Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells—specificity and promiscuity

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ABSTRACT Copolymer 1 (Cop 1) is a synthetic basic random copolymer of amino acids that has been shown to be effective in suppression of experimental allergic encephalomyelitis and is being tested as a candidate drug for multiple sclerosis. It has been previously demonstrated that Cop 1 is immunologically cross-reactive with the autoantigen myelin basic protein (BP) and competitively inhibits the response to BP of T-cell lines and clones of different major histocompatibility complex (MHC) restrictions, of both mouse and human origin. In the present study we demonstrated the direct binding of Cop 1, using its biotinylated derivative, to MHC molecules on living antigen-presenting cells. Binding of biotinylated BP and peptide p84–102 (an immunodominant epitope of BP) was also demonstrated. Cop 1 and BP bound in a promiscuous manner to different types of antigen-presenting cells of various H-2 and HLA haplotypes. The specificity of the binding was confirmed by its inhibition with either the relevant anti-MHC class II antibodies or unlabeled analogs. Cop 1 exhibited the most extensive and fast binding to antigen-presenting cells. In addition, Cop 1 inhibited the binding of biotinylated derivatives of BP and of p84–102 to the MHC class II molecules and even displaced these antigens when already bound. Thus, these results suggest that Cop 1 indeed competes with BP for MHC binding and, thereby, inhibits T-cell responses to BP. The binding of Cop 1 to different DR alleles, probably because of its multiple MHC binding motifs, may indicate its potential as a broad-spectrum drug for multiple sclerosis.

Myelin basic protein (BP) is a major autoantigen involved in the induction of experimental allergic encephalomyelitis (EAE), a cell-mediated autoimmune disease of the central nervous system (1). EAE serves as an animal model for human demyelinating diseases including multiple sclerosis (MS) (2). Although the myelin components that act as the autoantigen in MS have not been identified yet, recent studies suggest that T-cell reactivity to BP may be of significance in this disease (3).

We have previously reported that suppression of EAE in various animal species may be induced by copolymer 1 (Cop 1), a synthetic basic random copolymer of amino acids (4–8). The immunological cross reactivity of Cop 1 with BP was conclusively established at the humoral level, using monoclonal antibodies to either BP or Cop 1 (9), and at the cellular level (10). Studies in mice suggest two possible mechanisms for Cop 1 activity in EAE. (i) Induction of antigen-specific suppressor cells: Cop 1 was found to induce suppressor T cells specific to BP that mediate prevention of clinical EAE (7, 11). (ii) Competition with BP for binding to major histocompatibility complex (MHC) class II molecules. Cop 1 can competitively inhibit the response to BP of murine T-cell lines and clones specific to different epitopes of BP and involving different H-2 restrictions (12). We have shown (13) that Cop 1 can inhibit the response to BP of various specific human T-cell lines and clones, and, similarly, BP inhibited T-cell clones specific to Cop 1, irrespective of their DR restriction. The inhibition was demonstrable only in the presence of antigen-presenting cells (APCs), indicating that the site of competition between BP and Cop 1 is probably the MHC class II binding site.

To demonstrate the direct binding of Cop 1 and BP to MHC molecules on APCs and to study the specificity, affinity, and time course of these interactions, we have now employed a fluorometric method shown earlier to be effective for studying peptide binding to class I and class II MHC molecules (14–16). The binding of peptides to MHC molecules on live target APCs has been demonstrated using iodinated peptides (17) or nonradioactive biotinylated peptides and fluorescently labeled avidin (16, 18). Whereas both methods do not require the isolation of the individual MHC alleles, the use of biotinylated peptides to monitor their binding to the MHC is advantageous, since it measures only peptides bound to the surface MHC molecules on intact cells (14, 16, 19). This approach can be utilized (20, 21) to demonstrate the binding not only of short peptides but also of larger molecules. Thus, by using biotinylated poly(L-Tyr-L-Glu)-poly(L-Ala)--poly(L-Lys), a synthetic multichain polypeptide antigen of L-amino acids, and poly(D-Phe-D-Glu)-poly(D-Pro)--poly(D-Lys), a synthetic polymer composed of D-amino acids, this method was exploited to measure equilibrium and kinetics of binding to the MHC and the processing requirements (20, 21).

