Evidence that macrophages are programmed to die after activating autologous, cloned, antigen-specific, CD4+ T cells*

The bone marrow produces large numbers of monocytes daily, but the mechanisms balancing production and elimination are unknown. In this report we demonstrate that macrophages (MΦ) undergo apoptosis after activating autologous CD4+ cells. Since apoptosis is a genetically programmed response, these results argue that MΦ death can be part of a normal immune response. This event may have relevance to monocyte/MΦ homeostasis, as well as immune response regulation and host defenses to intracellular organisms.

1 Introduction

Numerous reports document that cultured CD4+ T cells can kill Ia+ APC in an antigen- and MHC-specific fashion. Most of these experiments use transformed lines of different cell lineages as APC [1-9]. Relatively few have used MΦ as APC. However, these reports suggest that MΦ also die when cultured with autologous or syngeneic antigen-specific CD4+ T cell lines and antigen [2, 9-11].

Authors have argued that the killing of antigen-presenting MΦ is important, and could contribute to the elimination of cells infected with intracellular organisms [10, 11], downregulate immune responses [1, 2], and contribute to autoimmune processes [12, 13]. Macrophage death following CD4+ T cell activation also suggests one answer to questions regarding the fate of the approximately 1.2 x 10^10 monocytes produced each day by human bone marrow [14, 15]. However, the mechanisms leading to MΦ death are unknown, and, since cultured T cells are required, whether the MΦ killing has in vivo significance or is an in vitro phenomenon has been questioned [1, 16].

In this report we have asked whether the mechanism of MΦ killing is apoptosis or necrosis. Necrosis is a relatively nonspecific mechanism for cell death, caused in part by the release of molecules such as perforin and serine esterases from activated CTL [3, 17-19]. In contrast, apoptosis is a programmed response of the target cell. Evidence that MΦ die by apoptosis would argue that the MΦ was programmed to "self-destruct" on command from the T cell. The existence of an apoptotic pathway for MΦ elimination would imply that a "suicide" response is sufficiently important to the host to be genetically programmed into the repertoire of MΦ functions, and would thus argue for an in vivo significance of T cell-mediated MΦ killing.

To characterize mechanisms of MΦ killing, cloned, antigen-specific human CD4+ T cells were cultured with autologous MΦ and antigen, and the MΦ examined for changes characteristic of apoptosis or necrosis. The results demonstrate that MΦ undergo apoptosis after activating these cells.

2 Materials and methods

2.1 T cell and MΦ isolation

Peripheral blood mononuclear cells were isolated as described [20, 21]. T cells were separated by rosetting with aminophytothioauronium bromide (Sigma, St. Louis, MO)-treated sheep erythrocytes [22]. Macrophages were purified by adherence to plastic petri dishes and eluted with lidocaine [21]. Macrophage yield was usually 10% of the starting number of PBMC.

2.2 Cloned T cell lines

The cloned T cell lines used in this report have been previously described. All are CD4+, proliferate in response to autologous APC plus tetanus toxoid (TT), do not respond to autologous APC alone, and were derived from an HLA-DR 4,4 donor [13, 20, 23]. The cell lines were maintained in 16-mm wells (Costar, Cambridge, MA) containing 2 ml RPMI/20% AB serum/IL-2 (supernatant from the MLA-144 T cell line) [24] by adding IL-2 containing media two to three times per week and 10^6 irradiated (2000 rad) autologous PBMC and TT diluted 1:50 once a week.

2.3 Proliferation and cytotoxicity assays and MHC typing

Proliferation assays using cloned T cells were performed as described [20, 21]. Proliferation assays using freshly isolated T cells were performed by mixing 10^5 T cells with 5 x 10^4 irradiated autologous MΦ plus TT in 200 μl RPMI supplemented with 10% fetal calf serum, using round-bottom microtiter plates. Proliferation was assessed 6 days later by measuring [3H]dThd (New England Nuclear, Boston, MA) incorporation [20, 21]. Cytotoxicity assays were performed as previously described [13], using a modification of the method of Ottenhoff [10].
otherwise noted, MΦ were directly adhered from PBMC, and were assumed to represent 10% of the PBMC [10]. All proliferation and killing assays were performed in quadruplicate, and results are presented as the mean ± SEM of the quadruplicate determinations.

