Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9528–9532, November 1991
Immunology

Cross-reactions and specificities of monoclonal antibodies against myelin basic protein and against the synthetic copolymer 1

(experimental allergic encephalomyelitis/autoimmunity/multiple sclerosis/polyclonal antibodies)

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Contributed by Michael Sela, July 19, 1991

ABSTRACT **Antibody cross-reactivity is here demonstrated between basic protein (BP), the encephalitogenic molecule of myelin, and copolymer 1 (Cop 1), the synthetic amino acid copolymer, which has a suppressive effect on experimental allergic encephalomyelitis and is effective in reducing the number of relapses in exacerbating-remitting multiple sclerosis. This cross-reactivity is conclusively established using mouse monoclonal antibodies (mAbs). About a third of anti-rat BP mAbs and most of anti-mouse BP mAbs cross-reacted with Cop 1. This cross-reactivity could be demonstrated with anti-BP mAbs of different specificities. In addition, several anti-Cop 1 hybridomas cross-reacted with BP. This cross-reactivity was verified in several assay systems, including competitive inhibition experiments. Moreover, some anti-BP mAbs and anti-Cop 1 mAbs reacted in a heteroclitic manner and favored the cross-reactive antigen over the immunogen. In contrast to the mAbs, no cross-reactivity could be demonstrated with the antisera of immunized mice. This observation may reflect the different B-cell populations expressed in the mAb response as compared to the polyclonal response. Thus, the use of mAbs has uncovered specificities that are not evident in antisera and has revealed pronounced cross-reactivity between BP and Cop 1 at the B-cell level. These results further establish the immunological interrelationships between Cop 1 and BP, demonstrated earlier at the T-cell level.**

Copolymer 1 (Cop 1) is a synthetic basic copolymer of L-alanine, L-glutamic acid, L-lysine, and L-tyrosine in a molar residue ratio of 6.1:1.9:4.7:1.0 (1). Cop 1 was demonstrated to be active in the specific suppression of experimental allergic encephalomyelitis (EAE), an autoimmune neurological disease induced by myelin basic protein (BP). EAE serves as an animal model for human demyelinating diseases including multiple sclerosis (2). The suppression of EAE by Cop 1 was demonstrated in several species including primates (1, 3–6). 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 BP. This cross-reactivity was established at the level of T-cell-mediated immunity (8, 9). Cop 1 was found to induce suppressor T cells specific to BP that mediate protection against EAE (5) and to specifically block the *in vitro* response to BP of murine T-cell lines and clones (10). These results suggest two possible mechanisms for Cop 1 activity in EAE: (*i*) induction of antigen-specific suppressor cells and (*ii*) competition with BP for binding to the major histocompatibility complex.

EAE is a classical T-cell-mediated disease in which $CD4^+$ T-cell lines of the T_H1 phenotype were demonstrated to transfer the disease (11). Although antibodies are not essential factors in EAE, autoreactive anti-BP antibodies also play a role in the disease process, and antibodies may either contribute to or ameliorate the disease (2, 12, 13). It was therefore of interest to study in detail the cross-reactivity between BP and Cop 1 at the humoral level, especially as the determinants of BP that elicit the antibody response are distinct from those inducing the T-cell response (2). In earlier studies we demonstrated (8), using polyclonal guinea pig anti-Cop 1 antibodies, a slight degree of cross-reactivity between Cop 1 and bovine BP (BBP). Monoclonal antibodies (mAbs), which are homogenous monospecific reagents, were demonstrated to be a valuable tool in dissecting antigenic determinants and in studying structural immunological relationship between different molecules. Hence, we utilized hybridoma technology to further delineate and establish the cross-reactivity between BP and Cop 1. We report here the production of mAbs specific to BP and Cop 1. Several of the mAbs manifested a reciprocal cross-reactivity between the two antigens. Some of the mAbs even had a heteroclitic activity; i.e., they favored the cross-reactive antigen over the immunogen. These findings corroborate our previous results (8, 9) on cellular cross-reactivity and establish a clear cut immunological similarity between BP and Cop 1.