Herein we report the binding of biotinylated Cop 1 and BP to MHC class II of different haplotypes on live APCs of mouse and human origin. The results revealed that Cop 1 bound very efficiently and apparently indiscriminately to a variety of MHC class II molecules on APCs (degenerate binding). In addition, Cop 1 inhibited the binding of intact BP and its major epitope (residues 84–102 or p84–102).

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Olac (Bicester, England). SJL/J and (BALB/c × SJL/J)F1 mice were obtained from The Jackson Laboratory. Mice were used at the age of 6–12 weeks.

Antigens. BP was isolated from spinal cords of human and mouse white matter as described (22). The synthetic peptide p84–102 of the human BP (DENPVVHFFKNIVTPRTP) was used as a hapten.

Abbreviations: APC, antigen-presenting cell; BP, basic protein; Cop 1, copolymer 1; EAE, experimental allergic encephalomyelitis; EBV, Epstein–Barr virus; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NCC, mononuclear cell; MS, multiple sclerosis; PE, phycoerythrin.

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was synthesized by the Merrifield solid-phase method (23), using the peptide synthesizer model 430A of Applied Biosystems, and purified by HPLC. Cop 1 is a synthetic random copolymer prepared by polymerization of the N-carboxyanhydrides of l-Ala, γ-benzyl-l-Glu, Nε-trifluoroacetyl-l-Lys, and L-Tyr (4). The end product is a mixture of acetate salts of random polypeptides with amino acid composition of Ala (4.1-5.8 residues), Glu (1.4-1.8 residues), Lys (3.2-4.2 residues), Tyr (1 residue). Two Cop 1 batches obtained from Teva Pharmaceutical Industries (Petch Tikva, Israel) were used throughout the studies, batches 29,028 and 03,992, with average molecular weights of 5550 and 8,600, respectively. Poly(l-lysine) (degree of polymerization, 150) was purchased from BioMakor (Kiryat Weizmann, Rehovot, Israel).

Antibodies. Monoclonal anti-DR, anti-DQ, and anti-class I antibodies were obtained from Serotec (Oxford). Cell lines producing the monoclonal antibodies mouse anti-mouse I-A\(^d\) (MK-D6), I-A\(^a\) (7-27-71), and K\(^d\)D\(^d\) (12-2-25) were obtained from American Type Culture Collection. The monoclonal antibodies were kindly provided by Dr. G. Eisenbach (Lea Institute, Israel). Anti-I-A\(^a\) serum was prepared in our laboratory. Phycoerythrin (PE)-conjugated streptavidin was purchased from Jackson Immunoresearch.

Epstein-Barr Virus (EBV)-Transformed B-Cell Lines. These lines were prepared from monoclonal fraction of peripheral blood cells according to Brenner et al. (24) from MS patients (donors: A, DR3 (DRB1*0301); E, DR2, W6 (DRB1*1401, 1501); L, DR7 (DRB1*0701); I, DRw13 (DRB1*1301, 1502); M, DRw11 (DRB1*1104); P, DR4 (DRB1*0403, 0405); R, DR7,w11 (DRB1*0701, 1104); and SS, DR2.7 (DRB1*0701, 1501)). In addition, monoclonal cells (MNCs) were prepared from peripheral blood of MS (SS) and normal (EG) DR2 (DRB1*1501, 1502) donors.

Biotinylation of the Antigens. Biotinylation of Cop 1, BP, and p84–102 was performed at 0°C with biotin-N-hydroxysuccinimide (Sigma) as described (14). Unreacted biotin was removed by dialysis. Analysis of the biotinylated derivatives (25) showed that the average 52% of the Cop 1 molecules, 46% of the BP, and 100% of p84–102 carried the biotin moiety.

