Two Distinct Cytolytic Mechanisms of Macrophages and Monocytes Activated by Phorbol Myristate Acetate

Tejune Chung and Yoon B. Kim
Department of Microbiology and Immunology, University of Health Sciences/The Chicago Medical School, North Chicago, Illinois

Pulmonary alveolar macrophages (PAM) and peripheral blood monocytes (PBMO) of the miniature swine can be converted to cytolytically active effector cells by treating with phorbol myristate acetate (PMA) as determined by enhancement of cytotoxicity to various target cells. Kinetics of the PMA-activated PAM and PBMO in cytotoxicity show that the effective PMA concentration ranges from 10 to 1,000 ng/ml. Induction of cytotoxic macrophages and monocytes occurred as early as 30 min and to their maximum cytotoxicity after 1 hr exposure to PMA and the enhanced cytotoxic activity persisted up to 24 to 40 hr when PMA was removed by washing after 1 hr exposure, but prolonged exposure to PMA for more than 6 hr resulted in a drastic decrease of cytolytic activities suggesting the prolonged exposure to PMA causes macrophages and monocytes to become refractory to PMA stimulation. Target cells displayed varying degrees of cytotoxic sensitivity to the PMA-activated PAM and PBMO; PRBC, SRBC, and K562 were sensitive, WEHI-164 and U937 were relatively sensitive, and SB was very resistant to these activated effector cells. The mechanisms of PMA-induced cytotoxicity could largely be divided into two categories. One was the H2O2 mediated killing as shown by complete reduction of cytotoxicity after adding catalase in the assay. The other was the proteases mediated cytotoxicity, which could be blocked by protease inhibitors, Phenyl methyl sulfonyl fluoride (PMSF), and N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK). H2O2 was the only mediator produced in large enough quantities from PBMO to kill target cells, whereas PAM could produce both mediators (H2O2 and proteases). PRBC, SRBC, and K562 appeared to be killed by H2O2 produced by PAM and PBMO. In contrast, U937 and WEHI-164 appeared to be killed by proteases in PAM mediated cytotoxicity but by H2O2 in PBMO-mediated cytotoxicity. These results suggest that the observed cytolytic mechanisms can be differed by type of target cells as well as the source of mononuclear phagocytes within the individual animal.

Key words: macrophages and monocytes, cytolytic mechanisms, phorbol myristate acetate, porcine or swine

INTRODUCTION

In light of the potential importance of macrophages and monocytes in host defense mechanism [1], we have begun to investigate the effector mechanisms of macrophages and monocytes against various target cells. We have reported previously that the lytic mechanism involved in the nonspecific cytotoxicity generated by exposing pulmonary alveolar macrophages (PAM) to immobilized immune complexes or immune complexes in suspension in conjunction with cytochalasin B (both of which prevent the internalization of immune complex-bound Fc receptor) is mediated by hydrogen peroxide; whereas the lytic mechanism involved in antibody-dependent cellular cytotoxicity (ADCC) operates through a peroxide independent mechanism [24, 25]. This finding suggests that mononuclear phagocytes can perform their lytic functions in various different ways depending on the mode of activation and on the type of target cells. Several secretory products of mononuclear phagocytes have been identified that may play a role in this lytic process. These include reactive oxygen intermediates (ROI), [19], neutral proteases [2], tumor necrosis factor [16], complement component [9].

Phorbol myristate acetate (PMA), known for its potent tumor promoting activity in vivo, has a number of pleiotropic effects on cells in culture, including their differentiation and enhanced replication and secretion [31]. When PMA is added to mononuclear phagocytes, it produces dramatic changes in cell shape, spreading, endo-

Received August 25, 1987; accepted June 10, 1988.

