Human Neutrophil-Mediated Lysis of Ovarian Cancer Cells

By Alan Lichtenstein, Miriam Seelig, Jonathan Berek, and Jacob Zighelboim

Because of recent questions concerning the sensitivity of human tumor cells to neutrophil-induced oxidative injury, we studied six freshly obtained human ovarian cancer (OC) specimens. Stimulation of neutrophils (PMNs) by phorbol myristate acetate (PMA) did not result in OC cytolysis during the first nine hours of incubation. However, three of six specimens were significantly lysed by stimulated PMNs when assay length was increased to 18 hours. Cytotoxicity was mediated by PMN production of reactive oxidative intermediates (ROIs). Presentation of ROIs to OC targets as preformed or enzymatically generated molecules in cell-free systems duplicated the enhanced lysis at 18 hours (as compared with six hours). Since addition of catalase at three or six hours did not inhibit enhanced lysis at 18 hours (achieved by PMNs or in cell-free systems), it appears that an initial ROI-mediated lethal event occurs early, but longer incubations are required for the event to become manifested as cell death. These data suggest that shorter assays may underestimate the potential of PMNs as effector cells against human tumor cells.

Most investigations on human cell-mediated responses to tumor growth have focused on the role of killer lymphocytes and macrophages. In contrast, less support has accumulated for participation by neutrophils (PMNs) in the anti-tumor process. Although PMNs can readily lyse continuously cultured leukemia-lymphoma targets in vitro, there is no evidence that they can affect freshly obtained tumor cells from patients as has been shown for activated lymphocytes and macrophages.

A recent report contributed further doubt about the potential role of PMNs by demonstrating an inherent resistance of nonleukemic human tumor cells to oxidative cytolysis, the major mechanism of target injury induced by PMNs. The concentration of H2O2 causing 50% lysis (LD50) of nine human target cell types ranged from 2 to 20 mmol/L, ~100-fold higher than those of murine tumor targets. These data raised the question of whether human PMNs could generate lytic concentrations of H2O2 (for human tumors) when maximally stimulated.

Since the above investigations were performed with continuously passaged targets that may have become altered during long-term culture, in the current study, we evaluated freshly explanted human ovarian cancer (OC) cells as a more relevant target for PMNs. OC cells are well suited for short-term culture and use as targets in cell-mediated cytotoxicity assays. In addition, previous research has documented the role of tumor-lytic PMNs in murine OC, and other investigators have demonstrated a temporal correlation between OC tumor cytoreduction and PMN influx after intraperitoneal (IP) therapy in humans. The results confirm the relative resistance of human tumor cells to PMN-induced oxidative lysis when short-term assays are used (three to 9 hours). However, a significant tumor-lytic effect on three of six fresh OC targets was detected when assay durations were increased to 18 hours. Lysis was mediated by reactive oxygen intermediates (ROIs).

Materials and Methods

Reagents. The following reagents were purchased from Sigma (St Louis): EDTA, superoxide dismutase (SOD), catalase, phorbol myristate acetate (PMA), D-mannitol, thiourea, urea, methimazole,aminotriazole,xanthine,xanthine oxidase (XO), and ferrous sulfate. Dimethylsulfoxide (DMSO) was obtained from Mallinkrodt (St Louis). Deferoxamine was a gift from Ciba-Geigy (Suffern, NY). PMA was stored in ethanol at −20°C at 10−4 mol/L.

Fresh OC specimens. All primary tumor specimens were either serous or mucinous adenocarcinomas. Tumor cells were isolated from either peritoneal fluid or solid tumors. Solid tumors were minced with a scalpel and teased apart with needles. Cells thus freed were isolated from the tissue fragments by passage through no. 100 mesh stainless-steel screens. The same procedure was repeated until cell release was no longer significant. Cells obtained in this manner were pooled and washed twice in Hanks’ balanced salt solution (HBSS). The tumor cells were separated from contaminating RBCs or lymphocytes by a discontinuous Ficoll-Hypaque gradient. The cell suspension was overlayered on the gradient (formed with 50%, 80%, and 100% Ficoll-Hypaque) and centrifuged at 500 g for 30 minutes. Peritoneal lymphocytes were found in the 100% Ficoll fraction, and RBCs sedimanted to the bottom. Tumor cells were isolated at the 80% Ficoll interface, and epithelial cells were present at the 50% interface. After harvest, the tumor cells were washed three times in HBSS. The cells were then maintained as monolayer cultures on plastic in RPMI 1640 media supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, NY), 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, and 1% penicillin-streptomycin. The tumor cells were harvested by overlaying the monolayer with a solution of 0.25% trypsin and 0.02% EDTA. After five to ten minutes of incubation, the cells were removed from the plastic by tapping the flasks sharply. They were washed three times and resuspended.

Effector cells. Human PMNs from normal donors and two patients with the X-linked form of chronic granulomatous disease (CGD) were studied. Approval was obtained from the UCLA and Wadsworth VA Institutional Review Boards for these studies. Patients and volunteers were informed that blood samples were obtained for research purposes and that their privacy would be protected. Both patients with CGD had nondetectable cytochrome b. PMNs were isolated as previously described, and RBCs were

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removed by hypotonic water lysis. Purity of PMN suspensions was
>97% and viability was >96%.