MATERIALS AND METHODS

Mice. SJL/J and (SJL/J × BALB/c)F_1 mice 4–6 weeks of age were obtained from The Jackson Laboratory.

Antigens. BP was isolated from spinal cords of mouse, rat, guinea pig, bovine, and human white matter as described (14). Cop 1, a random copolymer 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, was synthesized at the Weizmann Institute, Bio-Yeda (Rehovot, Israel) or at Teva (Petach-Tikva, Israel) and characterized as described (1).

Immunization. SJL/J mice were injected with rat BP (RBP), mouse BP (MBP), or Cop 1 at 200 μg per mouse in enriched complete Freund's adjuvant (containing H37Ra at 4 mg/ml) into four footpads. Three or four booster injections were given intradermally and intraperitoneally at 2-week intervals with the same dose of antigen in phosphate-buffered saline (PBS). Ten days after the last injection, mice were bled and the sera were tested for antibody levels in an RIA. Spleen cells of responding mice were taken for fusion.

Cell Fusion. Four days before fusion mice were given a booster injection intraperitoneally. Splenic lymphocytes from immunized mice were fused with the NSO/1 murine plasmacytoma cells, in a ratio of 5:1, respectively, using 41% (wt/vol) polyethylene glycol 1500 (Serva) according to Eshhar (15). Culture supernatants were screened by solid-phase RIA. Positive hybridomas were grown and cloned either by

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Abbreviations: BP, basic protein; BBP, bovine BP; HBP, human BP; GPBP, guinea pig BP; MBP, mouse BP; RBP, rat BP; BSA, bovine serum albumin; Cop 1, copolymer 1; EAE, experimental allergic encephalomyelitis; mAb, monoclonal antibody.

soft-agar cloning or under limiting dilution. Hybridomas were propagated in (SJL/J × BALB/c) mice that were pretreated with Pristane, and ascites fluids were collected 10–20 days after cell inoculation (15).

RIA. Several variations of RIA were used throughout the study to determine antibody responses. Results are expressed as the mean ^{125}I bound for triplicate samples. Standard deviation for triplicate samples were within 10% of the mean value.

Solid-phase RIA. Flexible plastic microtiter plates were coated with BP or Cop 1 (1–10 μg per well). After a 16-hr incubation at room temperature, plates were washed three times and saturated for 2 hr with PBS containing 2% (wt/vol) bovine serum albumin (BSA), 0.05% Tween 20, 0.1% sodium azide, 10 mM EDTA, and heparin at 5 units/ml. The antibody solution (50 μl) to be assayed (diluted serum, hybridoma supernatant, or ascites fluid) was added to the wells for a 2-hr incubation and then wells were washed. To assess the quantity of antibodies bound to the antigen, ^{125}I-labeled goat anti-mouse Fab antibody (1×10^5 cpm per well) was added for overnight incubation at 4°C. After extensive washing, plates were dried, wells were cut out of the plates, and radioactivity was measured in a γ counter.

Reverse-solid-phase RIA. In this method the plates were coated with goat anti-mouse Fab antibodies (10 μg per well; Bio-Yeda) by overnight incubation at 4°C, followed by incubation with tested antibodies for 2 hr at room temperature. Finally, iodinated BP or Cop 1 (1×10^5 cpm per well) was added to the antibodies and incubated overnight at 4°C (15).

RIA in solution. Ascites fluid, 100 μl diluted in PBS/lysozyme (0.5 mg/ml), was bound to ^{125}I-labeled BP or Cop 1 (3×10^4 cpm) for 1 hr at 37°C. To precipitate the bound antigen, 100 μl of goat anti-mouse immunoglobulin serum was added. After 1 hr, the tubes were centrifuged and the amount of radioactivity in the supernatant and in the precipitate was measured.

Competitive Inhibition of Solid-Phase RIA. Inhibition of antibody binding to antigen was performed as follows. Solutions containing various concentrations of the tested inhibitor (25 μl) were added to plates precoated with a limiting amount of antigen (as described above), followed by 25 μl of the antibody dilution that was found to give 50–75% binding. After a 2-hr incubation, ^{125}I-labeled anti-mouse Fab was added, followed by the procedure described for the RIA binding assay.

