Role of Extracellular Adenosine Triphosphate in the Cytotoxic T-Lymphocyte-Mediated Lysis of Antigen Presenting Cells

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The lysis of antigen presenting cells (APCs) by cytotoxic T lymphocytes (CTLs) may be one mechanism whereby an immune response is downregulated by Staphylococcus superantigens. Disappearance of monocytes/macrophages from staphylococcal enterotoxin A (SEA)-activated peripheral blood mononuclear cell (PBMC) cultures, but not from control PBMC cultures was seen by flow cytometry. Recently, adenine triphosphate (ATP) has been described as an effector molecule in CTL-mediated lysis of some murine tumor target cells. We have also shown that ATP caused the lysis of human macrophages, and that treatment of cells with interferon-gamma (IFN-gamma) rendered macrophages significantly more sensitive to ATP than untreated cells. To show that this purine nucleotide may play a role in modulating the immune system, we generated human CTLs that were stimulated with SEA, and used them as effector cells against SEA-pulsed autologous macrophages. CTLs were found to specifically lyse SEA-pulsed macrophages, while control, unpulsed, macrophages were unaffected. The addition of hexokinase, an enzyme that hydrolyzes ATP, significantly abrogated the killing of SEA-pulsed cells during the assay. In examining the mechanism of cytotoxicity, electron microscopy showed that macrophages incubated with both ATP and CTLs underwent necrosis, rather than apoptosis. From these results, it is suggested that ATP is released from CTLs during antigen presentation, and that IFN-gamma-activated macrophages, which are inherently more sensitive to this mediator, are readily lysed and therefore removed from circulation, thus downregulating an immune response.

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CTL/APC interactions, without the constraints of antigen specificity.

MATERIALS AND METHODS

Materials. ATP (#A3284, tissue culture grade) and hexokinase (#H4502, sulfate-free) were obtained from Sigma Chemical Co (St Louis, MO). KN-62 (soulsolubilized in dimethylsulfoxide [DMSO]) was obtained from Calbiochem (LaJolla, CA). Human rIFNγ was kindly provided by Genentech Corp (South San Francisco, CA) and human rGM-CSF was a generous gift from Immunex (Seattle, WA). Human rIL-2 was a generous gift from Hoffman LaRoche (Nutley, NJ). SEA was purchased from Toxin Technology (Sarasota, Fl.). All media and reagents contained less than 0.1 ng/mL of endotoxin as determined by the Limulus amebocyte lysate assay (MA Biologics, Walkersville, MD).

Preparation of human leukocytes. PBMC were isolated from leukocyte buffy coats, obtained from normal volunteers at the Southwest Florida Blood Bank, as previously described. PBMC were suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 5% heat-inactivated human AB serum (Flow Laboratories, McLean, VA), 2 mmol/L L-glutamine, 10 U/mL penicillin, 100 μg/mL streptomycin, 5 mmol/L HEPES buffer, and 5 × 10−5 M 2-mercaptoethanol, and will subsequently be referred to as complete medium.

Preparation of monocytes. PBMC were incubated on gelatin-coated tissue-culture flasks for 1 hour at 37°C to allow adherence of monocytes. Nonadherent cells were recovered by washing of the flasks with warm medium and the adherent cells were cultured with fresh medium in the presence of 1,000 U/mL of IFNγ or GM-CSF for 4 days. Macrophages activated with IFNγ are termed MIFN, and cells incubated with GM-CSF are referred to as MCFS. Culture-derived macrophages were then recovered by incubating the monolayer with cold phosphate-buffered saline (PBS), followed by vigorous pipetting. Viability of cells was greater than 90% by trypan blue exclusion, and typically less than 5% of cells were contaminating lymphocytes, as determined by flow cytometry using anti-CD2 and anti-CD19 monoclonal antibodies (MoAbs).

Percoll density gradient centrifugation. Plastic nonadherent cells were further depleted of adherent cells and B cells by incubation on nylon wool columns for 30 minutes at 37°C. The cells passing through the columns were then placed on a six-step discontinuous density gradient, as previously described, for separation of LGL and T cells. T-cell–enriched populations were located and recovered from fractions 4 through 7. For generation of SEA-activated CTLs, T lymphocytes were incubated at a concentration of 2 × 106 cells/mL with 10 ng/mL of SEA and 2 U/mL interleukin 2 (IL-2) for 4 days in 25-cm2 tissue-culture flasks in the presence of 1% monocytes, washed twice in medium, and used as effector cells in a cytotoxicity assay (see below).