Reprint requests: Yoon Bern Kim, Department of Microbiology and Immunology, University of Health Sciences/The Chicago Medical School, 3333 Green Bay Road, North Chicago, IL 60064.
cytosis, and the release of lytic mediators and regulators. In cytotoxicity of PMA-activated macrophages and monocytes, enhancement of oxidative burst mechanism triggered by PMA has been initially reported to be the primary mechanism of tumoricidal activity [13, 18]. Recently, Colotta et al. [7] have demonstrated that PMA-activated monocytes can kill drug-treated WEHI-164 target cells by proteolytic enzymes. Also importance of ROI-independent mechanism in tumor cytosis was implicated by others [3, 15]. But those two distinct mechanisms of cytotoxicity of activated PAM and PBMO have not been demonstrated clearly in one system at the same time. We believe it to be valuable to know the optimal activation conditions of mononuclear phagocytes that will produce either ROI or protease.

In this report we have used macrophages and monocytes of the miniature swine kept in a controlled environment [23]. The advantage of using these cells is that collection of pure cell populations in large quantities without prior stimulation is relatively easy and that immunologic similarities between human and pig will make it possible to project our study to human application.

We have examined the cytolytic mechanisms of the PAM and PBMO activated by PMA. Our results presented here strongly suggest that studying the cytolytic mechanisms of macrophages and monocytes of the same animal using different types of target cells including malignant and normal cells at the same time may be the best way to understand the functional nature of these mononuclear phagocytes.

MATERIALS AND METHODS

Animals

Young adult specific pathogen free (SPF) miniature swine maintained in a clean environment were used in these experiments [23].

Pulmonary Alveolar Macrophages (PAM)

PAM were collected as previously described elsewhere [23]. Briefly, phosphate-buffered saline, pH 7.2, was injected intratracheally into swine sacrificed by CO₂ anesthesia. The lavage fluid was removed, and the lavage cells were washed with balanced salt solution (BSS). This routinely yielded PAM that were 90-95% pure.

Peripheral Blood Monocytes (PBMO)

Peripheral blood mononuclear cells were separated from heparinized venous blood of healthy adult swine by standard Ficoll-Hypaque density gradient centrifugation at 400 x g for 45 min at room temperature [4]. Mononuclear cells were collected at the interface, washed with BSS, and suspended in RPMI-1640 medium supplemented with 25 mM Hepes, 2 mM L-Glutamine, 100 U penicillin/ml, 100 µg streptomycin/ml, and 10% heat inactivated FBS at a concentration of 5 x 10⁶ cells/ml. The mononuclear cells were incubated for 1 hr at 37°C in 100-mm plastic tissue culture dishes (Falcon; Oxnard CA) precoated with autologous serum for 15 min at 37°C [10]. After incubation, nonadherent cells were removed by extensive washing with cold BSS. Adherent cells were detached by treatment for 10 min with EDTA (Versene 1:5,000; Gibco Laboratories) at room temperature followed by gentle scraping with a rubber policeman in the presence of FBS. Collected cells were washed and resuspended in complete medium, and used as effector cells in cytotoxic assay. Viability as assessed by trypan blue dye exclusion was over 90%.

Cell Lines and Cells

The following murine and human cell lines were employed in this study: WEHI-164, a chemically induced fibrosarcoma of BALB/c mice; U937, a human histiocytic lymphoma line; K562, a human erythroleukemia line, SB, a normal human B cell line adapted for in vitro culture. In parallel with those permanent cell lines, normal cells were also used as targets in ⁵¹Cr-release assay: chicken red blood cells (CRBC), pig red blood cells (PRBC), and sheep red blood cells (SRBC).

Reagents

The PMA (4 β-phorbol 12 β-myristate 13 α-acetate; Sigma, St. Louis) was dissolved in 15% dimethyl sulfoxide (DMSO, Calbiochem-Behring, La Jolla, CA) at a concentration of 1 mg/ml and stored in small aliquots at—80°C. The final concentration of DMSO in working solution was less than 1%, a concentration that has no demonstrative effect on viability and function of cultured cells. Catalase (2890 U/mg protein), Phenyl methyl sulfonyl fluoride (PMSF), N-α-p-Tosyl-L-Lysine Chloromethyl Ketone (TLCK), bovine pancreatic trypsin inhibitor (BPTI), Soybean trypsin inhibitor (STI) were all purchased from Sigma Co.