Determination of mAb Isotype. Hybridoma supernatants to be assayed were added to antigen-coated plates as described above for the solid-phase RIA. Goat anti-mouse immunoglobulin isotype sera (Meloy Laboratories) (2 μg/ml) were added for 2 hr, followed by overnight incubation of ^{125}I-labeled rat anti-goat immunoglobulin.

Iodination. Affinity-purified goat anti-mouse Fab antibodies and rat anti-goat IgG (Bio-Yeda) were iodinated according to Greenwood and Hunter (16). The iodinated proteins were separated from free iodine on a Sephadex G-25 (coarse) column using 1% BSA as a carrier protein in PBS. BP and Cop 1 were iodinated similarly but with ten-fold lower concentrations of chloramine-T and sodium metabisulfite. The separation step on the Sephadex column was performed in 0.1 M HCl supplemented with 1% BSA.

RESULTS

Reactivity of Murine Polyclonal Antibodies to BP and to Cop 1. Murine polyclonal antibodies against MBP, RBP, or the synthetic copolymer Cop 1 were raised by immunizing SJL/J mice. Specific antibodies to BP could be obtained with either the heterologous RBP or the homologous MBP. The titers of anti-BP antibodies measured in a solid-phase RIA were similar in the two antisera, they did not differentiate between BP from various species, and reacted similarly with RBP, MBP, and BBP (Fig. 1 *A* and *B*). Immunization with Cop 1 yielded specific antibodies with higher titers than did immunization with BP (Fig. 1*C*). We tested for cross-reactivity between BP and Cop 1 using these polyclonal antibodies in a solid-phase RIA. No significant cross-reactivity could be detected between BP and Cop 1 either with whole serum or with purified antibodies. Anti-BP serum bound only to BP, whereas its reactivity with Cop 1 was identical to the reactivity of normal serum (Fig. 1 *A* and *B*). Similarly, the sera of mice immunized with Cop 1 recognized solely Cop 1 and showed no significant binding to BP (Fig. 1*C*).

Reactivity of mAbs to BP and Cop 1. The humoral reactivity to BP and Cop 1 was extensively studied by using a series of fusions with NSO plasmacytoma cells of spleen cells from SJL/J mice immunized with RBP, MBP, or Cop 1. Fusion of cells from mice immunized with RBP resulted in 48 out of 600 (8%) hybridoma lines of different isotypes that reacted with BP but not with the nonrelevant antigen BSA. Upon hybridization using anti-Cop 1 lymphocytes, we also found a considerable number of hybridomas (50 of 600 or 8%) that bound Cop 1 and were of various isotypes. Two fusion experiments were performed to obtain antibodies against self MBP; but only 15 lines out of 1200 (1.25%) reacted with MBP. All of them except one expressed the IgM isotype.

Representative hybridoma lines were further cloned, and the reactivity of the mAbs was tested using a panel of BPs of various species [RBP, MBP, guinea pig BP (GPBP), BBP, and human BP (HBP)]. The anti-BP mAbs could be differ-