MHC typing was performed by the University of Michigan Tissue Typing Laboratory as described [22].

### 2.4 Immunofluorescence and flow cytometric analysis

Cells stained with anti-CD14 (OKM1) or anti-CD2 (OKT11, Ortho Pharmaceuticals, Raritan, NJ) and goat anti-mouse IgG-FITC (Sigma) were analyzed on an EPICS C flow cytometer [20]. Indirect immunofluorescence was performed as described previously [25].

### 2.5 Antibodies, cytokines and chemicals

Antisera specific for HLA-B loci were purchased from Pel-Freeze (Brown Deer, WI). An Ab to nonpolymorphic portions of class II determinants (9.49) and control ascites were generous gifts from Dr. Robert Todd III [26], and an Ab to class I determinants was purchased from Cappel Laboratories (Cochranville, PA). Anti-lymphocyte function antigen-1 (LFA-1; anti-CD11a) and anti-ICAM-1 were obtained from the American Type Culture collection and used as culture supernatant. Anti-LFA-3 was kindly contributed by Dr. Timothy Springer [27]. TNF-β was purchased from Biosource (Camarillo, CA), IFN-γ from Boehringer Mannheim (Indianapolis, IN), and neutralizing antibodies to human IL-4 from R&D Systems (Minneapolis, MN). Cycloheximide was purchased from Sigma.

### 2.6 Serine esterase assays

The procedure used was a modification of that described by Pasternak and Eisen [28]. Briefly, cells were washed three times in cold (4°C) PBS containing 100 μg/ml BSA (Sigma). The cells were then lysed by suspending at 10 × 10⁵ - 20 × 10⁹/ml in PBS supplemented with 0.5% Nonidet P-40. The mixture was incubated for 20–30 min at 4°C with frequent vortexing, then the nuclei were sedimented at 15,000 × g for 15 min at 4°C. Aliquots of cellular extract (10 μl) were then added to 200 μl of the substrate reaction mixture [2 × 10⁻⁴ M N-benzoyloxy carbonyl-lysine thiobenzyl ester, Calbiochem, La Jolla, CA, and 2.2 × 10⁻⁴ M 5,5'-dithiobis-(2-nitrobenzoic acid), Sigma], in 0.1 M Tris/HCl, pH 8.0) in flat-bottom microtiter wells. The mixture was incubated at room temperature for 30 min and then absorbance measured at 405 nm using a Titertek Multiskan (Flow Laboratories, McLean, VA). All determinations were performed in triplicate.

### 2.7 Northern analyses

RNA was isolated as previously described [13]. Samples of 10–20 μg (quantitated by UV spectrometry and ethidium bromide staining) were electrophoresed through 1% agarose, 3% formaldehyde gels and capillary-blotted onto nylon membranes (Gene Screen Plus, New England Nuclear). The membranes were hybridized with a [32P]-cDNA of the entire human perforin coding sequence, kindly donated by Dr. Ko Okumura [29].

### 2.8 Electron microscopy

At least 10⁶ cells were fixed in 1 ml of 4% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 for 1 h. The samples were washed twice in 0.1 M cacodylate, pH 7.3, then dehydrated in a series of graded alcohols (30–100%) and finally in propylene oxide. The cell pellets were then infiltrated with increasing concentrations of Epon resin: propylene oxide then embedded in pure Epon at 65°C overnight. Plastic sections (1 μm thick) were cut on an American Optical Ultracut ultramicrotome and stained with toluidine blue. Areas of interest were identified microscopically. Thin sections were obtained and stained with uranyl acetate and lead citrate and examined on a Philips 400T transmission electron microscope. Enumeration of the percentage of dead cells was done by photographing the entire thick section of the cell pellet at 40 ×, making an enlarged photographic montage of each sample and, with light microscopy (100 ×) verification, identifying those cells which contained pyknotic nuclei. At least 500 cells were counted for each determination.