Direct Binding of Biotinylated Antigens to APCs. Splenic adherent cells, EBV-transformed B cells, MNCs, and the control F10.9 clone of B16 mouse melanoma cells (26), which was obtained from Lea Eisenbach (1 × 10\(^6\) cells per sample), were incubated with biotinylated Cop 1, BP, or p84–102 in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin at 37°C, followed by incubation with PE-streptavidin at 4°C for 30 min. BP of mouse origin was incubated with mouse APCs, whereas human BP was used with APCs of human origin. After each incubation, the cells were washed three times with the above solution. Thereafter, cells were analyzed by flow cytometry using FACScan (Becton Dickinson). In each analysis, 5000 cells were examined.

For examining the specificity of binding, monoclonal antibodies specific for the respective human or mouse class II or class I molecules or an excess (2- to 10-fold) of unbiotinylated antigen was incubated with the biotinylated peptide for 20 hr. Percent inhibition was calculated as \((1 - \text{percent binding in the presence of inhibitor}/\text{percent binding in the absence of inhibitor}) \times 100\).

RESULTS

Direct Binding of Cop 1, BP, and p84–102 to APCs from Mouse and Human Origin. We used biotinylated Cop 1, BP, and p84–102 to examine their direct binding to MHC molecules on APCs from both mouse and human origin. Binding was measured by means of PE-streptavidin and flow cytometry analysis. Fig. 1 shows histograms of fluorescence intensities of various types of APCs of both mouse and human origin after incubation with the different biotinylated antigens for 1 hr at 37°C. Human APCs (Table 1) were incubated with macrophages obtained from BALB/c (H-2\(^b\), SJL/J (H-2\(^d\)), and their F\(_1\) hybrids (H-2\(^b\)-2\(^d\)). The binding, as expressed by both percentage of labeled cells and the mean fluorescence intensity (MFI), was very similar irrespective of the H-2 haplotype (Fig. 1 A–C). Furthermore, APCs from naive and sensitized mice bound biotinylated Cop 1 to the same extent (87% vs. 83% in naive vs. sensitized mice, respectively).

The double-reciprocal plot analysis. In all cases, no differences were observed when MNCs from a normal individual (90% cell staining) or a MS patient (91% cell staining) or the EBV-transformed B-cell line derived from this MS patient (87% cell staining) served as APCs. Consequently, EBV-transformed B-cell lines derived from eight MS patients of different HLA haplotypes were used to study the restriction of Cop 1 binding. As can be seen (Fig. 1 E–I), Cop 1 bound to all APCs used with the described inhibitors. In all cases, 50 μg (90 μM) of biotinylated Cop 1 stained >85% of all the cells, with a 100-fold increase in the MFI above background.

Biotinylated BP also bound to the various APCs of both mouse (e.g., Fig. 1D) and human (e.g., Fig. 1M) origin. The level of cell staining by 50 μg (27 μM) of BP was >75%, with a 20-fold increase in MFI. p84–102 of the mouse BP bound to all the APCs tested (e.g., Fig. 1N), with the exception of mouse BALB/c (H-2\(^b\)) APCs. However, biotinylated p84–102 (20 μg, 110 μM) stained not more than 60% of the different cells with a 5- to 10-fold increase in MFI. In the presence of equimolar concentrations of Cop 1, BP, and p84–102 (110 μM), the fraction of B cells of the EBV-transformed line from MS donor L that were stained was 94%, 72%, and 58%, with 130-, 40-, and 10-fold increase in MFI, respectively. Thus, Cop 1 exhibited the most efficient and the most extensive binding to various APCs.

Cop 1 bound to the various APCs in a dose-dependent manner. The double-reciprocal plots of the binding data were linear (data not shown), thus allowing the calculation of an apparent \(K_d\) of Cop 1 binding to EBV-B cells and MNCs from MS donor (15 nM). For BP and p84–102, the calculated \(K_d\) values were 6 nM and 22 nM, respectively. Similar \(K_d\) values were obtained for Cop 1 and BP by using APCs of different DR haplotypes.