Cytotoxicity Assay

Tumoricidal activity was measured by using 18 hr ⁵¹Cr release assay [24]. Various target cells were used for every experiment to compare the cytotoxic responses. Target cells, suspended in 0.2 ml complete medium, were labeled with 100 to 200 µCi ⁵¹Cr for 60 to 120 min at 37°C in 5% CO₂ in air, followed by washing three times with BSS. Assays were performed in triplicate against 1 x 10⁴ target cells per well in 96-well flat bottom tissue culture plates (Costar, Cambridge, MA) in a final volume of 200 µl. In direct cell mediated assays, effector cell numbers were adjusted at effector to target (E:T) ratios of 20:1 for malignant target cells and 1:1 for RBC target cells, unless otherwise specified. To assay
soluble cytolytic factors of the PAM and PBMO that had been prestimulated by PMA, cell free supernatants were pre-equilibrated in a humidified, 5% CO2 atmosphere to assure correct pH before the addition of target cells. After 18 hr at 37°C, the plates were centrifuged at 250 x g for 7 min, and 100 μl of supernatants were harvested from each well and counted in an automatic gamma counter. The percentage of cytolysis was calculated as:

\[
\text{% specific } ^{51}\text{Cr release} = \frac{\text{experimental cpm} - \text{SR cpm}}{\text{MR cpm} - \text{SR cpm}} \times 100
\]

where spontaneous release (SR) was measured in the wells containing media alone and maximum release (MR) were determined by lysis with NP-40 detergent. Data are presented as percentage of mean specific lysis ± SD. In the series of experiments reported here, spontaneous release was less than 10% for RBC target cells and not more than 25% for malignant targets.

\section*{Measurement of H2O2 Production of the Activated Cells}

H2O2 production from the PMA-activated macrophages and monocytes were quantitated according to the methods reported by Pick et al. [20, 21], which were based on the H2O2 mediated and horseradish peroxidase HRPO) dependent oxidation of phenol red to a product whose absorbance was read at 610 nm. Briefly, macrophage or PBMO monolayers were established in 96-well bottom plates at a concentration of 1 x 10^5 cells/well and cultured with PMA in various time intervals from 1 hr to 18 hr. After preincubation with PMA, monolayer cells were washed with phenol red free BSS twice and 0.01 ml of phenol red solution (PRS) was added per well. The PRS was prepared just before assay by adding 0.56 mM (0.2 g/L) phenol red and 19 U/ml of HRPO to PRS solution containing 140 mM NaCl, 10 mM potassium phosphate buffer of pH 7.0, and 5.5 mM dextrose, which was kept in 4°C cold room. Column No. 1 was left without cells and filled with 100 μl PRS per well. The second column contains cells covered with 100 μl PRS, but 10 μl of 1 N NaOH were added to each well to bring the pH to alkalinity and induce cell death. The subsequent rows contain wells covered with 100 μl PRS in the absence of stimulant or to which the stimulant under study were added. The plates were incubated at 37°C for 5 hr and the reaction stopped by adding 10 μl 1 N NaOH per well. This will terminate the production of H2O2 and bring the pH of the reaction mixture to the alkaline region required for reading of absorbance at 610 μm. The plate was then transferred to the Microelisa reader II (Dynatech Laboratories, Alexandria, VA) equipped with a 610 nm wave length filter and absorbances were measured.