### 2.9 Isolation and electrophoresis of fragmented DNA

Cells were washed three times and then lysed with 10 mM Tris, 1 mM EDTA, pH 7.5 (TE) containing 0.2% Triton X-100 (TTE). The chromatin was removed by centrifugation at 15,000 for 10 min, and fragmented DNA was precipitated from the cytosol with ethanol and sodium acetate [23] followed by centrifugation at 15,000 × g 15 min. The pellet was suspended in 10 vol TE buffer containing 150 mM NaCl and 1 mg/ml proteinase K, incubated at 37°C for 4 h, extracted with an equal volume of salt-saturated phenol, then again with chloroform and isoamyl alcohol (24:1). The aqueous phase was precipitated with ethanol/acetate, resuspended in TE, and DNA fragments separated by electrophoresis through 1.2% agarose. The gel was then stained with ethidium bromide and visualized under UV light.

### 3 Results

#### 3.1 Characterization of autologous MΦ killing by CD4⁺, TT reactive T cell clones

The optimal number of T cells required and the kinetics of MΦ lysis were determined initially. Autologous MΦ adhered from 10⁶ PBMC were labeled with ⁵¹Cr and increasing numbers of cloned T cells (TT44G) were added together with TT diluted 1:50. ⁵¹Cr release was measured 18 h later (Fig. 1a). A small but significant amount of lysis (11 ± 3%, p < 0.05 relative to control cultures without T cells) was detected using 2.5 × 10⁴ T cells, corresponding to an effector:target ratio of approximately 2.5:1. The percent lysis increased linearly over the range tested, with an effector:target ratio of approximately 20:1 giving 55 ± 4% lysis. Since results in murine systems demon-
A.

with TT diluted autologous APC. (A) The indicated number of cloned T cells were added to approximately $10^4$ 51Cr-labeled autologous MΦ together with TT diluted 1:50. 51Cr release was measured after 18 h. (B) Cloned T cells (2 x 10^5) were added to approximately $10^4$ 51Cr-labeled autologous MΦ together with TT diluted 1:50. Percent cytotoxicity was determined at the times indicated. (C) Cloned T cells (2 x 10^5) were added to approximately $10^4$ 51Cr-labeled autologous MΦ together with TT diluted 1:50 and mAb to nonpolymorphic portions of class II (1:10^4-10^5, as ascites), class I (1-100 ng/ml) determinants, or control ascites (1:10^4-10^5). Anti-LFA-1 was as added as culture supernatant diluted 10^-3-10^-5. Maximum inhibition is shown, and results are expressed relative to maximum killing.

strated that MΦ pretreatment with IFN-γ was required for killing, similar experiments were performed using untreated MΦ or MΦ pretreated for 18 h with 1-10000 U/ml rLFN-γ and an effector:target ratio of 25:1. No significant difference in the killing of treated or untreated MΦ was observed (64 ± 4 % vs. 63 ± 6 %, mean ± SD, untreated vs. 10000 U/ml IFN-γ). The requirement for IFN-γ in the murine system was attributed to its effect on MΦ Ia expression [2, 11], and it is possible that the constitutive expression of HLA-D products on human monocytes obviates the requirement for IFN-γ treatment.

To determine the kinetics of the cytotoxic response, the same cloned T cell line was cultured with autologous MΦ and TT as before, using an effector:target ratio of approximately 20:1. In the experiment shown (Fig. 1b), minimal 51Cr release was detected at 1, 3 or 5 h, but was readily detected at 18 h. Serial repeats of this experiment using two other cloned lines also demonstrated that no significant 51Cr release was detectable at 6 h (9.5 ± 7.5 %, mean ± SEM of two experiments). This is consistent with other reports of MΦ killing [2, 10].

The requirement for class I and II MHC molecules and LFA-1 in the cytolytic response was tested by adding mAb specific for these ligands to 18-h killing assays similar to those described above. Relative to cultures containing control ascites, mAb specific for LFA-1 and class II MHC determinants produced significant (p < 0.05) inhibition, while mAb to class I MHC determinants gave no inhibition (Fig. 1c). This is also consistent with previous reports in the murine [2] and human [9] systems.

### 3.2 Requirement for cell-cell contact in MΦ killing

The requirement for self determinants and antigen was determined by comparing 51Cr release from autologous and allogeneic MΦ with and without antigen (Table 1). In these experiments, autologous and allogeneic macrophages were isolated by adherence, then labeled with 51Cr and cultured for 18 h with three cloned lines, with or without TT, using an effector:target ratio of 25:1 (Table 1). All three lines lysed TT-bearing autologous MΦ, but not autologous MΦ without TT, and none gave significant lysis of allogeneic MΦ with or without antigen. These results suggest that MΦ cytosis is restricted to autologous, antigen-bearing MΦ.