For characterization of CTL phenotype, rabbit-antimouse IgG-coated immunobeads were used essentially as described. Briefly, 3-day SEA-stimulated CTLs were incubated at 4°C for 30 minutes with anti-CD6 or anti-CD4 antibodies. Cells were then washed with PBS supplemented with 1% FCS and then incubated with washed immunobeads at 4°C for 30 minutes, with agitation. CD8- or CD4+ T cells were then recovered by magnetic adherence, washed and then cultured overnight in fresh medium to allow capping of the antibody-magnetic bead complex to occur, which were then removed by magnet. All antibodies were purchased from AMAC, Inc (Westbrook, ME) and immunobeads were from Advanced Magnetics (Cambridge, MA). The phenotype of purified lymphocytes was verified by flow cytometry, and there was typically less than 5% of contaminating populations present.

Fluorescence-activated cell sorting analysis of SEA-activated PBMC. PBMC were cultured at a concentration of 2 × 106 cells/mL in medium alone or in the presence of 10 ng/mL of SEA and 2 U/mL IL-2 for 4 days in Teflon (Fisher Scientific, Pittsburgh, PA) beakers, to allow for complete recovery of adherent cells. Cells were then stained with LeuM3-fluorescein isothiocyanate (FITC) (anti-CD14; Becton Dickinson, Mountain view, CA) or with FITC-conjugated isotype control antibody (IgG2a) and then analyzed on the FACScan flow cytometer (Becton Dickinson). Only live cells were analyzed as determined by exclusion of propidium iodide–stained cells.

Measurement of CTL- and ATP-mediated cytotoxicity. A 5-hour 51Cr-release assay was used to measure the effect of CTLs or ATP on cytokine-treated macrophages, and was performed essentially as previously described. Macrophages were labeled with sodium [35Cr] chromate (Amersham Corp, Arlington Heights, IL) for 1 hour in 0.5 mL of medium and washed once with PBS. SEA, at a concentration of 100 ng/mL, in 2 mL of PBS, was added to indicated target cells, and then incubated for 30 minutes at 37°C. Cells were washed twice with PBS to remove unbound SEA and then added to serial dilutions of CTLs or ATP in microtiter wells at 5 × 103 cells/well in a final volume of 0.2 mL in each well. When using ATPase solutions, the indicated amount of enzyme, or its respective carrier salt, was added directly to assay wells just before the addition of target cells. Control wells in which no effector cells were added were also prepared to verify that enzyme solutions had no direct effect on the viability of targets. All determinations were done in triplicate, and the SEM of all assays was calculated and was typically < 5% of the mean or less. Student’s t-tests were performed to identify significant differences between treatments. Lytic units were calculated and were defined as the number of effector cells per 1 × 106 required to lyse 20% of the target cells.

Electron microscopy. For the morphologic studies, MIFN were incubated for 15 minutes in medium alone or in the presence of 1.25 mmol/L ATP for 5 to 15 minutes. Additionally, cocultures of purified SEA-activated CTLs and SEA-pulsed MIFN were incubated at an effector/target (E/T) ratio of 4/1 for 2 hours. Cells were then fixed with 1% glutaraldehyde in 0.1 mol/L phosphate buffer, postfixed with 1% osmium tetroxide, and embedded in resin according to routine techniques. For electron microscopy, thin sections were mounted on nickel grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope.

RESULTS

Depletion of macrophages from SEA-activated PBMC cultures. The central focus of these studies was to determine whether ATP was involved in homeostasis of the immune response, as a mediator of CTL lysis of APCs. Therefore, preliminarily, it was important to determine if macrophages were eliminated from cultures of activated PBMC, as a means of downregulating an immune response. Figure 1 depicts the flow cytometric analysis of both control (Fig 1, A and B) and SEA-activated (Fig 1, C and D) PBMC cultures that were stained with anti-CD14-FITC antibodies (the scattergram is included to define the monocyte/macrophage-gated population, seen in Fig 1, B and D). The bar above the histograms indicates positively stained cells, as determined by FITC-labeled isotype control antibodies. As shown, PBMC that were cultured in medium alone contained 18% monocytes/macrophages (Fig 1A), as identified by anti-CD14 staining of the entire PBMC population. In the monocyte/macrophage-gated population, 80% of cells were posi-
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PBMC

PBMC + SEA/IL-2

A

B

C

D

Fig 1. Elimination of macrophages from SEA/IL-2–activated PBMC. PBMC were cultured for 4 days in medium alone or in the presence of SEA and IL-2, recovered, and stained with anti-CD14-FITC MoAbs. A and C are ungated PBMC populations and B and D are gated to include the monocytes/macrophages, as outlined in the scattergrams. The abscissa is expressed as log₁₀ fluorescence units.