Comparing Specificity of Biotinylated Cop 1, BP, and p84–102. Cop 1 and BP did not bind to the F10.9 clone of B16 mouse melanoma cells, which do not express MHC class I or II molecules (<2% of bound cells, Fig. 1P). In addition, the binding of these antigens to L-EBV-B cells in the stationary phase, which led to limited expression of HLA determinants, was low (29% compared to 90% of the regular Cop 1 binding, Fig. 1O). On the other hand, poly(l-lysine), either preincubated with the APCs or coincubated with Cop 1 or BP, did not inhibit their binding to human APCs (data not shown). These results suggest that the binding of the biotinylated antigens is to MHC molecules and not to other cell surface molecules and that the interaction is specific and not due to the positive charge of the antigens. The specificity of the binding of biotinylated Cop 1, BP, and p84–102 to the class II DR molecules on APCs of human origin was confirmed by its inhibition with anti-DR, but not with anti-DQ or anti-class I (ABC) antibodies (Table 1). Similarly, the binding to APCs of mouse origin was inhibited by anti-I-A, but not by anti-H-2K or anti-H-2D, antibodies (Table 2). It should be noted that both anti-I-A\(^a\) and anti-I-A\(^d\) completely inhibited the binding. The cause for this observation may be related to the existence of the class II molecules as heterodimers (27) on the (BALB/c × SJL/J)F\(_1\) cells. To ascertain this, biotinylated Cop 1, 1, and p84–102 bind to the same sites as their unlabeled analogs, we incubated the
EBV-transformed B-cell lines with these biotinylated antigens and with an excess of unbiotinylated antigens. Table 1 demonstrates that each unlabeled antigen inhibited considerably the binding of its biotinylated derivative, suggesting that they interact with the same binding sites. We have also shown that biotinylated Cop 1 is presented by the MHC molecules to T cells similarly to the unmodified antigen. Thus, biotinylated Cop 1 stimulated a Cop 1-sensitive mouse T-cell line as efficiently as the unmodified antigen. In addition, Cop 1 and its biotinylated form caused similar levels of specific lysis of pulsed EBV-B cell line by a CD4⁺ T-cell clone derived from the same donor in a class II-restricted manner (data not shown).

Kinetics of Binding of Biotinylated Antigens to APCs. We attempted to analyze the time course of the binding of Cop 1, BP, and p84–102 to the surface of APCs. The binding of Cop 1 was detected immediately (i.e., within 5 min) after its addition to the various APCs, whereas BP binding was first detected after 15 min of incubation. The binding of p84–102 was first detected after 2 hr of incubation (Fig. 2). Each antigen exhibited a time-dependent increase in the MFI during the incubation period (results not shown). The immediate binding of Cop 1, the short incubation required for BP binding, and the observation that the time courses of binding of Cop 1 and BP were similar at 37°C and on ice (data not shown) suggest that these antigens are not processed before binding to the MHC molecules on the APCs.

Mutual Inhibition of Binding to APCs of Cop 1, BP, and p84–102. Since Cop 1, BP, and p84–102 bind to the HLA-DR family.