The apparent restriction of MΦ killing to antigen-bearing autologous MΦ, as well as the inhibition with mAb to LFA-1, suggested that physical contact may be required for MΦ killing. The requirement for physical contact between the T cells and MΦ was addressed with co-culture experiments. TT37L cells were cultured with DR 4,4 APC, DR 4,4 and DR 4,6 APC, or DR 4,6 APC alone. Control cultures demonstrated that the DR 4,6 APC could present TT to autologous (DR 4,6) T cells, as measured by proliferation assays, but not to the DR 4,4 clones lines (11 000 ± 1300 vs. ±000 ± 1500 cpm maximum responses, using TT, diluted 1:25-1:100 and 1.25 x 10^4 - 5.0 x 10^5 MΦ). For the coculture experiments, the APC preparations were irradiated (2000 rad) to prevent activation of T cells other than TT37L, and TT was added to all cultures. The cultures were maintained with IL-2 as described in Sect. 2.2, and twice a week one half the media was removed, and replaced with fresh IL-2-containing media. After 9-14 days the wells were examined by phase-contrast microscopy. In cultures initially containing TT37L, autologous APC and TT, numerous lymphoblasts but no MΦ were visible. Cultures containing TT37L, allogeneic APC and TT, contained abundant MΦ but no lymphoblasts. However, in cultures originally containing TT37L and equal numbers of autologous and allogeneic MΦ, lymphoblasts and MΦ were seen. Similar results were seen using a second TT-reactive T cell line, TT36C (data not shown).

### Table 1. Cloned, antigen-specific CD4+ cells kill antigen-bearing autologous macrophages

<table>
<thead>
<tr>
<th>T cell line</th>
<th>Percent specific lysis</th>
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<tr>
<td>B8,37 DR4,6 MΦ</td>
<td>MΦ+TT</td>
</tr>
<tr>
<td>TT37L</td>
<td>0 ± 1</td>
</tr>
<tr>
<td>TT36C</td>
<td>0 ± 1</td>
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<tr>
<td>TT18I</td>
<td>16 ± 8</td>
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a) Cells (2.5 x 10^5) from the T cell lines were cultured with 10^4 51Cr-labeled MΦ of the indicated MHC phenotype. All T cell lines were derived from the B55 DR4,4 donor. 51Cr release was measured 18 h later.

b) p < 0.05 relative to autologous MΦ without TT.
The MHC type of the remaining MΦ was identified by immunofluorescence. TT37L was challenged with autologous and allogeneic APC, but coverslips were first placed in the bottoms of the wells. After 1 week the coverslips were removed, nonadherent cells rinsed off, and the macrophages stained with antisera specific for autologous (HLA-B 55) or allogeneic (HLA-B 8) MHC alleles. All the remaining MΦ stained with antisera specific for the HLA-B allele of the allogeneic MΦ, while none of the surviving cells stained with antisera specific for the autologous HLA-B allele (data not shown). These experiments indicate that allogeneic, but not autologous MΦ remain when cultured with TT37L, TT and IL-2, and suggest that under these conditions TT37L preferentially kills autologous MΦ. These results support previous studies demonstrating that killing requires physical contact between the MΦ and the T cell [5,30]. To examine further whether soluble factors could contribute to MΦ killing, supernatants were collected from 10^6 antigen-activated, cloned, IL-2-dependent T cells cultured in media without IL-2 for 48 h. Supernatants from similar cultures have previously been shown to yield significant amounts of secreted cytokines in as little as 12 h [31]. Addition of serial dilutions (1:4 – 1:32) of the supernatant to the cultured MΦ for 18 h induced no statistically significant CR release at any dilution (8 ± 6 %, mean ± SEM of maximum killing from two experiments), further suggesting that the MΦ death is not due to soluble factors.

