Inhibition of Bu-6 proliferation response to RBP. ●, Response to RBP without inhibitors; ▲, response to RBP with various concentrations of Cop 1; ◆, response to RBP in the presence of TGA at 5 μg per culture; ■, response to RBP in the presence of D-Cop 1 at 5 μg per culture. (*B*) Inhibition of BP-induced IL-2 secretion by Bu-6. IL-2 secretion induced by RBP without inhibitors (●) and with various concentrations of Cop 1 (▲) is shown. (*C*) Specificity of Cop-1 inhibition. The response in the absence of Cop 1 to RBP (●), MBP (▲), and Con A (◆) and the response in the presence of Cop 1 at 5 μg per culture to RBP (○), to MBP (△), and to Con A (◇).

The effect of Cop 1 was then tested on the other three BP-specific T-cell clones of H-2^u origin, as well as on the PPD-specific T-cell clone of H-2^u origin, PPU-4. The results, summarized in Table 2, demonstrate that in all four BP-specific clones, the responses to BP are inhibited by Cop 1 to various degrees. For comparison, the inhibitory effect of Cop 1 is given in Table 2 at a constant Cop 1/BP ratio of 5:1. In those clones which respond to both RBP and MBP—i.e., clones Bu-6 and -20—Cop 1 inhibits these two responses, while activation of the clones by Con A is not affected by Cop 1. In all cases TGA and D-Cop 1 did not inhibit the response to BP. In addition, Cop 1 did not inhibit the response of the PPD-specific T-cell clone PPU-4.

The inhibition of T-cell activation by Cop 1 is demonstrable also when measuring IL-2 secretion (Fig. 2). As observed in the proliferation assay, Cop 1 specifically blocked IL-2 secretion in response to BP in all BP-specific clones, whereas IL-2 secretion triggered by Con A was not affected. Furthermore, Cop 1 did not interfere with IL-2 secretion induced by PPD in the PPD-specific T-cell clone PPU-4 (data not shown).

The Effect of Cop 1 on BP-Specific, H-2^k- and H-2^{s,k}-Restricted T-Cell Lines. The effect of Cop 1 was tested also in T-cell lines of other H-2 types—i.e., H-2^{s,k} and H-2^k, listed in Table 1. In these BP-specific T-cell lines, two patterns of response to Cop 1 were observed. In B-SK-LNI, B-SK-SpI, and B-K-Sp cells, Cop 1 inhibited the proliferative response to RBP. On the other hand, in the B-SK-SpII T-cell line, Cop 1 alone was capable of inducing proliferative response. Furthermore, when this cell line was cultured with both RBP and Cop 1, an additive proliferative response, rather than inhibition, was observed (Fig. 2). TGA caused neither inhibition nor enhancement. The response to Con A of all these T-cell lines was not affected by Cop 1, and Cop 1 also had no effect in the PPD cell line of H-2^{s,k} origin—i.e., PP-SK-Sp (data not shown).

The dose–response curve of the inhibition by Cop 1 in these T-cell lines, as demonstrated in Fig. 3 for the B-SK-LN line, illustrates similar characteristics of dose-dependent inhibition as in the H-2^u clones. Inhibition of the proliferative response by 50% was obtained at a 5-fold molar excess of Cop 1, with a maximal inhibition of 79.5% (Fig. 3*A*). Among these T-cell lines only cell line B-SK-LN was found to release IL-2 in response to T-cell activation. Cop 1 inhibited the IL-2 release induced by RBP, up to 80% with a 4-fold molar excess of Cop 1, whereas TGA had no effect (Fig. 3*B*).

Fig. 2 summarizes the effect of Cop 1 both on the proliferative response and on the IL-2 secretion induced by RBP, in all the T-cell lines and clones described in this work. As can be seen, eight BP-specific lines and clones with different specificities and H-2 restrictions were affected by Cop 1. In seven of them Cop 1 specifically inhibited the response to BP, and in one Cop 1 was able to induce proliferation. In those T-cell lines and clones which can be induced to secrete IL-2, the effect of Cop 1 on the RBP-induced IL-2 secretion mirrors that observed in the proliferation response.

Table 2. Specificity of Cop 1 inhibition of the proliferative response to BP of B10.PL (H-2^u) T-cell clones