Table 2. Binding of biotinylated antigens to APCs of (BALB/c × SJL/J)F1 in the presence of various inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% binding of biotinylated antigen</th>
<th>p84-102</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>94 (0)</td>
<td>81 (0)</td>
</tr>
<tr>
<td>Anti-I-A</td>
<td>0 (100)</td>
<td>0 (100)</td>
</tr>
<tr>
<td>Anti-I-1-2</td>
<td>5 (95)</td>
<td>7 (91)</td>
</tr>
<tr>
<td>Anti-I-1-3</td>
<td>93 (1)</td>
<td>80 (1)</td>
</tr>
<tr>
<td>Anti-I-1-4</td>
<td>86 (9)</td>
<td>76 (6)</td>
</tr>
<tr>
<td>Anti-I-1-5</td>
<td>84 (10)</td>
<td>79 (2)</td>
</tr>
<tr>
<td>Anti-I-1-6</td>
<td>94 (0)</td>
<td>93 (1)</td>
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NT, not tested. Biotinylated Cop 1 (75 μM), human BP (81 μM), or p84–102 (110 μM) was incubated with the L-EBV-B cells in the presence of various inhibitors for 20 hr. Background binding in the absence of biotinylated antigens was 10%. Results are expressed as mean values of percent binding from two to five experiments. SD values were <10%. Percent inhibition is shown in parentheses. Boldface type indicates the homologous inhibition studies.
molecules on APCs, we decided to examine whether these antigens compete for MHC binding. Thus, we incubated L-EBV-B cells with biotinylated Cop 1, BP, or p84–102 and unlabeled antigens for 20 hr at 37°C (Fig. 2B and Table 1). The results showed that the binding of BP was inhibited by Cop 1 and p84–102; however, Cop 1 inhibited the binding of BP at lower molar concentrations than p84–102 (Fig. 3A). The binding of p84–102 was more efficiently inhibited by Cop 1 and BP than by p84–102 itself (Fig. 3B). The binding of Cop 1 was inhibited by Cop 1, BP, and p84–102 at similar efficiencies. However, the level of inhibition by BP was somewhat lower than by Cop 1 or p84–102 (Fig. 3C).

We next examined whether the blocking of HLA-DR determinants on the surface of APCs would lead to the inhibition of Cop 1 binding. Preincubation of L-EBV-B cells with either BP or p84–102 for 3 hr at 37°C resulted in inhibition of 78% and 58%, respectively, of the immediate biotinylated Cop 1 binding. However, with long incubation time (at 37°C), the binding of Cop 1 increased and the binding of BP and p84–102 declined (63% decrease within 1 hr), suggesting that Cop 1 displaced these antigens from the HLA class II binding site. In contrast, preincubation of the APCs with biotinylated Cop 1 caused no change in its binding even after a further 20 hr of incubation with BP and p84–102; namely, Cop 1 could not be displaced from the HLA class II binding site.

**DISCUSSION**

The main findings of the present study are as follows. (i) Cop 1 binds to several APCs of mouse and human origin, irrespective of their I-A/DR restrictions (degenerate/promiscuous binding, Fig. 1). (ii) This binding is specific to MHC class II (I-A/DR) molecules (Tables 1 and 2). (iii) This binding is characterized by its high efficiency (Fig. 1) and fast rates (Fig. 2). Furthermore, Cop 1 competes with BP and its major epitope p84–102 for MHC binding (Fig. 3) and can even displace bound BP. These observations provide a mechanism for the reported inhibition of murine and human T-cell responses to BP by Cop 1 (12, 13).

The role of BP in disease induction in the EAE system is well established, and BP is also implicated as a candidate autoantigen in the pathogenesis of MS (3). In EAE, there is usually one major encephalitogenic epitope that is recognized by BP-sensitized cells, expressing species and strain specificity (1). In MS, several laboratories demonstrated responses to multiple epitopes of BP (28–31), although T cells from different MS patients were also shown to interact preferentially with the distinct BP epitope, p84–102 (32). These immunodominant peptides of BP were previously shown to bind to purified MHC class II molecules of both murine and human origin (33, 34). We have now observed the binding of p84–102 to MHC molecules on living APCs by using a biotinylated analogue. The results show that this peptide bound to human cells of all the DR haplotypes tested, whereas in the murine system binding was to the I-Aβ but not to the I-Aβ haplotype. The DR specificity of p84–102 binding has been shown using purified DR molecules (34), and similar allele-specific binding to murine I-A molecules was demonstrated for its smaller segment, namely, residues 89–101 (35).