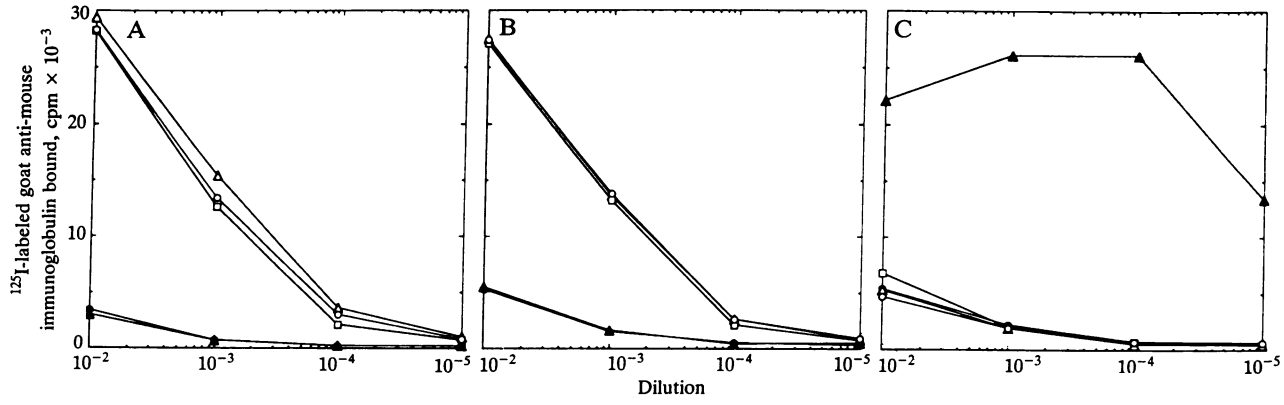

FIG. 1. Reactivity of murine antisera. Mouse antisera anti-RBP (*A*), anti-MBP (*B*), and anti-Cop 1 (*C*) were incubated in a solid-phase RIA with MBP- (○), RBP-(△), BBP-(□), or Cop 1-(▲) coated plates and the reactivity was compared to the reactivity of normal mouse serum (●) with these antigen-coated plates.

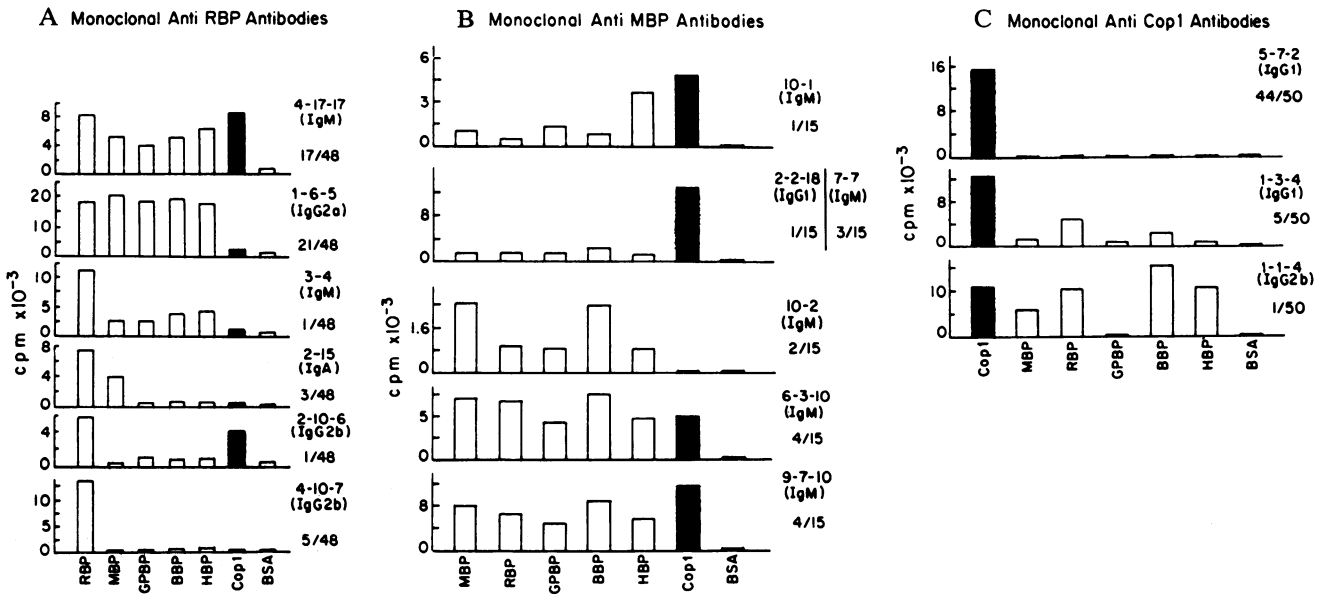

FIG. 2. Specificity of mAbs. mAbs anti-RBP (*A*), anti-MBP (*B*), and anti-Cop 1 (*C*) were reacted in a solid-phase RIA with plates coated with BP of various origins (open bars), Cop 1 (solid bars), or BSA (bars to the right). For each response pattern, the results obtained with a representative hybridoma are illustrated. The isotype of the representative hybridoma and the incidence of hybridomas exhibiting this specificity pattern are indicated.