Comparison of CTL- and ATP-mediated lysis of SEA-pulsed macrophages. Our previous studies using extracellular ATP to lyse human macrophages had shown increased sensitivity of MIFN to ATP-mediated cell death, as compared with MCSF, or macrophages incubated for 4 days in medium alone. Untreated macrophages were not routinely included in these assays because no significant differences in their responses to ATP were noted between MCSF and control cells in preliminary studies, and cytokine-treated macrophages were typically more viable than cells incubated in medium alone; ≈90% viability seen for the former cells, and 75% for the latter.

To determine whether ATP played a role as a soluble mediator in the CTL-mediated lysis of APCs, it was essential to fully characterize the lysis of SEA-pulsed macrophages by both agents. For this experiment, MIFN and MCSF were pulsed with SEA (or incubated in PBS alone as a control), and used as target cells in a 5-hour ⁵¹Cr-release assay (Fig 2). For these studies, although sensitivity to lysis was noted at 10 ng/mL SEA (data not shown), a concentration of 100 ng/mL of SEA was used because preliminary studies had determined this to be the optimal concentration. As shown in Fig 2A, only macrophages that had been pulsed with SEA served as targets for SEA-activated CTLs, with little lysis noted for unpulsed cells. In experiment 1, lysis of MCSF was only 9 ± 1 lytic units in the absence of SEA, and increased to 77 ± 2 lytic units when incubated with this superantigen. Similarly, lysis of MIFN alone was only 17 ± 1 lytic units, which was elevated to 313 ± 12 lytic units when cells were pulsed with SEA. Also of importance was the evidence that MIFN were significantly more sensitive to CTL killing than MCSF.

To ascertain whether pulsing of macrophages with SEA caused cells to become more sensitive to ATP, this purine nucleotide was used as the effector molecule in Fig 2B. Unlike lysis by CTLs, there was no significant difference in
the ATP-mediated lysis of macrophages, whether or not they were coated with SEA. Taken together, Fig 2 indicates that MIFN are more easily lysed by both ATP and CTLs. More importantly, lysis of macrophages does not occur unless antigen is presented to CTLs, which presumably triggers the release of soluble mediators.

Characterization of the phenotype of CTLs that lyses APCs. Because both CD4\(^+\) and CD8\(^+\) CTLs are reported to be effective lytic units.

In A, SEA-sensitized CTLs were used as effectors against SEA-pulsed MIFN. Numbers are the mean \(\pm\) SEM of percent specific lysis of triplicate determinations.

Because MIFN are found to be significantly more sensitive to lysis by extracellular ATP than MCSF,\(^{29}\) and because CTLs reportedly secrete ATP upon ligation of the TCR,\(^{16,17,19}\) the possibility that ATP released by CTL-mediated lysis of antigen-presenting MIFN was explored. To directly determine whether ATP was the lytic agent, 2 U/mL of hexokinase, which hydrolyzes ATP, was added to the cytotoxicity assays. In addition, the equivalent amount of sodium citrate that was found in the hexokinase preparation was added to the controls to determine whether CTLs were directly inhibited by the solvent. As shown in Fig 4, the addition of hexokinase inhibited the lysis of SEA-pulsed MIFN, but not SEA-pulsed RPMI 8866 cells. This human lymphoblastoid B-cell line was chosen because it was HLA-DR\(^-\), and could present antigen, but was resistant to lysis by extracellular ATP.
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To further define the mechanism by which CTLs kill SEA-pulsed macrophages, we preincubated 31Cr-labeled target cells with the macromolecular synthesis inhibitor actinomycin D, which was shown in a previous report to inhibit the killer cell-induced apoptosis of a murine tumor target cell, YAC-1.33 As a positive control in our assay system, we included the ATP-resistant target cell, RPMI-8866. In preliminary experiments, lysis of RPMI-8866 by SEA-induced CTLs occurred when these target cells were preincubated with SEA, similar to those results seen with macrophages (see below). Because these cells are not sensitive to ATP up to concentrations of 5 mmol/L, their lysis by CTLs must be mediated via a mechanism distinct from ATP-related events. As shown in Fig 7, the lysis of SEA-pulsed MIFN by either CTLs or ATP was not affected by their preincubation with actinomycin D. Alternatively, lysis of SEA-pulsed RPMI 8866 by CTLs was 33% ± 2% in medium, as compared with 17% ± 1% in the presence of actinomycin D, an inhibition of 49%. Additionally, there was no lysis of RPMI-8866 cells by ATP, either in the presence or absence of actinomycin D. Furthermore, incubation of RPMI-8866 cells with IFNγ did not generate sensitivity of those cells to ATP (data not shown). These results indicate that the mechanism of lysis of RPMI-8866 by CTLs differs from that of MIFN, and displays characteristics of killing via apoptosis. On the other hand, lysis of MIFN, whether by CTLs or ATP, does not involve macromolecular synthesis.

