Antigen-specific Deletion of Cloned T Cells Using Peptide-Toxin Conjugate Complexed with Purified Class II Major Histocompatibility Complex Antigen*

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In a previous report, we showed that cloned T cells incubated with soluble, cognate major histocompatibility complex (MHC) II-peptide complex internalized the peptide moiety of the complex. Here, we report antigen-specific deletion of cloned T cells by treatment with soluble, cognate MHC II-(peptide-toxin) complexes. Toxin (doxorubicin or mycophenolic acid) was attached to synthetic AcMBP(1-14)Ala4 peptide, an analog of the natural acetylated NH2-terminal segment, AcMBP(1-14), of rat myelin basic protein (MBP). IAα-restricted, AcMBP(1-14)-specific AJ1.2 and 4R3.9 cloned murine T cells were killed by IAα-(AcMBP(1-14)Ala4-toxin). No killing resulted from incubating AJ1.2 and 4R3.9 cells with irrelevant MHC II-(peptide-toxin) or treating IEβ-restricted, pigeon cytochrome c-specific A.E7 cloned murine T cells with IAα-(AcMBP(1-14)Ala4-toxin). T cell receptor-mediated T cell uptake of the peptide-toxin moiety of relevant complex was blocked by anti-T cell receptor-α/β antibody and by excess toxin-free complex. LD50 determinations revealed that cognate MHC II-(peptide-toxin) killed T cells much more effectively than did peptide-toxin conjugate alone. Finally, T cell uptake of peptide-toxin and intracellular release of toxin occurred after incubation with relevant MHC II-(peptide-toxin) containing radiolabeled toxin. These findings, which provide the first evidence that cloned T cells can be deleted in vitro, may have significant clinical relevance for antigen-specific therapy of autoimmune or other T cell-mediated diseases.

The trimolecular model of antigen presentation posits that antigenic stimulation of a T cell by an autologous APC requires noncovalent association between TCRs on the responding T cell surface and APC membrane-associated complexes consisting of the stimulatory antigenic peptide combined with molecules of the restricting MHC class II antigen (1-5). A number of accessory molecules on both the APC and T cell also interact during the presentation of antigen (6, 7). Although this model of antigen presentation is well accepted, the fate of the components of the TCR/MHC II-peptide complex following antigen stimulation and disengagement of the APC and T cell is unknown. Recent results from our laboratory indicated that interaction of purified relevant MHC II-peptide complexes with TCRs on cloned T cells in vitro leads to T cell internalization of only the peptide moiety of the complex (8). Based on this novel observation, the present study was undertaken to determine if cloned T cells could be deleted in vitro, using purified complexes of cognate MHC II-(peptide-toxin) containing an intracellularly cleavable peptide-toxin bond.

The association of T cells and particular MHC class II molecules with several autoimmune diseases has been well established (9-12) and provides a rational basis for development of antigen-specific therapies. Toxin conjugates of acetylcholine receptor using the plant toxin, ricin, have been employed for selective in vitro elimination of specific lymphocytes involved in triggering and progression of experimental autoimmune myasthenia gravis (EAMG) in animals (13, 14). Recently, in vitro treatment of EAMG with acetylcholine receptor conjugated with another plant toxin, gelsemium, also has been reported (15). Moreover, antigen-specific and MHC II-restricted T cell killing in vitro requiring 125I-labeled antigen and a nonspecific soluble factor of adherent cells has also been reported (16).

In contrast to large toxin molecules like ricin and gelsemium, the smaller doxorubicin and mycophenolic acid molecules act intracellularly by intercalating into DNA (17) or inhibiting DNA synthesis (18), respectively. Doxorubicin, a glycoside antibiotic (19) and analog of daunorubicin which differs from the latter by a smaller doxorubicin and mycophenolic acid molecules act intracellularly by intercalating into DNA (17) or inhibiting DNA synthesis (18), respectively. Doxorubicin, a glycoside antibiotic (19) and analog of daunorubicin which differs from the latter by a single hydroxylation site (20), is an important anticancer agent (21). DNA has been considered to be the primary target for the cytotoxic action of this drug on susceptible cells (22). Mycophenolic acid is a novel immunosuppressive agent (23) distinct from cyclosporin A and FK506 in activity (24). In this study, doxorubicin or mycophenolic acid was coupled to the COOH terminus of MBP peptide analogs via an intracellularly cleavable disulfide or ester linkage, respectively. The peptide-doxorubicin or peptide-mycophenolic acid conjugate was combined with purified MHC II molecules, and the resulting MHC II-(peptide-toxin) complexes were used for antigen-specific deletion of cloned T cells in vitro.

