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). IAk-restricted, AcMBP(1-14)-specific AJ1.2 and 4R3.9 cloned murine T cells were killed by IAk-(AcMBP(1-14)Ala4-toxin). No killing resulted from incubating AJ1.2 and 4R3.9 cells with irrelevant MHC II-(peptide-toxin) or treating IEK-restricted, pigeon cytochrome c-specific A.E7 cloned murine T cells with IAk-(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 with soluble, cognate MHC II-(peptide-toxin) complexes, 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 vivo treatment of EAMG with acetylcholine receptor conjugated with another plant toxin, gelsenin, 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 gelsenin, the smaller doxorubicin and mycophenolic acid molecules act intracellularly by intercalating into DNA (17) or inhibiting DNA synthesis (18). 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 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 IAk-AcMBP(1-14) complexes, were obtained from the Laboratory of Dr. Patricia Jones, Stanford University, Stanford, CA. Cloned A.E7 T cells restricted for IEk 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,
 murine lidine, 2,2-dithiodipyridine (Aldrithiol), and diisopropylethylamine.

AJ1.2 and 4R3.9 cloned T cells (2.5\&r 48 h of stimulation, the cells were split 1:19 into complete RPMI

IAk, droxysuccinimide ester, and dimethylsulfoxide were purchased from Pierce Chemical Co. and mycophenolic acid, bromoacetic acid N-hy-

peptide and its analog, AcMBP(1-14)Ala4, were cultured in RF"I1640

mice and pigeon cytochrome c (pCyt C) at a final concentration of

in 1

Doxorubicin hydrochloride (20

ylsulfonyl fluoride. Each fraction was neutralized with 1

lysed cells was detergent-extracted in a buffer containing 10 m~ '&is-

Ac-ASAARASQRHGSKY-NH2 and

analog, OVA(32&336)Cy~~~~, representing the sequence,

and resuspended in medium containing 10% fetal bovine serum.

beads by the standard cyanogen bromide coupling method (27, 28).

were removed by subjecting cloned T cells to

19% methazin density gradient centrifugation, followed by two

Cells were stimulated

charges. The loaded column was washed with 10 bed

or A20.1.11 cells (26), respectively. Affinity columns were pre-

Affinity columns were prepared by immobilizing 10-2.16 monoclonal antibody (specific for IA\& and IA\& or MK-D6 monoclonal antibody (specific for IA\& on Sepharose 4B beads by the standard cyagen bromide coupling method (27, 28).

Prior to use in experiments, the cells were subjected twice to 19% metrazin density gradient centrifugation, washed in RPMI 1640, and reuspended in medium containing 10% fetal bovine serum.

Preparation of Marine IA\& or IA\& were purified from Nonidet P-40 extracts of a membrane fraction obtained from CH27 cells (25) or A20.1.11 cells (26), respectively. Affinity columns were prepared by immobilizing 10-2.16 monoclonal antibody (specific for IA\& and IA\& or MK-D6 monoclonal antibody (specific for IA\& on Sepharose 4B beads by the standard cyanogen bromide coupling method (27, 28).

Briefly, a high speed (10,000 \times g) membranes were collected by centrifugation and resuspended in medium containing 1% n-yl-p-5glucop~an~ide

Concentrated using an Amicon Centriprep-10 concentrator. Affinity-pu-

ties-doxorubicin conjugates were confirmed by mass spectroscopic anal-

Synthesis and Purification of Peptide-Mycophenolic Acid Con-

jigate—Mycophenolic acid (32 mg, 100 \mu mol) and 12 mg (50 \mu mol) of bromoacetic acid N-hydroxysuccinimide ester were dissolved in 200 \mu l of dry Me2SO containing 9 \mu l of diisopropylthiophosphorylthio. After standing the solution for 15 min, 2-mercaptoethanol was added, followed by

The excess 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 proliferation assay, washed T cells were plated in triplicate in a 96-well microtiter plate at a cell density of 105 cells/200 \mu l well in each of 4 5-unit interleukin 2 (IL-2) units in 200 \mu l RPMI 1640 containing 1% n-yl-p-glucop~an~ide, and 10 units of penicillin/streptomycin. The cells were examined at this stage for percent viability by staining with acridine orange/ethidium bromide (.0003%0.001%) and viewing under an epifluorescence microscope.

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Analysis of Trichloroacetic Acid Extracts of T Cells Incubated with IA*- (Peptide-1,4-Doxorubicin) Complex—An equivalent amount of radio-labeled IA*-AcMBP(1-14)Ala4Cys14,37-1,4-doxorubicin or IA*-AcMBP(1-14)Ala4Ala4Cys14,37-1-doxorubicin complex (10,000 cpm) was incubated with $10^9$ 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, 300 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 μl of 80% aqueous acetone and 1 μl of acetonitrile. Samples of the trichloroacetic acid extract were then applied onto a silica gel TLC plate and developed with the upper phase of butanol: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-mercaptobutyrimido doxorubicin.