The MΦ loss in cultures containing cloned T cells, autologous MΦ and TT was presumably due to cytolysis, but if the T cells caused the MΦ to become nonadherent, they might not be recognized by phase-contrast microscopy. To exclude this possibility, the nonadherent cells were stained with OKM1 or anti-CD2 1 week after addition of irradiated autologous APC plus TT and then analyzed by flow cytometry. The nonadherent cells were 97–99 % CD2^+ , and no OKM1^+ cells were found, although OKM1^+ cells were easily identified in irradiated PBMC (89 % of the monocyte population, gated by forward and 90° light scatter) and in mixtures of cloned T cells and PBMC analyzed the same day (66 % of the monocyte population). OKM1^+ cells were similarly sought in the nonadherent fraction of cultures containing T cells and allogeneic MΦ. Again none were found, suggesting that the MΦ were not becoming nonadherent.

3.3 Mechanisms of MΦ killing

Current opinion maintains that electron microscopic ultrastructural analysis is the most accurate test for distinguishing apoptosis from necrosis [32]. Necrosis is characterized morphologically by “ballooning” and rupture of the cytoplasmic membrane and dissolution of cytoplasmic structures and organelles such as mitochondria, while the nucleus remains intact [32,33]. In contrast, apoptosis is characterized by early nuclear condensation and DNA degradation, which occurs before cytoplasmic disruption [32-36]. Macrophages, purified by adherence, were cultured alone or with equal numbers of TT18X cells. TT diluted 1:50 was added to all cultures. At 0, 2, 4 and 6 h cells were harvested, fixed in OsO_4, then examined by electron microscopy (Fig. 2). At 0 and 2 h no changes were seen in MΦ cultured alone or with T cells. However, at 4 and 6 h, 25–30 % of MΦ cultured with T cells and antigen showed changes characteristic of apoptosis, with nuclear condensation and relative preservation of organelles such as mitochondria. Less than 5 % of MΦ cultured alone showed these changes at 4 and 6 h. If should be noted that these changes preceded 51Cr release, similar to other reports of cells undergoing apoptosis [4].

Another characteristic of cells undergoing apoptosis is that the DNA is fragmented into nucleosome polymers [32, 34].
To determine if DNA from MΦ cultured with T cells and antigen shows similar fragmentation, MΦ were cultured alone or with the TT18X cloned line and antigen for 6 h. In these experiments, IL-2 was included in the media to prevent apoptotic death of the T cells [37]. The cells were then washed, lysed, the chromatin sedimented by centrifugation, then DNA extracted from the cytosol and separated by electrophoresis through agarose gels (Fig. 3). Macrophages cultured alone released very little DNA. The T cell lines cultured alone routinely showed evidence for fragmented DNA which was not of nucleosome size, and presumably comes from the irradiated PBMC used to stimulate the lines. However, DNA from T cells cultured with MΦ demonstrated the characteristic banding pattern seen in cells undergoing apoptosis. Similar bands were seen using TT45C and TT181 as the CTL. These results further suggest that MΦ death induced by autologous CD4+ T cells involves apoptosis.

Since physical contact between T cells and MΦ appears to be required to induce apoptosis, it was possible that cross-linking two or more ligands on the MΦ was responsible, as shown in other reports [38]. However, using goat anti-mouse Ig to cross-link saturating amounts (as determined by flow cytometry) of mAb to HLA-D, ICAM-1, class I MHC, LFA-1 and LFA-3 alone or in various combinations did not reproducibly induce MΦ death in standard 18-h killing assays. In addition, TNF, reported to induce apoptosis in some systems [36], did not decrease MΦ survival when cultured with MΦ for 18 h at 1-100 ng/ml. Finally, a recent report indicates that IL-4 may contribute to MΦ death [39]. However, the addition of neutralizing anti-IL-4 at concentrations of 0.8-10 µg/ml had no significant inhibitory effect on MΦ death relative to control antibodies (41 ± 25% inhibition vs. 57 ± 16% inhibition of killing, anti-IL-4 vs. control at 10 µg/ml). At this time, the signals inducing MΦ death in this system remain unknown.

4 Discussion

The data presented demonstrate that antigen-presenting MΦ die after culture with autologous, cloned, CD4+, antigen-specific T cells. Other have reported MHC- and antigen-specific MΦ killing by CD4+ T cells in human and murine systems [2, 9-11], and it seems reasonable to propose that this is a fairly general phenomenon.