MATERIALS AND METHODS

Cells, Antibodies, and Chemicals—Murine T cell clones, AJ1.2 and 4R3.9, which respond to IAα-AcMBP(1-14) complexes, were obtained from the Laboratory of Dr. Patricia Jones, Stanford University, Stanford, CA. Cloned A.E7 T cells restricted for IEβ in association with a peptide segment of pigeon cytochrome c (pCyt C(81-104)) were obtained from the laboratory of Dr. R. H. Schwartz. The hybridoma cell lines,
10-2.16, which produces monoclonal antibodies (IgG2b) against murine IA^, and MK-D6, which produces monoclonal antibody (IgG2a) against murine cell membrane-associated myelin basic protein (containing 32-36 residues). The structure of the intermediate product was confirmed by mass spectroscopy. Lysophospholipids, pure N^4(4-(2-pyridyl)diethybutyrimidazolide)-doxorubicin (2.5 mg) was dissolved in 0.5 ml of degassed water in a 15-ml polypropylene centrifuge tube. To this solution, 1 mg of 0.5 ml of degassed water of HPLC-grade methanol (solvent B) was added, and 0.5 ml of methanol (solvent A) was added with vortex mixing. After 6 h at room temperature in the dark, the disulfide-linked peptide-doxorubicin conjugate was purified by gel filtration. The solution was centrifuged at 4°C for 24 h at 10,000 g, and the supernatant was concentrated by lyophilization. The purified conjugate was dissolved in water and stored at 4°C until used. For radiolabeling, ethyl acetate was removed from the dried residue, 1 ml of degassed water was added, and the mixture was centrifuged at 10,000 g for 5 min. Then, 1 ml of 10% acetic acid was added to a final concentration of 0.01% tri- fluoroacetic acid, and the mixture was washed with heptane, dried, and stored at 4°C. For radiolabeling, ethyl acetate was removed from the dried residue, 1 ml of degassed water was added, and the mixture was centrifuged at 10,000 g for 5 min. Then, 1 ml of 10% acetic acid was added to a final concentration of 0.01% tri- fluoroacetic acid, and the mixture was washed with heptane, dried, and stored at 4°C. The purified MBP peptide-mycophenolic acid conjugate was purified by mass spectroscopy.

Synthesis and Purification of Peptide-Mycophenolic Acid Conjugate—Mycophenolic acid (32 mg, 100 μmol) and 12 mg (50 μmol) of bromoacetic acid N-hydroxysuccinimide ester were dissolved in 200 μl of dry MeSO containing 9 μl of disopropylthethylamine. After standing at room temperature overnight, the solution was mixed to a final concentration of 5 μg ml^(-1) of mycophenolic acid attached to the Ne-amino group of the position 13 amino residue of MBP peptide analogs. Finally, the structure and homogeneity of the purified MBP peptide-mycophenolic acid conjugate were confirmed by mass spectroscopy.

Preparation of IA-(Peptide-Toxin) Complexes—Affinity-purified IA^ or IA^ (100 μg) was incubated with a 50-fold molar excess (167 μg) of AcMBP(1-14)Ala^ or AcMBP(1-14)Ala^ mycophenolic acid conjugate at 37°C for 48 h in a total volume of 1 ml. The excess unbound peptide-toxin conjugate was removed by dialyzing the complex three times against 3 liters of RPMI 1640 medium at 4°C. The absence of free peptide-toxin in the final preparation was confirmed by TLC analysis as described elsewhere (27, 28). For control experiments, an equivalent amount of peptide-toxin was incubated and dialyzed under identical conditions in the absence of IA^ or IA^ molecules.