entiated by their different patterns of cross-reactive responses. Some mAbs, represented by anti-RBP 4-17-17 and 1-6-5 (Fig. 2*A*) and anti-MBP 6-3-10 and 9-7-10 (Fig. 2*B*), reacted with all BP species tested to a similar degree, as did the polyclonal antibodies. However, in contrast to the anti-BP antiserum, the other anti-RBP mAbs reacted preferentially (e.g., mAbs 3-4, 2-15, and 2-10-6) or solely (e.g., mAb 4-10-7) with RBP, the immunizing antigen (Fig. 2*A*). Of the anti-MBP mAbs that differentiated between BPs, one (mAb 10-1) reacted preferentially with MBP and the other two (e.g., mAb 10-2) reacted preferentially with MBP and HBP. Interestingly, no mAb specific to autologous MBP could be detected (Fig. 2*B*).

Cross-Reactivity Between BP and Cop 1. Anti-RBP and anti-MBP mAbs were screened for reactivity with Cop 1. About one-third of the anti-RBP mAbs (14 of 48) and most of the anti-MBP mAbs (13 of 15) reacted with Cop 1 to various degrees (Fig. 2 *A* and *B*). Moreover, some of the mAbs that had originated from MBP-immunized mice (e.g., mAbs 10-1, 2-2-18, and 7-7) reacted in a heteroclitic manner and bound to Cop 1 better than to BP (Fig. 2*B*). The cross-reactivity with Cop 1 was not limited to a particular subgroup of mAbs but

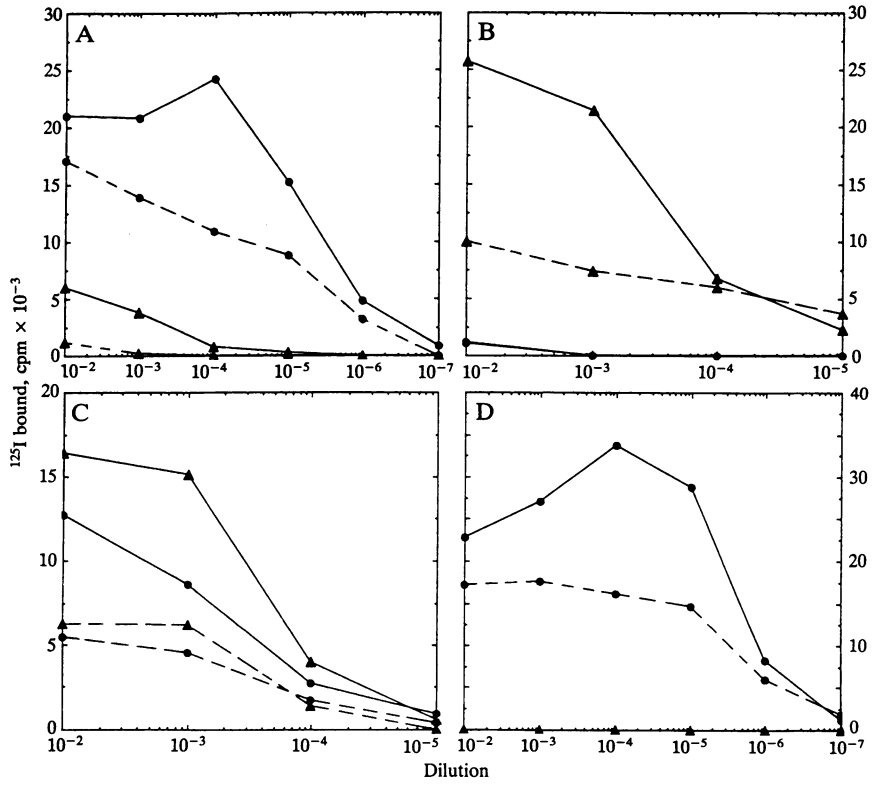

FIG. 3. Cross-reactivity of mAbs. mAbs (from ascites fluid) anti-MBP 2-2-18 (*A*), anti-RBP 1-6-5 (*B*), anti-Cop 1 1-1-4 (*C*), and anti-Cop 1 5-7-2 (*D*) were reacted in a solid-phase RIA (solid lines) and reverse RIA (dashed lines) with Cop 1 (●) and BP (▲) (MBP in *A*, RBP in *B*, and BBP in *C* and *D*).