\section*{RESULTS}

\subsection*{Optimal Conditions for Maximal Activation of PAM and PBMO by PMA}

Freshly collected PAM and PBMO of Minnesota miniature swine do not show any cytotoxic activities regardless of the length of the assay time or type of target cells, but pretreatment with PMA for as short as 30 min could convert them rapidly to cytotoxic effector cells. Figures 1 and 2 illustrate the relationship between the concentration of and pretreatment time with PMA and target cell lysis. These figures are representative of normal (Fig. 1) and transformed target cells (Fig. 2). Maximal cytolytic effect was obtained at 1 hr pretreatment of PAM with PMA, and effective concentration for peak activation ranged from 10 to 1,000 ng/ml. Neither PMA alone nor its solubilizing vehicle had an effect on the viability of the target cells, as indicated by the lack of significant isotope release through the range of concentrations used.
When pretreatment time was prolonged 6 to 12 hr, cytotoxicity was drastically decreased. This was not because prolonged exposure to PMA was toxic to effector cells as judged by viability testing with trypan blue dye exclusion method.

As illustrated in Figure 3, H$_2$O$_2$ production of the PMA-activated PAM was also highest at 1 hr pretreatment with PMA followed by decline of the H$_2$O$_2$ production, which correlated well with the cytotoxicity curve (Figs. 1, 2). These findings may suggest the important role of H$_2$O$_2$ in cytotoxicity mediated by PMA-activated PAM or PBMO, but the possibility of other mediators involved in cytotoxicity has not been eliminated. The pattern of cytotoxicity kinetics was basically the same for both PAM and PBMO, with the same range of concentration and pretreatment time with PMA required for full activation (PBMO data not shown); 1 hr pretreatment with 20 ng/ml of PMA was chosen as our standard activation method for analysis of cytolytic mechanisms of activated PAM and PBMO. Once the cell was fully activated, activated status was maintained for more than 24 hr (Fig. 4).

**Sensitivity of Target Cells to PMA Activated PAM and PBMO**

Various target cells were utilized to examine target cell differences in cytotoxicity of PMA-activated PAM and PBMO. As shown in Tables 1 and 2, the degree of % of specific cytolysis obtained using PMA activated effectors apparently reflected sensitivity differences of the target cells. PRBC, SRBC, and K562 were very sensitive targets, lysis observed was greater than 50% when exposed to PMA-activated effector cells. In contrast, SB cell line was resistant, and WEHI-164 and U937 were moderately sensitive with cytolytic ranges of around 20%.

Killing of PRBC, an autologous target, by PMA-activated mononuclear phagocytes indicates the indiscriminate nature of cytolyis, implying potentially harmful effects of macrophages and monocytes when this type of cytolytic mechanism is used as a major host defense mechanism in vivo.

**Effects of Catalase, PMSF, and Other Proteolytic Inhibitors on PAM and PBMO Mediated Cytotoxicity**

The role of cytolytic mediators produced by PMA-activated PAM and PBMO was evaluated with oxygen scavengers or protease inhibitors. Catalase (250 μg/ml) and PMSF (1 mM) had no effect on the target cell viability in the presence of unstimulated effector cells (data not shown). Catalase inhibited PMA-stimulated cytolyis against PRBC, SRBC, and K562 targets by greater than 90%. If catalase was added to the assay 1 hr after the addition of target cells, inhibition of cytotoxicity was reduced to less than 20% (data not shown). In contrast, the cytotoxicity of WEHI-164 and U937 targets were completely blocked by PMSF and TLCK, not by catalase, STI, or BPTI (Table 1). These data suggest that PAM activated by PMA can produce both H$_2$O$_2$ and proteases, which may be the principally involved molecular species in the cytolyis of various target cells dependent on susceptibility to either proteases and/or H$_2$O$_2$.