Assay for Proliferation of T Cells Exposed to IA-(Peptide-Toxin) Complexes—The IA-(peptide-toxin) complexes were mixed with 1 × 10^6 cloned AJ2, 4R3.9, or A.E7 T cells in a total volume of 1 ml in a 15-ml polypropylene tube and incubated at 37°C for 24 h. Unbound IA-(peptide-toxin) complexes were removed by washing the cells three times with 15 ml of T cell medium (RPMI 1640 containing 10% fetal bovine serum, 100 units ml^-1 of recombinant interleukin-2, and 1 mg ml^-1 of 500 μmol CIL) and reprecipitated with ethyl acetate.
Analysis of Trichloroacetic Acid Extracts of T Cells Incubated with IA*- (Peptide-14-Mercaptobutyrimidooxorubicin) Complex—An equivalent amount of radiolabeled IA*-AcMBP(1-14)Ala*Cys14,180-Mercaptobutyrimidooxorubicin (19,000 cpm) or IA*- (AcMBP(1-14)Ala3Ala3Cys14,180-Mercaptobutyrimidooxorubicin) complex (19,000 cpm) was incubated with 10⁴ 4R3.9 T cells in a total volume of 0.5 ml of RPMI 1640 medium at 37 °C for 5 h. Following incubation, the cells were washed three times with 10 ml of PBS, suspended in 300 μl of PBS, and counted in a γ counter. To this suspension, 500 μl of acetonitrile containing 10 μl of trichloroacetic acid were added. The samples were mixed well and centrifuged at 100,000 × g. The supernatant was recovered, the pellet was re-extracted with 1 ml of 80% aqueous acetonitrile containing 0.1% trichloroacetic acid, and the combined supernatants were vacuum-dried and dissolved in alcoholic acetic acid:water in the ratio of 10:1:3. The developed TLC plate was subjected to autoradiography, and the extent of intracellular disulfide bond cleavage was estimated by the percent of total counts recovered from the plate at the Rf value (0.9) for the cleavage product, 4-mercaptobutyrimidooxorubicin.

RESULTS AND DISCUSSION

Murine T cell clones, AJ1.2 and 4R3.9, were selected for their recognition of IA*-complexes containing the NH2-terminal nonapeptide segment, AcMBP(1-9), of rat MBP which is acetylated at the NH2 terminus (29). These cell lines also proliferate when stimulated with APC-associated IA* complexes with AcMBP(1-14) or the MBP peptide analog, AcMBP(1-14)Ala8, but not with the analog, AcMBP(1-14)Ala3Ala8, although both peptide analogs were found to bind equally well to IA*. The natural sequence of AcMBP(1-14) contains a lysine residue at position 4. Replacement of this lysine residue by alanine results in an increased binding of the peptide analog to IA* (30) which may explain the heteroclitic response observed for in vitro stimulation of cloned T cells with this analog (31).

Two toxins molecules, doxorubicin and mycophenolic acid, were covalently linked to the synthetic peptide analog, AcMBP(1-14)Ala3Cys14. For covalent attachment of these peptides to doxorubicin, the tyrosine residue at position 14 was substituted with cysteine during peptide synthesis. The three-step synthesis of peptide-doxorubicin conjugate containing an intracellularly cleavable disulfide linkage between the peptide and doxorubicin moieties is shown in Fig. 1. In the first step, a free sulphydryl group was generated at the amino group of doxorubicin hydrochloride by reaction with 2-iminothiolane. The resulting sulphydryl group was converted in the second step to a dithiopyridyl group, using 2,2'-dithiodipyridine. The intermediate product, N-(4-(2-pyridyl)dithiobutyrimido)doxorubicin, was purified by reverse-phase HPLC. Reductive cleavage of the HPLC-purified mixed disulfide intermediate with excess 2-mercaptoethanol gave the expected products upon HPLC analysis. In the final step of synthesis, a sulffide exchange reaction resulted in replacement of the thio pyridyl group with the sulphydryl moiety of the COOH-terminal cysteine residue of AcMBP(1-14)Ala3Cys14. Reductive cleavage of

Fig. 1. Synthesis and structure of peptide-doxorubicin conjugate. The structure of doxorubicin and peptide-doxorubicin conjugate are presented in the reaction scheme. The synthetic intermediates are 4-mercaptobutyrimido doxorubicin and 4-mercaptobutyrimido doxorubicin. The arrows below the final peptide-doxorubicin conjugate structure indicates the disulfide bond at which intracellular cleavage occurs.