Antigen	[^{3}H]Thymidine incorporation, cpm				
	Bu-6	Bu-10	Bu-19	Bu-20	PPU-4
—	199	199	126	417	1,272
Cop 1 (5 μg)	189	249	146	303	1,891
D-Cop 1 (5 μg)	240	267	195	364	NT
TGA (5 μg)	182	NT	134	683	NT
RBP (1 μg)	50,555	21,852	28,095	53,534	1,502
RBP + Cop 1	10,170 (80.2%)	2,315 (90.2%)	3,860 (86.6%)	32,212 (40.1%)	NT
RBP + D-Cop 1	61,285 (0%)	26,689 (0%)	28,317 (0%)	50,718 (5.3%)	NT
RBP + TGA	51,045 (0%)	NT	26,179 (6.8%)	50,398 (5.9%)	NT
MBP (1 μg)	42,336	181	535	49,247	NT
MBP + Cop 1	3,174 (92.9%)	NT	NT	19,599 (60.7%)	NT
PPD (1 μg)	200	213	195	513	43,053
PPD + Cop 1	NT	NT	NT	NT	49,740 (0%)
Con A (0.25 μg)	50,655	32,509	75,514	59,552	45,781
Con A + Cop 1	52,831 (0%)	29,899 (8.1%)	76,389 (0%)	63,459 (0%)	53,830 (0%)

NT, not tested. Values in parentheses represent percent inhibition.

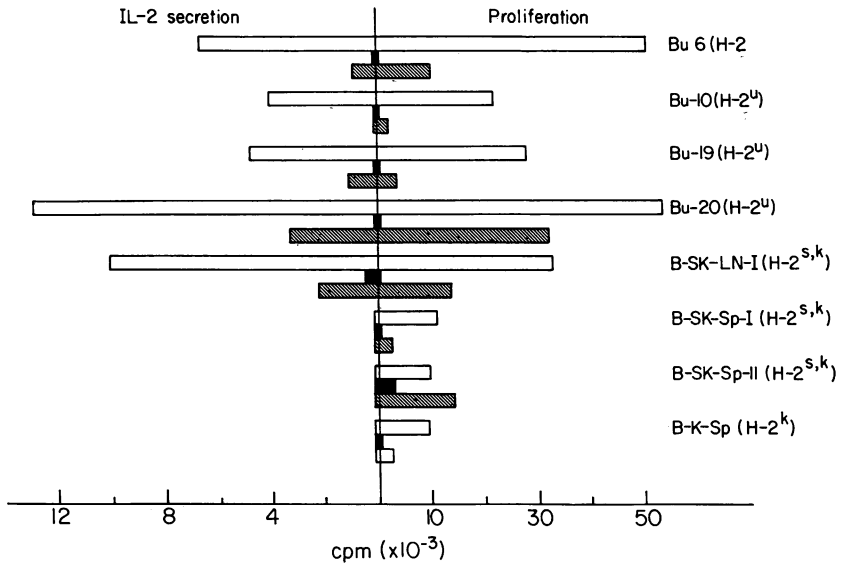

FIG. 2. Effect of Cop 1 on the proliferation response and IL-2 secretion of RBP-specific T-cell lines and clones. The responses to RBP alone (□), to Cop 1 alone (■), and to RBP plus Cop 1 (▨) are shown.

DISCUSSION

The study described here was undertaken to further establish whether Cop 1, which is an efficient suppressive agent in EAE and possibly in multiple sclerosis, is capable of blocking specific T-cell responses to BP. We have demonstrated (8) that Cop 1 can inhibit the response to BP of sensitized lymphocytes from strain 13 guinea pigs and rabbits. Furthermore, Cop 1 inhibited the *in vitro* induction of BP-specific cells from normal lymphocytes (10). In the present study, these observations were corroborated at the level of specific T-cell lines and clones of defined specificities and H-2 restrictions. Indeed, Cop 1 specifically inhibited the response to BP of several murine T-cell lines and clones, irrespective of their fine specificity or H-2 restriction.

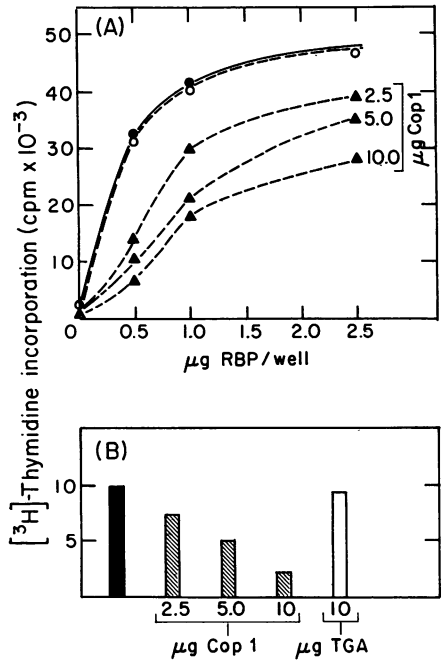

FIG. 3. Inhibition of BP-SK-LN cells activation by Cop 1. (*A*) Inhibition of the proliferation response to RBP. The responses to RBP without inhibitors (●), to RBP in the presence of Cop 1 (▲), and to RBP in the presence of TGA at 5 μg per culture (○) are shown. (*B*) Inhibition of IL-2 secretion in response to 2.5 μg of RBP per culture without inhibitors (■), in the presence of Cop 1 (▨), and in the presence of TGA (□) is shown.