The effects of catalase and PMSF on cytotoxicity by PMA-stimulated PBMO are presented in Table 2. PBMO mediated cytolyis of all types of target cells tested were
Cytolytic Mechanisms of PMA Activated PAM and PBMO

TABLE 1. Effects of Inhibitors on the Cytotoxicity of PMA-Activated PAM

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Inhibitorsb</th>
<th>PRBC</th>
<th>SRBC</th>
<th>K562</th>
<th>U937</th>
<th>WEHI-164</th>
<th>SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated PAM</td>
<td>None</td>
<td>1.3 ± 0.4</td>
<td>1.2 ± 0.7</td>
<td>0.3 ± 1.1</td>
<td>3.9 ± 1.1</td>
<td>1.8 ± 1.3</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>PMA-activated PAMc</td>
<td>None</td>
<td>80.8 ± 1.9</td>
<td>69.8 ± 3.4</td>
<td>68.5 ± 3.7</td>
<td>19.6 ± 1.2</td>
<td>22.5 ± 3.0</td>
<td>7.9 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
<td>3.7 ± 1.5</td>
<td>3.7 ± 0.7</td>
<td>1.8 ± 1.7</td>
<td>23.1 ± 0.9</td>
<td>23.8 ± 3.2</td>
<td>-0.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>PMSF</td>
<td>59.4 ± 8.7</td>
<td>54.7 ± 2.6</td>
<td>56.7 ± 2.4</td>
<td>1.8 ± 0.4</td>
<td>2.8 ± 0.6</td>
<td>5.0 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>TLCK</td>
<td>ND</td>
<td>ND</td>
<td>51.1 ± 2.1</td>
<td>5.6 ± 0.7</td>
<td>7.3 ± 1.7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>BPTI</td>
<td>ND</td>
<td>ND</td>
<td>72.3 ± 1.9</td>
<td>21.5 ± 0.5</td>
<td>21.6 ± 2.3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>STI</td>
<td>ND</td>
<td>ND</td>
<td>54.9 ± 2.1</td>
<td>18.6 ± 0.7</td>
<td>23.9 ± 2.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

*18-hr 51Cr-release assay with E/T ratios of 1:1 for PRBC and SRBC, and 10:1 for K562, U937, WEHI-164, and SB.

*Inhibitors: Catalase (250 μg/ml), PMSF (2 mM), TLCK (0.2 mM), BPTI (150 μg/ml) and STI (150 μg/ml) were added in the assay mixture.

*PAM were stimulated with PMA 20 ng/ml for 1 hr at 37°C and washed with BSS before assay.

TABLE 2. Effects of Inhibitors on the Cytotoxicity of PMA-Activated PBMO

<table>
<thead>
<tr>
<th>Effectors</th>
<th>Inhibitorsb</th>
<th>CRBC</th>
<th>PRBC</th>
<th>SRBC</th>
<th>K562</th>
<th>U937</th>
<th>WEHI-164</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated PBMO</td>
<td>None</td>
<td>0.5 ± 0.3</td>
<td>2.3 ± 1.1</td>
<td>0.7 ± 0.3</td>
<td>3.1 ± 2.3</td>
<td>1.8 ± 0.7</td>
<td>3.6 ± 1.8</td>
</tr>
<tr>
<td>PMA activated PBMOc</td>
<td>None</td>
<td>37.4 ± 2.2</td>
<td>88.2 ± 1.6</td>
<td>45.6 ± 1.5</td>
<td>16.3 ± 1.6</td>
<td>13.9 ± 0.1</td>
<td>28.7 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
<td>0.3 ± 0.7</td>
<td>8.3 ± 2.4</td>
<td>0.3 ± 1.1</td>
<td>3.7 ± 0.8</td>
<td>2.4 ± 0.9</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>PMSF</td>
<td>37.2 ± 2.1</td>
<td>53.5 ± 2.1</td>
<td>39.8 ± 1.8</td>
<td>14.1 ± 2.5</td>
<td>20.4 ± 0.4</td>
<td>30.1 ± 2.7</td>
</tr>
</tbody>
</table>

*18-hr 51Cr-release assay with E/T ratios of 1:1 for PRBC, CRBC, and SRBC, and 10:1 for K562, U937, and WEHI-164.

*Inhibitors: Catalase (250 μg/ml) and PMSF (2 mM) were added in the assay mixture.