RESULTS AND DISCUSSION

Murine T cell clones, AJ1.2 and 4R3.9, were selected for their recognition of IAk 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 IAk complexed with AcMBP(1-14) or the MBP peptide analog, AcMBP(1-14)Ala4, but not with the analog, AcMBP(1-14)Ala3Ala4, although both peptide analogs were found to bind equally well to IAk. 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 IAk (30) which may explain the heterodetic response observed for in vitro stimulation of cloned T cells with this analog (31).

Two toxin molecules, doxorubicin and mycophenolic acid, were covalently linked to the synthetic peptide analog, AcMBP(1-14)Ala4Cys14. 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)dithiobutryrimido)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 disulfide exchange reaction resulted in replacement of the thiopyridyl group with the sulphydryl moiety of the COOH-terminal cysteine residue of AcMBP(1-14)Ala4Cys14. Reductive cleavage of Mycophenolic acid

![Diagram of peptide-doxorubicin conjugate](image1)

**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 N(4-(2-pyridyl)dithiobutyrirnido)doxorubicin. The arrow below the final peptide-doxorubicin conjugate structure indicates the disulfide bond at which intracellular cleavage occurs.

![Diagram of peptide-mycophenolate conjugate](image2)

**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 mycophenoloyxacetic acid N-hydroxysuccinimide ester. The arrow in the final peptide-mycophenolic acid conjugate structure indicates the ester bond that undergoes intracellular hydrolysis.
the mixed disulfide bond in HPLC-purified AcMBP(1-14)-
Ala4Cys14-doxorubicin conjugate with excess dithiothreitol pro-
duced the expected mercaptopeptide and 4-mercaptopurinyl-
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, OVA(324-336)Cys136.

Peptide-mycophenolic acid conjugate containing an intracel-
ularly cleavable ester bond between peptide and toxin was
prepared in a two-step synthesis (Fig. 2). In the first step, an
active N-hydroxysuccinimide ester derivative of mycophenolic
acid was generated by reaction with bromoacetic acid N-hy-
droxy succinimide ester. In the second step, the active ester
product, mycophenoloyxacetic acid N-hydroxysuccinimide es-

ter, was then linked by amide bond formation to the Ne-amino

group of the position 13 lysine residue of AcMBP(1-14)Ala4.

The resulting peptide-mycophenolic acid conjugate was puri-

fied by reverse-phase HPLC, and the structure was confirmed

by mass spectroscopy.

The toxin conjugates of the MBP and OVA peptide anal-
gos were complexed with affinity-purified IA4 and IA4, re-
spectively, and the resulting purified complexes were incubated with the T
cell clones. Only T cells incubated with cognate IA4-(peptide-
doxorubicin) or IA4-(peptide-mycophenolic acid) complexes
were killed (Fig. 3). Untreated T cells and T cells incubated with
either purified IA4 alone, IA4 complexed with unmodified
AcMBP(1-14)Ala4, or IA4-(OVA(324-336)Cys136-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 IA4-(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 IgG 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 the 4R3.9 T cells with IA4-(AcMBP(1-14)Ala4Cys14-doxorubicin) complex in the presence of IA4-(AcMBP(1-14)Ala4Cys14-doxorubicin) complex containing no conjugated toxin (Fig. 4, lane 5). Finally, to dem-
strate that irrelevant T cells are not killed by similar concen-
trations of these MHC II-(peptide-toxin) complexes, A,E7