Effect of a calmodulin inhibitor on CTL- and ATP-mediated lysis of SEA-pulsed target cells. In examining the mechanism whereby ATP causes the lysis of macrophages, our preliminary studies had identified a role for calmodulin-linked systems. In those experiments, calmodulin antagonists were found to significantly inhibit the killing of macrophages by ATP.34 If ATP mediated the CTL-induced lysis of SEA-pulsed MIFN, then calmodulin inhibitors should be effective in our assays. For these experiments, KN-62, an inhibitor specific for multifunctional Ca2+/calmodulin-dependent protein kinase II (CaM kinase II),35 was added to cytotoxicity assays and assessed for its ability to block CTL- and ATP-mediated lysis of SEA-presenting MIFN and RPMI-8866. As shown in Fig 8, KN-62 almost completely blocked the

Fig 4. Effect of hexokinase on CTLs killing of SEA-pulsed APCs. SEA-pulsed MIFN and SEA-pulsed RPMI 8866 tumor cells were added to C89 CTLs at the indicated effector/target ratios in medium alone or in the presence of hexokinase (ATPase) at a concentration of 2 U/mL. Numbers are the means of triplicate determinations, and represent one of three experiments performed with similar results. (A), MIFN; (△), MIFN + ATPase; (●), 8866; (○), 8866 + ATPase.
Fig 5. Effect of ATP on ultrastructure of MIFN. The macrophages that were incubated in medium alone exhibited preserved microvilli, intact nuclei, and intact cytoplasmic organelles (A). MIFN that were exposed to 1.25 mmol/L of ATP for 5 to 15 minutes exhibited intracellular edema, disappearance of microvilli, and destruction of cytoplasmic organelles: early necrosis (B) and advanced necrosis (C) are shown. As a latest event, there is rupture of the cytoplasmic membrane (D) and destruction of mitochondrial cristae (E). The nuclear changes characteristic of apoptosis were not seen at any phase of ATP-induced cell death.
Fig 6. CTL-induced necrosis of MIFN. SEA-activated CTLs and SEA-pulsed MIFN were cocultured for 2 hours at a ratio of 4/1. The APCs, surrounded by CTLs, exhibit characteristics of necrosis, with intracellular edema and disorganization of cytoplasmic organelles. The inset depicts an enlargement of a necrotic macrophage, showing destruction of mitochondrial cristae.

ATP-mediated lysis of MIFN, from a control level of 49% ± 2% specific lysis to 3% ± 1% lysis with 5 μg/mL of inhibitor. KN-62 was found to also block the CTL-mediated lysis of MIFN, with lysis of 42% ± 1%, 33% ± 1%, 25% ± 2%, and 16% ± 1% in the presence of 0, 5, 10, and 20 μg/mL of KN-62, respectively. However, KN-62 had no significant effect on the CTL-mediated lysis of RPMI-8866. It should be noted that DMSO, the vehicle for KN-62, had no effect on either the ATP- or CTL-mediated killing of either target cell at the concentrations used in these assays. Additionally, KN-62 was not directly toxic for CTLs, MIFN, or RPMI-8866, as determined by trypan blue exclusion or by the spontaneous release of 51Cr from target cells at the concentrations used in these assays.