In addition to peptide binding, we have now observed the binding of native BP and Cop 1 to all the APCs tested, independent of their MHC haplotype, and both exhibited a very fast binding. The binding of Cop 1 was immediate and BP required 15 min of incubation (Fig. 2). It was, however, specific to MHC class II haplotypes and was not due to a mere electrostatic interaction or binding to other cell surface molecules, as indicated by the following observations: (i) BP and Cop 1 did not bind to cells with low or no expression of MHC class II molecules. (ii) Poly(L-lysine) did not affect the binding. (iii) Prior BP and peptide binding inhibited the immediate binding of Cop 1. (iv) Binding was specifically inhibited by anti-class II antibodies. This inhibition was quantitatively complete in the murine system using splenic adherent cells and anti-I-A antibodies, whereas for human EBV-transformed B-cell lines, the inhibition by anti-HLA-DR antibody was only 44–52%, similar to the observation of Busch et al. (38). This difference in the levels of inhibition between mouse and human APCs may be due to the higher levels of MHC class II molecules on the surface of EBV-transformed B-cell lines (36).

The fast rate of Cop 1 and BP binding suggests that these two antigens do not undergo processing before binding to MHC molecules. Indeed, preliminary results showed similar binding rates even when incubation was performed on ice. Furthermore, the binding was apparently unaffected by various protease inhibitors (data not shown). Our results thus support previous observations that class II molecules, in contrast to class I, can bind determinants of multichain polypeptide (21) or intact protein (37). These findings may be explained by the distinct models of peptide binding to class
II molecule, as seen in its three-dimensional structure (27). Whereas both ends of the binding groove are open in the latter (27), in MHC class I molecules, both ends are closed and involved in peptide binding (38).

The apparent $K_d$ values calculated for the binding of p84–102, BP, and Cop 1 to the different DR molecules were very similar (1–10 nM). However, for Cop 1, a mixture of peptides of different sizes that may carry multiple epitopes per molecule, the calculated $K_d$ value is only an average and peptides with higher and lower affinities and avidities may coexist. In all cases, the percentage of Cop 1-bound cells and the MFI were very high compared to BP and p84–102. These findings, in addition to the observation that all three reactants (namely BP, p84–102, and Cop 1) competed with each other for binding to the MHC but only Cop 1 could displace the others when already bound, are indicative of the high avidity that characterizes Cop 1 binding.

Of interest is the observation that Cop 1 and BP bind to APCS of mouse and human origin, with different H-2 and HLA haplotypes. For BP this finding is in line with the work of Vialli et al. (34) who demonstrated that synthetic peptides spanning the entire BP molecule were all bound to at least one DR molecule and that at least three BP peptides exhibited degenerate binding to three or more DR molecules. The ability of Cop 1 to interact with a large number of MHC molecules may be due to the fact that Cop 1, which is a mixture of polypeptides, contains different sequences that are able to bind to different MHC determinants. An alternative explanation is that Cop 1 contains a promiscuous class II binding motif(s). The phenomenon of promiscuity (binding to multiple DR alleles) has been described using endogenous and exogenously added peptides (39–42). A tentative DR motif was recently proposed to consist of an aromatic or hydrophobic residue in position 1, followed by Ser, Thr, Ala, Val, Ile, Leu, Pro, or Cys in position 6 and by a hydrophobic residue in position 9 (41). Cop 1, composed of Tyr, Ala, Lys, and Glu, therefore, may contain several such motif(s), providing multiple interaction sites.

Of particular importance is the finding that among the HLA-DR alleles shown to bind Cop 1 are also the DRB1*1501 haplotype (Fig. 1F), tightly linked to MS susceptibility in Caucasian MS patients (43, 44), and the DR4 haplotype (Fig. 1J), which has been reported to associate with MS in Sardinian (45) and Arab (46) populations of MS patients. The promiscuous binding of Cop 1 to the various MHC molecules may explain the unrestricted in vitro inhibition by Cop 1 of various murine and human BP-specific clones (12, 13) and its in vivo EAE-suppressive activity in various animal species (4–8). Since several lines of evidence have suggested that BP is a pathogenic factor in MS (3), the ability of Cop 1 to compete with BP and with the human immunodominant p84–102 for binding to various DR molecules may be a significant element in the beneficial effect of Cop 1 in MS patients.

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