The inhibition by Cop 1 is specific to BP-induced responsiveness and is not due to toxic factors or nonspecific binding to BP, as indicated by the following observations. (*i*) Cop 1 did not inhibit the response of the same cells to mitogen or the response of the PPD-specific T-cell line and clone to PPD. (*ii*) The effect of Cop 1 can be partially reversed by increasing the concentration of BP in the culture. (*iii*) Cop 1 inhibited the response triggered not only by BP but also with the peptide pR1-11 in responsive clones (preliminary results; data not shown). (*iv*) D-Cop 1 and TGA, which were both demonstrated (2, 9) to be ineffective in EAE suppression, did not inhibit the response to BP.

Cop 1 inhibited the response of several T-cell lines and clones which probably respond to different epitopes of the basic protein—i.e., they represent different T-cell receptor (TCR) specificities. For the four H-2^u-restricted BP-specific clones, two (Bu-6 and -20) respond to the same epitope, pR1-11, which is the encephalitogenic determinant in H-2^u strains of mice, as demonstrated by Zamvil *et al.* (16). Those two clones are inhibited by Cop 1 but with different efficiencies (Table 2), which may indicate differences in the affinity of the TCR for BP. The other two clones, Bu-10 and -19, respond to yet undefined epitopes of the BP. These two clones differ in their response to BBP, which might reflect response to different determinants. Therefore, we have four clones which represent at least three TCR specificities and they are all inhibited by Cop 1. For BP-specific lines derived from H-2^{s,k} and H-2^k mice, their exact epitope specificity has not yet been determined; however, they represent at least two specificities as reflected by the differences in response to MBP. One possibility might be the T-cell epitopes characterized by Sakai *et al.* (18) for H-2^s clones—i.e., within residues 89–101 of RBP. Of four lines, one was capable of proliferating in response to Cop 1, whereas the other three lines were inhibited by Cop 1 to various degrees (Fig. 2). These *in vitro* findings with the BP-specific T-cell lines and clones are in accord with the observed *in vivo*-suppressive activity of Cop 1 in a variety of species (mice, guinea pigs, rabbits, and monkeys) (2–6) which respond to different encephalitogenic determinants on BP (19).

Cop 1 had a similar effect on the two antigen-induced responses of T cells—i.e., proliferation and IL-2 secretion. Whereas antigen-induced proliferation activity has no known

in vivo correlate, certain lymphokine secretions including IL-2 have been shown (B. Tartakovsky, O. Axelrod, and E. Mozes, personal communication), by using (T,G)-A--L proliferative and helper clones, to be associated only with helper clones and possibly play a role in the helper activity [where (T,G)-A--L is the branched-chain polypeptide poly(Tyr,Glu)-poly(DL-Ala)--poly(Lys)]. Thus, the *in vitro* effect of Cop 1 on IL-2 secretion may have an *in vivo* significance.

The mechanism by which Cop 1 specifically inhibits the response of T-cell lines and clones with different TCRs to BP is not yet clear. The TCR recognizes only peptide fragments bound to a self major histocompatibility complex (MHC) molecule. A given T cell is both peptide-specific and MHC-restricted (21). It appears that class II MHC molecules possess only one major peptide binding site that has high affinity but paradoxically low specificity for peptides (22). The binding of a peptide to class II MHC molecule is a necessary but not sufficient condition to promote immunity. It has been demonstrated by Guillet *et al.* (23) that peptides, capable of being presented by a given class II MHC molecule, compete with regard to T-cell activation. Whereas it is hard to envisage specific binding of Cop 1 to totally different TCRs, a possible explanation to the observed effect of Cop 1 on the T-cell response to BP may be the binding of Cop I or a Cop 1-derived peptide to the relevant MHC molecule. Such binding may induce either inhibition of the T-cell response to BP or activation, as observed in some cases. We have so far no direct evidence to support this theory and additional studies are required to understand the molecular basis of this phenomenon and to establish whether it holds also for other H-2 and MHC restrictions. We have demonstrated (8) either stimulation or inhibition of BP response in guinea pigs and rabbits. In humans, Burns *et al.* (20) reported that in four BP-specific T-cell lines, isolated from peripheral blood lymphocytes of normal individuals, Cop 1 induced neither stimulation nor antigen specific immune tolerance to BP.

The results presented here, demonstrating specific *in vitro* inhibition of the T-cell response to BP by Cop 1, indicate that Cop 1 may be effective *in vivo* in EAE suppression, not only because of selective stimulation of suppressor T cells, as has been demonstrated (5), but also by specific inhibition of BP-specific effector T cells. Further studies are required to correlate the *in vitro* inhibitory effect of Cop 1 with the *in vivo* efficiency in disease suppression. Furthermore, studies with human BP-specific lines may help to establish whether this suggested mechanism may be relevant also in multiple sclerosis.

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