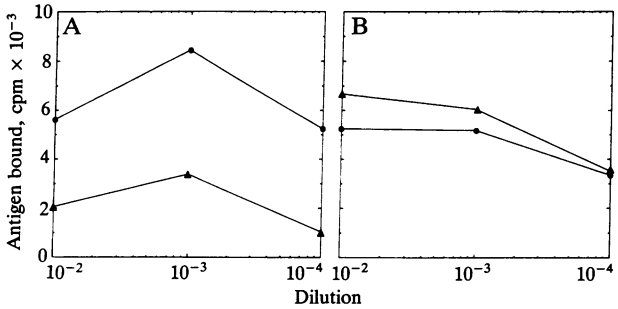

FIG. 4. Cross-reactivity of mAbs using RIA in solution, ascites fluid containing anti-MBP 2-2-18 (*A*) and anti-Cop 1 1-1-4 (*B*) and ^{125}I-labeled BBP (▲) or ^{125}I-labeled Cop 1 (●). Specific antigen bound was calculated by reducing the nonspecific binding obtained with control NSO ascites fluid.

was demonstrated with mAbs of different specificities, as exhibited by their distinct patterns of response with the various BPs. This was the case for the anti-RBP (Fig. 2*A*) and anti-MBP (Fig. 2*B*) mAbs.

The mAbs to Cop 1 were screened for their response with the homologous Cop 1 and for their cross-reactivity with BP of various species. Most of the anti-Cop 1 hybridomas reacted specifically with Cop 1 and did not react with any of the BPs tested. Nevertheless, some cross-reactive hybridomas (6 of 50) that recognized BP were found, and one of them, mAb 1-1-4, bound to BBP somewhat better than to Cop 1 (Fig. 2*C*).

The cross-reactivity described so far was demonstrated in a solid-phase RIA in which the mAbs reacted with antigen-coated plates. Additional assays were employed to investigate this cross-reactivity. The mAbs were tested also in a reverse-phase RIA, where they were immobilized to the plate and then allowed to react with soluble iodinated BP and Cop 1. As can be seen in Fig. 3, the mAbs showed the same pattern of response in both RIA versions. Thus, anti-RBP 1-6-5 and anti-Cop 1 5-7-2 mAbs showed restricted specificity to the immunizing antigen BP and Cop 1, respectively, whereas anti-MBP 2-2-18 and anti-Cop 1 1-1-4 mAbs bound to BP and Cop 1. Moreover, the heteroclitic reactivity of these two antibodies was shown in both assays (Fig. 3 *A* and *C*).

Cross-reactivity between Cop 1 and BP was also evident when the RIA was performed in solution, thus excluding the possibility of nonspecific adsorption. As in the previous RIA assays, anti-MBP 2-2-18 and anti-Cop 1 1-1-4 mAbs revealed cross-reactive patterns with a heteroclitic response (Fig. 4).

We further established the specificity of the mAbs in inhibition experiments in which both Cop 1 and BP were used to inhibit binding to the immunizing antigen. Binding of anti-RBP 1-6-5 to BP and anti-Cop 1 5-7-2 mAbs to Cop 1 could be inhibited only with the homologous antigen (i.e., BP or Cop 1, respectively); however, anti-MBP 2-2-18 and anti-Cop 1 1-1-4 mAbs were inhibited by both BP and Cop 1 (Fig. 5). Cop 1 was much more effective than BP (≈100-fold) in inhibiting the reactivity of anti-MBP 2-2-18 mAb (Fig. 5*A*).

DISCUSSION

The present study has conclusively established the existence of humoral immunological cross-reactivity between the naturally occurring encephalitogenic BP and the synthetic copolymer Cop 1, which has been shown to exert a specific suppressive effect on EAE (1, 3–6). The cross-reaction was revealed using monoclonal anti-BP and anti-Cop 1 antibodies and was demonstrated using several different methods: direct and reverse RIA (Figs. 2 and 3), RIA in solution (Fig. 4), and inhibition experiments (Fig. 5). The same patterns of cross-activity were observed for all Cop 1 preparations, regardless of their source.