*PBMO were stimulated with PMA 20 ng/ml for 1 hr at 37°C and washed with BSS before assay.

TABLE 3. Comparison of the Cytolytic Activities of Supernatants Generated From PAM and PBMO Stimulated With PMA and IFN-γ Plus LPS

<table>
<thead>
<tr>
<th>Source of cells</th>
<th>Stimulating agents</th>
<th>WEHI-164</th>
<th>U937</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM</td>
<td>PMA</td>
<td>3.5 ± 1.2</td>
<td>5.2 ± 1.3</td>
</tr>
<tr>
<td>PBMO</td>
<td>PMA</td>
<td>1.5 ± 1.3</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td>PAM</td>
<td>IFN-γ + LPS</td>
<td>28.0 ± 4.0</td>
<td>26.7 ± 3.2</td>
</tr>
<tr>
<td>PBMO</td>
<td>IFN-γ + LPS</td>
<td>28.7 ± 2.3</td>
<td>19.8 ± 1.0</td>
</tr>
</tbody>
</table>

*Cytolytic activities of culture supernatants against WEHI-164 and U937 were measured by mixing directly 100 μl of supernatant with 100 μl of 51Cr-labeled target cells for 18-hr 51Cr-release assay.

*bPAM or PBMO were cultured with 20 ng/ml of PMA for 18 hr and collected culture supernatant.

*PAM or PBMO were primed with 100 U/ml of IFN-γ for 18 hr and triggered with 5 μg/ml of LPS for 5 hr, and collected culture supernatant.

Fig. 4. Maintenance of the activated state of PMA-pretreated PAM. After pretreating the PAM with 20 ng/ml of PMA for 1 hr and washed with BSS, the activated PAM were cultured further in fresh media for varying time periods and assayed cytotoxicity against various target cells for 18-hr 51Cr-release assay.

completely blocked uniformly by addition of catalase in the assay, but PMSF had no effect or a slight effect only on PRBC and SRBC target lysis indicating H2O2 as the principal lytic mediator. None of these compounds influenced the cytolytic activity of unstimulated monocytes or the viability of target cells (data not shown).

Taken together, our results indicate that H2O2 is the only mediator produced in large enough quantities from PBMO to kill target cells, whereas PAM could produce both mediators (H2O2 and proteases), thus PAM appears more versatile for using several different lytic mediators. PRBC, SRBC, and K562 were lysed by H2O2 produced by PAM or PBMO. In contrast, U937 and WEHI-164 were lysed by proteases in PAM-mediated cytolysis and by H2O2 in PBMO-mediated cytolysis.
Effect of PAM and PBMO Culture Supernatants

To evaluate the possibility of cytolytic activity present in the cell free culture supernatants, we have collected culture supernatants from PAM and PBMO activated with PMA 25 ng/ml for 1, 3, 6, 12, and 18 hr. When we tested the direct cytolytic activity of the supernatants against WEHI-164 and U937 target cells, no visible killing could be obtained. In contrast to this result, when PAM or PBMO were primed with IFN-γ for 18 hr and then stimulated with LPS for 5 hr, these cells were found to secrete macrophage cytoplytic factor (MCF) into supernatants (Table 3). These results indicate PMA-activated PAM or PBMO did not produce MCF.