Fig. 2. Synthesis and structure of peptide-mycophenolic acid conjugates. The synthetic route and structure of mycophenolic acid and peptide-mycophenolic acid conjugate are shown. The intermediate synthetic product is mycophenolyl oxacetic acid N-hydroxysuccinimide ester. The arrows in the final peptide-mycophenolic acid conjugate structure indicates the ester bond that undergoes intracellular hydrolysis.
Complexes.

Antibody. The killing of T cells was substantially reduced in the further demonstrated in three different experiments the data

The resulting peptide-mycophenolic acid conjugate was purified as described under “Materials and Methods.” Cloned T cells were incubated at 37 °C, and proliferation was measured by the M1T assay described under “Materials and Methods.”

TABLE I

<table>
<thead>
<tr>
<th>Toxic</th>
<th>Toxin alone</th>
<th>Peptide-toxin conjugate</th>
<th>IA*-(peptide-toxin) complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.5 μM</td>
<td>5</td>
<td>≤0.3</td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>ND*</td>
<td>50</td>
<td>0.3</td>
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</table>

* Not done because of insolubility of mycophenolic acid in neutral aqueous solutions.

The specificity of T cell killing by MHC II-(peptide-toxin) was further demonstrated in three different experiments the data of which are presented in Fig. 4. To demonstrate that the killing of T cells is mediated by the T cell receptor, 4R3.9 cloned T cells were incubated with IA*-(AcMBP(1-14)Ala4Cys14-doxorubicin) complex in the presence of H57-595 anti-TCR-α/β monoclonal antibody. The killing of T cells was substantially reduced in the presence of the anti-TCR-α/β antibody (Fig. 4, lane 3). In a control experiment, an equivalent amount of isotype-matched hamster IgG2b antibody did not show any inhibition of T cell killing (Fig. 4, lane 4), demonstrating that the binding of the relevant MHC II-(peptide-toxin) complex and the uptake of the peptide-toxin moiety were TCR-mediated. Similarly, in a competition assay, T cell killing was inhibited by incubating 4R3.9 T cells with IA*-(AcMBP(1-14)Ala4Cys4-doxorubicin) complex in the presence of a 3-fold molar excess of IA*-(AcMBP(1-14)Ala4) complex. The TCR specificity was further demonstrated by incubating 4R3.9 cloned T cells with IA*-(AcMBP(1-14)Ala4Cys4-doxorubicin) complex in the presence of anti-TCR monoclonal antibody. Specificity was further demonstrated by incubating A.E7 cloned T cells (specific for pCyt C81-104) with IA*-(AcMBP(1-14)Ala4Cys4-doxorubicin) or IA*-(AcMBP(1-14)Ala4-doxorubicin) or MBP(1-14)Ala4) are described under “Materials and Methods.” Lane 1, untreated 4R3.9 T cells; lane 2, 4R3.9 T cells plus IA*-(AcMBP(1-14)Ala4Cys4-doxorubicin) complex; lane 3, 4R3.9 T cells plus IA*-(AcMBP(1-14)Ala4-doxorubicin) complex; lane 4, 4R3.9 T cells plus IA*-(AcMBP(1-14)Ala4-mycophenolic acid) complex. Each data point represents an average of eight determinations.

The mixed disulfide bond in HPLC-purified AcMBP(1-14)-Ala4Cys4-doxorubicin conjugate with excess dithiothreitol produced the expected mercaptopeptide and mercaptobutyrimido-doxorubicin as shown by reverse-phase HPLC analysis on a C18 column. Doxorubicin was similarly coupled via a mixed disulfide linkage to the nonstimulatory MBP peptide analog, AcMBP(1-14)Ala4Ala4Cys14, and to the ovalbumin peptide analog, OVA324-336/Cys135.