We have demonstrated (8, 9) a marked degree of cross-reactivity at the cellular level between BP and Cop 1. At the humoral level, cross-reactivity was obtained only for guinea pig anti-Cop 1 antiserum using the sensitive passive cutaneous anaphylaxis assay (8). In the present study, using murine polyclonal anti-Cop 1 and anti-BP antisera, no cross-reactivity could be demonstrated using either RIA (Fig. 1) or the passive cutaneous anaphylaxis technique (data not

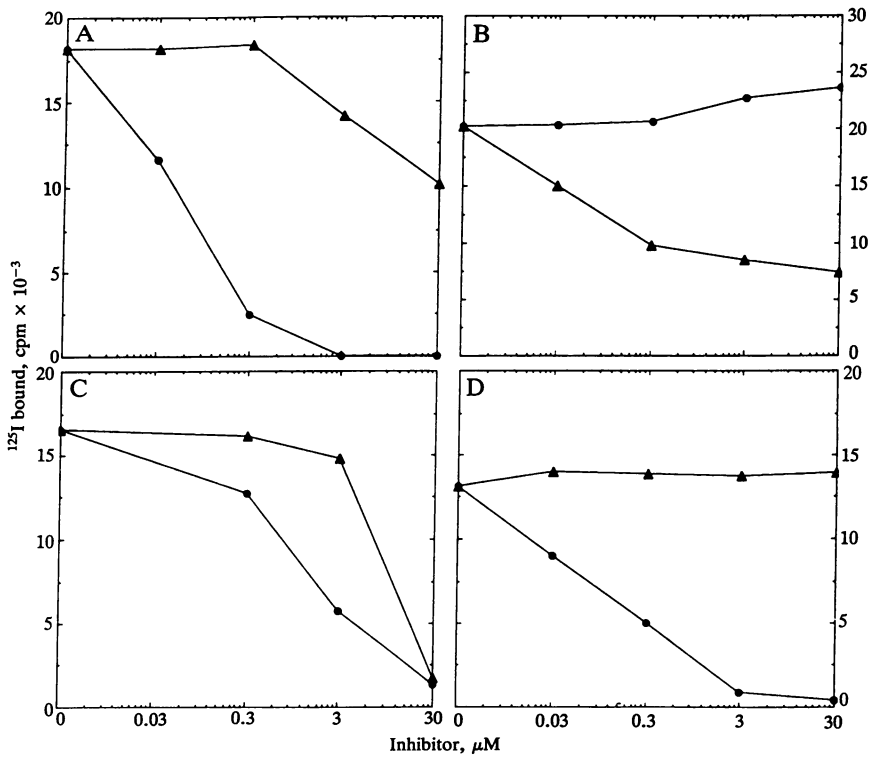

FIG. 5. Inhibition of specific binding of mAbs. The binding of mAbs anti-MBP 2-2-18 (*A*), anti-RBP 1-6-5 (*B*), anti-Cop 1 1-1-4 (*C*), and anti-Cop 1 5-7-2 (*D*) to the homologous antigen in solid-phase RIA was inhibited by Cop 1 (●) and BP (▲) (MBP in *A*, RBP in *B*, and BBP in *C* and *D*).

shown). This dissimilarity may result from differences between species or difference in the titers of the antibody.

In contrast to the lack of significant cross-reactivity between Cop 1 and BP with polyclonal antibodies, mAbs displayed a considerable degree of cross-reactivity manifested both in the number of cross-reactive mAbs (one-third of the anti-RBP mAbs and most of the anti-MBP mAbs) and in the heteroclitic response of some of these antibodies. An additional difference between polyclonal antibodies and mAbs is that mAbs can differentiate between BPs of different species (Fig. 2). These marked differences between the specificity of the antibodies in the serum and the mAbs may stem from several factors. The immune response as detected in the serum is the net result of immunoregulation *in vivo*, including suppressor cells that are especially active in down regulating reactivity to self antigens. The fusion technique makes it possible to detect a rare B-cell repertoire without these influences. By using the fusion technique, it is possible to detect rare B-cell clones or those with low affinity that are suppressed in the heterogeneous sera.