DISCUSSION

We have investigated PMA-activated cytolytic mechanisms of two different sources of mononuclear phagocytes of SPF miniature swine (PAM and PBMO) from the same animal simultaneously against variety of target cells (normal and transformed cells) using pretritrat optimal conditions. Some of the conflicting reported results may be due to PMA stimulation time and concentrations, different sources of mononuclear phagocytes, and/or to the target cell sensitivity differences, etc. Although it still remains in question whether the unstimulated resting monocytes and macrophages can exert cytolytic activities spontaneously without exogenous stimulants [10, 17, 18], unstimulated mononuclear phagocytes in our system do not show cytolytic activities at the E/T ratios used in our standard assay. Thus the activating capacity and cytolytic mechanisms of the effector cells could be determined by choosing appropriate activating agents. In this study we have chosen PMA as standard stimulant for several reasons: First, the activating capacity of PMA was strong, rapid, and easily reproducible. Regardless of what target cells were used, response curves to various concentrations and treatment time of PMA were similar. Minimum concentration of PMA required to obtain satisfactory cytolytic activity was 10 ng/ml. At 20 ng/ml, the cytolytic activity had already reached the plateau level (Figs. 1, 2). Second, the structure of PMA and its site of action in the cell has been extensively studied [8, 29]. Finally, PMA is known to bypass the prerequisite antigenic stimulation for full activation of mononuclear phagocytes [28]. This capacity of PMA to bring about a superactive state in macrophages and monocytes can be further emphasized by using various types of target cells. Sensitivity of target cells to various soluble factors produced by PMA-activated PAM and PBMO is variable; therefore studies utilizing several different target cells may give a better understanding of cytolytic mechanisms. Furthermore, contradicting reports [11, 12, 14, 16] regarding the inhibitory or stimulatory effects of phorbol esters on macrophage effector functions may be explained by the kinetic studies. As illustrated in Figures 1 and 2, PAM and PBMO can be activated to its maximum cytotoxicity after 1 hr prestimulation with PMA, but prolonged exposure to PMA for more than 6 hr resulted in a drastic decrease of cytolytic activities, which went down to baseline level after 18 hr exposure to PMA. Two different possibilities exist for this phenomenon. Most of the lytic mediators were exhausted within an 18-hr period by PMA or prolonged exposure to PMA will make the cells refractory to PMA activation. When PMA was removed by washing after optimal activation (1 hr) the activated state was maintained at its peak level for at least 24 hr (Fig. 4), suggesting the latter notion that on prolonged exposure to PMA, PAM, or PBMO might become refractory to PMA stimulation.

Oxidative burst mechanism for PAM- and PBMO-mediated cytotoxicity has been demonstrated [13, 18, 19]. As shown in our results, the kinetics for triggering H₂O₂ release by PMA (Fig. 3) is almost identical to that for eliciting cytotoxicity (Figs. 1, 2) provide indirect evidence for the importance of H₂O₂ in extracellular cytotoxicity. H₂O₂ mediated killing of target cells was active not only to transformed target cells such as K562 but also normal cells like PRBC, SRBC, or SB (Tables 1, 2). Moreover, we have reported previously that PAM activated by immobilized immune complex generated significant amounts of H₂O₂ in the media [24, 25], which was the cause of bystander cytotoxicity for autologous target cells by H₂O₂ may be an unwanted effect occurring in the process of in vivo tumor cell lysis by activated macrophages or monocytes.

Tumor cell killing by activated mononuclear phagocytes through oxygen-independent mechanism has also been reported [2, 3, 30]. The observation by Chen and Koren [5] of patients with chronic granulomatous disease, whose oxidative burst was absent or far below normal, yet retained the tumoricidal activity of monocytes, may be solid evidence that reactive oxygen metabolites are not the primary mediators of tumor cytolyis. Also, the introduction of actinomycin D-treated WEHI-164 target cells for short term assay (6 hr) of monocyte or macrophage mediated cytotoxicity [6, 33] has accelerated the study of ROI-independent mechanism of cytotoxicity. Colotta et al. [7] used various antiproteases to determine the possible role of proteolytic enzymes in mediating antitumor cytotoxicity and suggested that proteolytic enzymes were involved in actinomycin D-treated WEHI-164 targets by human PBMO. Adams and co-workers [2, 3] demonstrated a role for a novel neutral protease in activated macrophage killing of tumor cells in mice. Data reported here was based on an 18-hr assay using untreated target cells and suggests the role of protease for the killing of WEHI-164 and U937 targets by
activated macrophages (Table 1), not by activated monocytes (Table 2), in the porcine system.