The toxin conjugates of the MBP and OVA peptide analogs were prepared with affinity-purified IA* and IA4, respectively, and the resulting purified complexes were incubated with the T cell clones. Only T cells incubated with cognate IA*-(peptide-doxorubicin) or IA4-(peptide-mycophenolic acid) complexes were killed (Fig. 3). Untreated T cells and T cells incubated with either purified IA* alone, IA* complexed with unmodified AcMBP(1-14)Ala4, or IA*-(OVA324-336/Cys135-doxorubicin) complex were unaffected.

The specificity of T cell killing by MHC II-(peptide-toxin) was further demonstrated in three different experiments the data of which are presented in Fig. 4. To demonstrate that the killing of T cells is mediated by the T cell receptor, 4R3.9 cloned T cells were incubated with IA*-(AcMBP(1-14)Ala4Cys14-doxorubicin) complex in the presence of H57-595 anti-TCR-α/β monoclonal antibody. The killing of T cells was substantially reduced in the presence of anti-TCR-α/β antibody (Fig. 4, lane 3). In a control experiment, an equivalent amount of isotype-matched hamster IgG2b antibody did not show any inhibition of T cell killing (Fig. 4, lane 4), demonstrating that the binding of the relevant MHC II-(peptide-toxin) complex and the uptake of the peptide-toxin moiety were TCR-mediated. Similarly, in a competition assay, T cell killing was inhibited by incubating 4R3.9 T cells with IA*-(AcMBP(1-14)Ala4Cys4-doxorubicin) complex in the presence of a 3-fold molar excess of IA*-(AcMBP(1-14)Ala4-doxorubicin) complex in the presence of 100 μg of anti-TCR-αβ antibody; lane 4, 4R3.9 T cells plus IA*-(AcMBP(1-14)Ala4-doxorubicin) complex; lane 5, 4R3.9 T cells plus IA*-(AcMBP(1-14)-Ala4Cys4-doxorubicin) complex; lane 6, 4R3.9 T cells plus IA*-(AcMBP(1-14)Ala4-mycophenolic acid) complex; lane 7, A.E7 cells plus IA*-(AcMBP(1-14)Ala4Cys4-doxorubicin) complex; lane 8, A.E7 cells plus IA*-(AcMBP(1-14)Ala4-mycophenolic acid) complex. Each data point represents an average of eight determinations.

Finally, to demonstrate that irrelevant T cells are not killed by similar concentrations of these MHC II-(peptide-toxin) complexes, A.E7
Cloned T cells (32) restricted by IMPF with pCyt C(81-104) were incubated with an equivalent amount of IA4-(ACMBP(1-14)AlaCys14-doxorubicin) complex. As shown in Fig. 4, lanes 6, 7, and 8, no significant killing of AE7 cells was observed under identical experimental conditions.

The concentration of doxorubicin, ACMBP(1-14)AlaCys14-doxorubicin conjugate, or ACMBP(1-14)AlaCys14-mycophenolic acid conjugate required for 50% killing (LD50) of the T cell clones was determined in a dose-response study. These were compared with an estimation of the corresponding LD50 for T cell killing with the cognate IA4-(ACMBP(1-14)AlaCys14-doxorubicin) or IA4-(ACMBP(1-14)AlaCys14-mycophenolic acid) complex (see Fig. 3 for data representation). As shown in Table I, 50% killing of AJ1.2 T cells was observed after 24 h of incubation with relevant IA4-(peptide-toxin) complex at a concentration less than or equal to 0.6 μM. As expected, incubation of AJ1.2 T cells with uncomplicated peptide-doxorubicin or peptide-mycophenolic acid conjugate resulted in T cell killing, but achieving 50% cell killing under identical incubation conditions required 16 times (5 μM) or 160 times (50 μM) the concentration of the respective IA4-(peptide-doxorubicin) or IA4-(peptide-mycophenolic acid) complex. The concentration of free, lipophilic doxorubicin required for 50% cell killing was also analyzed and found to be 0.5 μM. The LD50 of free mycophenolic acid could not be accurately determined as a result of the insolubility of mycophenolic acid in aqueous medium.