FIG. 3. Treatment of cloned T cells with IA-(peptide-toxin)
complexes. Affinity-purified IA4 or IA4 (100 μg) was incubated with
167 μg (50-fold molar excess) of peptide-toxin conjugate, and the result-
ant IA-(peptide-toxin) complexes were purified as described under "Mate-
rials and Methods." Cloned T cells were incubated at 37 °C and
proliferation was measured by the MTT assay described under "Mate-
rials and Methods." Panel A represents specific killing of A,E7 cells by
IA4-(AcMBP(1-14)Ala4Cys14-doxorubicin) complexes. Lane 1, untreated
cells; lane 2, plus IA4; lane 3, plus relevant IA4-(AcMBP(1-14)Ala4Cys14-doxorubicin) complex; lane 4, plus irrelevant IA4-(AcMBP(1-14)Ala4Cys14-doxorubicin) complex; lane 5, plus irrelevant IA4-(OVA(324-336)Cys136-doxorubicin) complex. Panel B represents specific killing of 4R3.9 T cells by IA4-(AcMBP(1-14)Ala4Cys14-doxorubicin) complexes. Lane 1, untreated cells; lane 2, plus IA4-(AcMBP(1-14)Ala4 complex; lane 3, plus IA4-(AcMBP(1-14)Ala4Cys14-doxorubicin) complex; lane 4, cells plus irrelevant IA4-(OVA(324-336)Cys136-doxorubicin) complex. Panel C represents specific killing of A,E7 T cells with IA4-(AcMBP(1-14)Ala4-mycophenolic acid) complexes. Lane 1, untreated
cells; lane 2, cells plus IA4-(AcMBP(1-14)Ala4) complex; and lane 3, cells
plus IA4-(AcMBP(1-14)Ala4-mycophenolic acid) complex. Each data
point represents an average of six determinations.

Table I

| Toxin          | Toxin alone (μM) | Peptide-toxin conjugate (μM) | IC50 (μM) |
|----------------|------------------|-----------------------------|-----------|
| Doxorubicin    | 0.5 μM           | 5                           | <0.3      |
| Mycophenolic acid | ND*             | 50                          | 0.3       |

* Not done because of insolubility of mycophenolic acid in neutral aqueous solutions.
cloned T cells (32) restricted by IEP<sup>k</sup> complex with pCyt(C181-104) were incubated with an equivalent amount of IA<sup>k</sup>-AcMBP(1-14)Ala<sup>4</sup>Cys<sup>14</sup>-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)Ala<sup>4</sup>Cys<sup>14</sup>-doxorubicin conjugate, or AcMBP(1-14)Ala<sup>4</sup>-mycophenolic acid conjugate required for 50% killing (LD<sub>50</sub>) of the T cell clones was determined in a dose-response study. These were compared with an estimation of the corresponding LD<sub>50</sub> for T cell killing with the cognate IA<sup>k</sup>-AcMBP(1-14)Ala<sup>4</sup>Cys<sup>14</sup>-doxorubicin) or IA<sup>k</sup>-AcMBP(1-14)Ala<sup>4</sup>-mycophenolic acid (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 IA<sup>k</sup>-peptide-toxin complex at a concentration less than or equal to 0.3 μm. As expected, incubation of AJ1.2 T cells with uncomplexed peptide-doxorubicin or peptide-mycothephenolic 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 IA<sup>k</sup>-peptide-doxorubicin) or IA<sup>k</sup>-peptide-mycothephenolic acid complex. The concentration of free, lipophilic doxorubicin required for 50% cell killing was also analyzed and found to be 0.5 μm. The LD<sub>50</sub> of free mycothephenolic acid could not be accurately determined as a result of the insolubility of mycothephenolic 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)Ala<sup>4</sup>Cys<sup>14</sup>-doxorubicin or AcMBP(1-14)Ala<sup>4</sup>Ala<sup>6</sup>Cys<sup>14</sup>-doxorubicin conjugate was radiolabeled at the ketone carbonyl group of the doxorubicin moiety by reaction with 126<sup>l</sup>-labeled Bolton-Hunter hydrazide reagent, prepared as described under "Materials and Methods." Clone 4R3.9 T cells were incubated with IA<sup>k</sup>-AcMBP(1-14)Ala<sup>4</sup>Cys<sup>14</sup>,125<sup>I</sup>-doxorubicin) or IA<sup>k</sup>-AcMBP(1-14)Ala<sup>4</sup>Ala<sup>6</sup>Cys<sup>14</sup>,125<sup>I</sup>-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 IA<sup>k</sup>-AcMBP(1-14)Ala<sup>4</sup>Cys<sup>14</sup>,125<sup>I</sup>-doxorubicin) complex was internalized by the T cells (Table II). In a control experiment in which cells were exposed to irrelevant IA<sup>k</sup>-AcMBP(1-14)Ala<sup>4</sup>Ala<sup>6</sup>Cys<sup>14</sup>,125<sup>I</sup>-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 peptide-toxin molecules has been described (33, 34). To determine if free 4-mercaptopbutyryl doxorubicin was released by intracellular disulfide bond cleavage of the internalized peptide-doxorubicin moiety from T cell-bound IA<sup>k</sup>-peptide,125<sup>I</sup>-doxorubicin) complex, the trichloroacetic acid extracts were subjected to TLC. The bands at R<sub>f</sub> values corresponding to peptide,12<sup>I</sup>-doxorubicin and free 126<sup>I</sup>-4-mercaptopbutyryl 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 cells with toxin conjugates 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.

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