Fig 7. Effect of actinomycin D on lysis of SEA-presenting APCs by CTLs and ATP. SEA-pulsed MIFN and SEA-pulsed RPMI 8866 tumor cells were preincubated in medium alone (medium) or in the presence of 5 μg/mL actinomycin D (act D) for 30 minutes at ambient temperature, washed, and then used as target cells in a 5-hour 51Cr release assay. The effectors, as indicated, were either CTLs at an effector/target ratio of 20/1, or ATP at a concentration of 1.25 mmol/L. Numbers are means ± SEM of triplicate determinations and asterisks indicate significant differences (P < .05) of treatments compared with medium controls. (●), medium; (●●), ACT D.

DISCUSSION

In the present study, the CTL-induced lysis of SEA-pulsed APCs was used as a model system to explore the role of ATP, theorized to be released from stimulated CTLs, in the immune system. As expected, MIFN were far more susceptible to CTL-mediated lysis than MCSF. This is likely due to their increased MHC class II expression, which occurs after activation of macrophages with IFN-γ and, therefore, which would render these cells to be more efficient APCs.33 Concurrently, MIFN are inherently more easily lysed by the putative soluble mediator, ATP, which led us to the hypothesis that ATP was at least one mechanism by which CTLs could kill APCs, leading to downregulation of the immune response because of the elimination of highly active APCs. The mechanisms whereby CTLs lyse target cells is currently of intense interest. They can cause cell death via both apoptosis and necrosis, which appears to depend on the type of target cell and the mediator involved.33 Because perforin is thought to lyse cells via the formation of pores in the target cell membrane, its mode of action is likely caused by necrosis.7,9,30 In other studies, the ligation of the Fas antigen on target surfaces by CTLs results in apoptosis of the target cells, which led researchers to the hypothesis that perforin and the presence of the Fas ligand were the major mechanisms of CTL-mediated killing of target cells.14,15 However, we have previously shown that human macrophages were
Thus, ATP was explored as another mechanism of killing possibility that this soluble mediator could cause either DNA fragmentation assays were unsuccessful, despite overnight incubation of target cells (data not shown). Nonetheless, the mechanism is similarly ineffective in killing human APCs, an event that was inhibited by the presence of calmidazolium, a calmodulin inhibitor.38 Because of its ability to cause DNA fragmentation in certain susceptible cells, ATP has been implicated as a mediator in apoptosis, or programmed cell death.39,40 The responses of murine thymocytes to ATP are characteristic of apoptosis and include condensation of chromatin, blebbing of the cell surface, and breakdown of the nucleus and are accompanied by DNA fragmentation.24,45

In another study, Richardson et al31 reported that cloned CD4+ T cells killed autologous tetanus toxoid-presenting macrophages via apoptosis. Also in contrast with our results, the activation of macrophages with IFNγ had no effect on their sensitivity to killing by cloned T cells. Although it is not clear why these studies differ, it is possible that the source of T cells, freshly activated CD8+ CTLs in the present study versus cloned CD4+ T cells in the previous report, may indicate different pathways of APC elimination exist within different cell types. It is also possible that the antigen specificity of the CTLs may trigger alternative pathways of killing. Although SEA used in our experiments is a superantigen, we have similar results using Candida albicans as the antigen source (data not shown). Nonetheless, the manifestation of several mechanisms of lysis of APCs within T lymphocyte subpopulations emphasize the importance of the elimination of APCs from an immune response.

Although SEA appears to specifically bind to HLA-DR+ cells,22–24 the interaction between CD8+ cells and their targets is mediated by ligation of MHC class I. Although it seems contradictory that CD8+ CTLs more efficiently killed APCs, the lysis of SEA-presenting target cells by both CD4+ and CD8+ cells is reported.30,32 and it appears that the presence of HLA class II on the target cell is essential for efficient killing.46–48 In other studies, CTL lysis of class II-negative APCs can occur, suggesting that other ligands may be involved.49 In the present model, it appears that both CD4+ and CD8+ cells lyse SEA-pulsed MIFN, though the latter CTL is the more efficient.

The present studies show the possibility that more than one mechanism of killing of MIFN exists because lysis of SEA-pulsed target cells is not completely blocked by ATPases or calmodulin antagonists. However, it may be difficult to efficiently introduce these agents into the target cell/CTL junction, and further data are required to fully discriminate the signalling pathways involved in killing APCs.
The inhibition of CTL-mediated lysis of macrophages by KN-62 suggest a role for calmodulin as part of the signal cascade toward necrosis. However, it is possible that the CTLs are directly affected by this molecule, rather than the target cells. Nonetheless, these experiments do suggest that ATP is one part of lytic arsenal of CTLs, perhaps specifically designed for homeostasis of the immune response.

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