The internalization by T cells of peptide-toxin molecules from ternary TCR-(MHC II)-(peptide-toxin) complex was demonstrated using radiolabeled doxorubicin. ACMBP(1-14)AlaCys14-doxorubicin or ACMBP(1-14)AlaAlaAlaCys14-peptide-doxorubicin conjugate was radiolabeled at the ketone carboxyl group of the doxorubicin moiety by reaction with [125I]-labeled Bolton-Hunter diazide reagent, as prepared as described under "Materials and Methods." Cloned 4R3.9 T cells were incubated with IA4-(ACMBP(1-14)AlaCys14-doxorubicin) or IA4-(ACMBP(1-14)AlaAlaAlaCys14-doxorubicin) complexes at 37 °C for 5 h. The treated T cells were washed and lysed with acetonitrile/trichloroacetic acid in order to extract peptides. The amount of radioactivity recovered in the trichloroacetic acid extract indicated that 44% of the total radioactivity applied in the form of relevant IA4-(ACMBP(1-14)AlaCys14-doxorubicin) complex was internalized by the T cells (Table II). In a control experiment in which cells were exposed to irrelevant IA4-(ACMBP(1-14)AlaAlaAlaAlaCys14-doxorubicin) complex, the cell-associated radioactivity, as determined by trichloroacetic acid extraction, was only 5.9% of the total applied radioactivity.

Disulfide and ester bonds in prodrugs are generally known to be cleaved after uptake into target cells, and intracellular release of peptides from cell-internalized substances has been described (33, 34). To determine if free 4-mercaptobutyrylimido-doxorubicin was released by intracellular disulfide bond cleavage of the internalized peptide-doxorubicin moiety from T cell-bound IA4-(peptide-12S)-I-doxorubicin complex, the trichloroacetic acid extracts were subjected to TLC. The bands at RF values corresponding to peptide,125I-I-doxorubicin and free 125I-4-mercaptobutyrylimido-doxorubicin were excised and counted. As shown in Table II, 92.5% of the internalized peptide-doxorubicin conjugate was cleaved intracellularly at the disulfide bond joining toxin with peptide.

The killing of T and B lymphocytes by toxin conjugated to intact antigen molecule has been reported (13, 14), and the deletion of mature T cells by this method can be used to treat autoimmune diseases in animal models (15). The deletion of autoreactive T cells by soluble MHC II-(peptide-toxin) conjugates in which the toxin is linked to a T cell epitope of the antigen by an intracellularly cleavable linker may provide further specificity. The data may have relevance for development of therapies aimed at deletion of antigen-specific T helper cells in autoimmune and other T helper cell-mediated diseases.

Acknowledgments—We thank Dr. Patricia Jones for providing the AJ1.2 and 4R3.9 cloned murine T cell lines, Dr. R. H. Schwartz for providing the AE7 cloned murine T cell line, Dario Slavazza for synthesis and purification of peptides, David Pasmore for purification of MHC class II molecules, and Teresa Kendrick and Alicia Mizerek-Erhardt for culturing and maintaining the T cell clones.

REFERENCES


TABLE II

<table>
<thead>
<tr>
<th>Complex</th>
<th>T cell-associated radioactivity</th>
<th>Total applied radioactivity internalized by T cell</th>
<th>Cleavage of internalized peptide-toxin moiety of complex</th>
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<tbody>
<tr>
<td>IA4-(ACMBP(1-14)AlaCys14-doxorubicin)</td>
<td>8,467</td>
<td>44.2</td>
<td>92.5</td>
</tr>
<tr>
<td>IA4-(ACMBP(1-14)AlaAlaCys14-doxorubicin)</td>
<td>775</td>
<td>5.9</td>
<td>0</td>
</tr>
</tbody>
</table>
T Cell Deletion by Soluble MHC II-(Peptide-Toxir) Complex


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