The mechanisms of PMA induced cytotoxicity could largely be divided into two categories. One is the \( H_2O_2 \) mediated killing as shown by significant reduction of cytotoxicity after adding catalase in the assay. The other is the protease-mediated cytolysis, which could be abrogated by protease inhibitors. Our results clearly demonstrate that \( H_2O_2 \) is the principal mediator produced in a large enough amount from PMA-activated PBMO to kill target cells (Table 1), whereas PAM could produce both mediators (\( H_2O_2 \) and/or proteases), the principal mediators of PMA-stimulated PAM being determined by target cell phenotypes (Table 2). PRBC, SRBC, and K562 appear to be \( H_2O_2 \) sensitive and relatively resistant to proteases and are killed by \( H_2O_2 \) produced by PAM or PBMO. In contrast, U937 and WEHI-164 appear to be susceptible to proteases and relatively resistant to \( H_2O_2 \), and are killed by proteases in PAM-mediated cytolysis but by \( H_2O_2 \) in PBMO-mediated cytolysis. These apparent discrepancies of WEHI-164 and U937 sensitivity to \( H_2O_2 \) produced by PMA-activated PAM and PBMO (Tables 1, 2) may be explained by their susceptibility to proteases and relative resistance to \( H_2O_2 \). PMA-activated PBMO produce a large enough amount to kill WEHI-164 and U937, but PMA-activated PAM do produce \( H_2O_2 \) to kill \( H_2O_2 \) sensitive target cells such as PRBC, SRBC, and K562, but not enough \( H_2O_2 \) to kill WEHI-164 and U937. However, WEHI-164 and U937 are sensitive to proteases and PMA-activated PAM produce enough proteases to lyse them, but PMA-activated PBMO do not produce enough proteases to lyse WEHI-164 and U937.

In contrast to an oxidative burst mechanism, killing by protease seems to be rather selective for certain transformed cells. Considering the significance of this selective killing, elucidation of the precise role of neutral proteases in tumor cell lysis deserves further study.

In summary, we have shown that both oxidative burst mechanism and protease production could be activated simultaneously by the same stimulation of macrophages, but certain targets are killed by oxidative burst and others by protease, depending on the target sensitivity. We do not rule out synergy between oxidative and nonoxidative mechanisms of tumor cell injury. Possible existence of MCF resembling tumor necrosis factor were recently described in rodents [2, 22] and humans [27, 32]. These alternatives were investigated in PMA-activated PAM and PBMO by measuring cytotoxic activity of cell-free culture supernatants directly against target cells. The result (Table 3) indicates that PMA stimulation did not produce MCF in the porcine system, whereas MCF generation was possible by stimulating PAM or PBMO with LPS after priming with IFN-\( \gamma \). Whereas direct killing by PMA-activated PAM and PBMO is mediated by \( H_2O_2 \) and/or proteases (Tables 1, 2), cell-free culture supernatant of these activated effector cells did not kill the same target cells in the cytotoxic factor assay (Table 3). One possible explanation is that the amount of \( H_2O_2 \) or proteases produced is not enough in the culture supernatant, whereas concentrations in the microenvironment of effector-target conjugates may be high enough to exert the lysis. Alternately their biological activity may be inactivated by the serum proteins in the media. Thus this study points out the importance of the source of mononuclear phagocytes (stages of differentiation), target cell sensitivity to different lytic mediators, and activating stimulus for their cytolytic mechanisms. SPF and gnotobiotic miniature swine models provide an excellent animal model to study the development, differentiation, and activation of mononuclear phagocyte system and their cytolytic mechanisms.

ACKNOWLEDGMENTS

This work was supported by PHS Grant Number HL-36012, awarded by the National Heart, Lung and Blood Institute, DHHS. The authors wish to acknowledge the assistance of Mr. Terry Hardekopf in procuring animals for this study.

REFERENCES

9. Felgner, J., Shortlivan, H.V., Baptista, L.C., and Allison, A.C. Production of the complement cleavage product, C3a, by