It is of interest that most of the mAbs obtained in this research responded to "hidden epitopes" that were not seen by the sera. It is therefore possible that products of different B-cell populations are expressed in sera and in mAbs. The cells that actively secrete large amounts of immunoglobulins in the serum are the plasma cells that are mature undividing B cells selected by the antigen (17). In contrast, the B cells that succeed as fusion partners of the NSO myeloma line are proliferating antigen-activated B lymphocytes before they mature into plasma cells (18). In this regard it is significant that all the mAbs obtained (except one) in the MBP fusion that exhibited the highest incidence of cross-reactive antibodies with Cop 1 were of the IgM isotype. This suggests that indeed the mAbs resulted from B cells in early differentiation stages. In addition, the antibodies in the serum were secreted from circulating plasma cells whereas the mAbs were prepared from splenic lymphocytes. Differences between cells originating from spleen and blood had been demonstrated before in several systems (19, 20). All this may account for the variance between the polyclonal response and the monoclonal response.

A significant observation, which could be demonstrated in the various assays used in this study, was the heteroclitic reactivity exhibited by several mAbs—e.g., anti-MBP 2-2-18 and anti-Cop 1 1-1-4 (Figs. 2–5). This response was much more pronounced for anti-MBP mAbs than for anti-Cop 1 in the prevalence and the intensity of the heteroclitic effect. For anti-MBP 2-2-18 mAb, the heteroclitic effect favored Cop 1 over the immunogen by >100-fold (Fig. 5). This result suggests that mAb 2-2-18 probably recognizes a determinant that is not accessible to antibody in intact BP and is more widely present in the synthetic copolymer. Since Cop 1 is a synthetic random copolymer built from only four amino acids, repetitions of the same sequence may occur leading to higher prevalence of some epitopes on Cop 1 than on the natural antigen BP. Thus, more cross-reacting antibodies might bind to each molecule of Cop 1 resulting in high-avidity and heteroclitic reactivity. Hence in the competition experiments, Cop 1 was always more efficient than BP in inhibiting the binding of the cross-reacting mAbs to both BP and Cop 1. This was shown for anti-MBP 2-2-18 mAb (≈100-fold in comparison to BP, Fig. 5*A*) and even for anti-Cop 1 1-1-4 mAb (≈10-fold, Fig. 5*C*), which in the direct assays bound BP better than Cop 1 (Figs. 2*C*, 3*C*, and 4*B*). The higher efficiency of Cop 1 in competition suggests that Cop 1 contains more copies of the recognized epitope than does the autoantigen BP.

Myelin BP is a protein of ≈170 amino acids with no globular structure and exists in solution as a randomly coiled protein with local areas of ordered structure (21). A detailed immunochemical examination of polyclonal antibody and mAb responses to BP and its peptides demonstrated the existence of a large number of antigenic determinants, some of them sequential and some conformational (22). It is not surprising, therefore, that the anti-BP mAbs obtained in this study were of different specificities (Fig. 2). It is worth noting, however, that the occurrence of mAbs cross-reactive with Cop 1 was not limited to one specificity but could be demonstrated in mAbs of apparently different specificities (Fig. 2). This suggests that more than one determinant of BP is common between BP and Cop 1. Further studies are required to determine the nature of these cross-reactive determinants (sequential vs. conformational) and to identify them.

The present results establish humoral cross-reactivity between the encephalitogenic BP and the synthetic EAE-suppressing Cop 1 revealed by anti-BP and anti-Cop 1 mAbs. These results and the previously observed cross-reactivity at the cellular level may form the basis for understanding the mechanism of the suppressive activity of Cop 1. Response to myelin BP may also be implicated in multiple sclerosis as we have demonstrated (23) and as others have indicated (24–26). The cross-reactivity between BP and Cop 1 may, therefore, be of relevance also in multiple sclerosis.

We thank Bat-Sheva Ringel and Ada Wexler for their valuable assistance and Prof. I. Cohen for critically reviewing the manuscript. This research was supported in part by a grant from Teva (Petach-Tikva, Israel).

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