The mechanism of MΦ killing was addressed. Electron microscopic analysis, the isolation of characteristic DNA fragments from the cytosol of the cells, and the observation that DNA fragmentation precedes cellular membrane disruption all support the conclusion that the mechanism of death is apoptosis. Recent reports indicate that Zn2+ ions and protein synthesis inhibitors like cycloheximide can inhibit apoptosis [12, 32, 40]. ZnSO4 and cycloheximide also inhibited cytotoxicity in our system. However, in experiments in which MΦ were pretreated with these drugs, no inhibition was seen (unpublished data). This may be due to diffusion of the drugs out of the cells during the 18-h incubation [41]. Alternatively, the effects may have been on the T cells, and not all apoptotic events require protein and RNA synthesis [32].

Technical artifacts that could account for the apoptotic changes were considered but deemed unlikely. Others have reported that irradiation can lead to apoptosis in some cells [42]. However, in our system, unirradiated as well as irradiated MΦ were killed. In addition, the MΦ used in the electron microscopic analysis and in the DNA isolation experiments were not irradiated. Others have also reported that monocytes cultured alone, in the absence of cytokines, undergo apoptosis slowly [43]. It is unlikely that a similar phenomenon contributes to the MΦ death observed in this system, because all experiments contained controls in which MΦ were cultured alone under otherwise identical conditions, and no ultrastructural changes or DNA fragmentation was observed over the relatively short time course of these experiments (4-6 h).

Since apoptosis is a genetically programmed response, these results support the contention that the MΦ death is not necessarily "killing" by a CTL, but represents a predetermined response of the MΦ which occurs following the activation of a CD4+ T cell subset. The CD4+ T cell clones used in this study were shown to express perforin mRNA and serine esterases in amounts comparable to activated CD8+ cells (unpublished results). Despite this, no evidence for necrosis was found. Others have reported that some CD4+ T cell clones lacking perforin and serine esterase activity can also induce apoptosis in susceptible cells [3]. This suggests that the ability to induce apoptosis may be relatively common among CD4+ cells.

The apoptotic response could be important to the host. For example, MΦ are susceptible to infection by intracellular parasites like Listeria monocytogenes and Mycobacterium tuberculosis. Apoptosis may provide a mechanism to help eliminate such pathogens [9-11]. Macrophage apoptosis may also regulate immune responses. The death of MΦ following CD4+ T cell activation would prevent further T cell recruitment, and could serve to down-regulate immune responses [1]. In addition, MΦ apoptosis could also maintain MΦ homeostasis. Current estimates suggest that the human bone marrow produces approximately 7 × 10^6 monocytes/kg/h [14, 15]. In a 70-kg person, approximately 1.18 × 10^10 monocytes would be released into the bloodstream each day. Given an average monocyte radius of 6 µm [44] and assuming that the cells are spherical, this amounts to a packed cell volume of 10 ml produced daily. Without a mechanism for disposing of these cells, the MΦ accumulation would be rapidly apparent. Macrophage apoptosis could also provide an explanation for granulocyte/MΦ (GM)-CSF secretion by T cells [45], in that secreted GM-CSF would stimulate MΦ production to replace those lost following T cell activation.

Finally, it is possible that MΦ death could contribute to autoantibody formation and autoimmune disease. A recent report indicates that nucleosomal DNA released from apoptotic cells can be immunogenic, and may trigger antibodies to antigens such as DNA and histones [12]. Another report suggests that some lupus patients have a T cell subset which kills autologous MΦ [13]. We have recently found that autoreactive CD4+ T cells capable of killing syngeneic MΦ can induce anti-DNA antibodies in vivo (Quddus et al., manuscript submitted). It is possible that MΦ death occurring in lupus patients could contribute to some of the autoantibodies characteristic of this disease.
The authors would like to thank Ms. Robin Kunkel for preparing the electron photomicrographs, Ms. Clare Rogers and Ms. Kristine Weber for performing the flow cytometric analyses, and Drs. David Fox and Steven Kunkel for their critical reviews of this work. Dr. Timothy Springer is also thanked for his generous contribution of anti-LFA-3 monoclonal antibodies.

Received September 16, 1992; in final revised form March 17, 1993.

5 References