Chromium release assay. Tumor targets were labeled by incubating
10^6 cells with 50 μCi chromium-51 for one hour at 37°C. After being washed three times, targets (10^6 in 0.1 mL) were mixed
with PMN effectors (at different effector/target E:T ratios), H2O2,
xanthine and XO or H2O2, and EDTA-chelated ferrous sulfate
(ferrous sulfate chelated by a threefold molar excess of EDTA) in
0.1 mL in microtiter plates. PMA (10^{-7} mol/L) or inhibitors/
scavengers were added in appropriate concentrations. Samples were
run in quadruplicate. Plates were then centrifuged (1,200 rpm, ten minutes) to enhance cell contact. After incubation at
37°C in a 5% CO2 incubator for varying durations, plates were
centrifuged (1,200 rpm, ten minutes) and 0.1 mL supernatant
was removed and counted in a γ-counter. Percentage of specific lysis was
determined as follows: (cpm experimental group – cpm minimal
release/cpm maximal release – cpm minimal release) x 100.
Minimal release was determined by incubating targets alone with
PMA, and maximal release was determined by adding HCl to a
centration of 1 mol/L. Maximal release was always >90%
of incorporated counts. Minimal release is described in the Results
section. The LD50 for H2O2 concentrations was calculated as previ-
ously described.1 The assays were performed in RPMI media
supplemented with 5% FCS. The media and serum were specifically
selected for low endotoxin levels, and endotoxin contamination was
not detected by the Limulus assay (<0.125 eu/mL).

Statistics. The t test was used for all P value determinations.

RESULTS

PMN lysis of OC targets. Peripheral blood PMNs were
stimulated with PMA in endotoxin-free media and mixed
with chromatized targets for varying durations. Eight freshly
explanted OC targets were studied initially. Because of the
high spontaneous chromium release (>40% of incorporated
counts), two targets could not be tested. The spontaneous
release after 18-hour culture from the other six was usually
<30% (80% of experiments), and only these experiments
were used to compute the data shown in Fig 1. None of these
six fresh targets demonstrated >10% PMN lysis during the
first nine hours of incubation. However, substantial lysis of
three of the six specimens (nos. 204, 222, and 348) was
detected after 18-hour incubations. Spontaneous release was
too high to test lysis after 18 hours.

In all experiments at either 3, 6, 9, or 18 hours, targets
cultured with PMNs in the absence of PMA did not result in
significant lysis. In addition, incubation of targets (six to 18
hours) with PMA alone (no PMNs) did not elicit significant
chromium release. Furthermore, pretreatment of targets
with PMA for one to four hours followed by washing did not
increase the lysis of OC cells when either unstimulated
PMNs or PMA-stimulated PMNs were used as effector
cells. PMA-stimulated PMN lysis of OC cells at 18 hours
was the same as that obtained with no. 348 cells to cryopreserve; thus, further investigation of this target was impossible.

In vitro proliferation of the explanted OC cells was usually
maintained for only 2 to 3 months. During this time, we could
not grow sufficient numbers of no. 348 cells to cryopreserve;
thus, further investigation of this target was impossible.

In contrast, stimulated PMNs from two CGD patients were ineffective
(<3% lysis at all E:T ratios). Likewise, lysis of no. 222 cells
at 18 hours by control PMNs from two healthy donors was
19% ± 1.5% and 26% ± 2.3%, whereas CGD PMNs were
ineffective (lysis <4% at all E:T ratios).

Figure 2 summarizes experiments utilizing inhibitors/
scavengers of ROIs (at least three experiments for each
inhibitor). In all cases, the added reagents in concentrations
shown were not toxic to targets when added alone. As shown,
catalase (but not heat-inactivated catalase) markedly inhib-
it lysis of both targets. When the same concentration of
catalase was added at three or six hours after initiation of the
assay, the detected lysis at 18 hours was unaltered. Thus,
H2O2-induced events resulting in enhanced lysis are com-
pleted in the first three hours of the assay. SOD had no effect
on no. 204 but inhibited lysis of no. 222. Heat-inactivated
SOD had no effect on the lysis of no. 222 (not shown).

Neither did myeloperoxidase (MPO) inhibitors methimazole
and aminotriazole have any effect, indicating that peroxi-
dase-catalyzed reactions of PMNs are not critical. Several
known scavengers of hydroxyl radicals (mannitol, DMSO,
thiourea) significantly inhibited PMN cytotoxicity against
no. 222 but had little effect on no. 204. In concurrent
experiments, urea demonstrated no significant effect. Since
this agent is biochemically related to thiourea but is incapabe
of scavenging hydroxyl radicals,8 these control assays

Fig 1. Time course of PMN-induced lysis of OC cells: E:T
ratio = 25:1.
Fig 2. Effect of ROI scavengers on lysis of OC cells. Data are percentage of inhibition, mean ± SD of at least three experiments for each inhibitor. Inhibition determined from lysis at an E:T ratio of 25:1. Inhibitor concentrations: catalase 5,000 U/mL, SOD 200 U/mL, methimazole 1 mmol/L, aminotriazole 25 mmol/L, mannnitol 50 mmol/L, DMSO 750 mmol/L, thiourea 50 mmol/L, urea 50 mmol/L, and deferoxamine 1 mmol/L. Open bars: no. 204. Hatched bars: no. 222. Significant (P < .05) depression of lysis of no. 204 by catalase; significant depression of lysis of no. 222 by catalase, SOD, mannnitol, DMSO, thiourea, and deferoxamine.

Further support a role for these moieties in lysis of no. 222 cells. Furthermore, the iron chelator deferoxamine also significantly inhibited lysis of no. 222 cells. Concentrations of 1 mmol/L were most effective, but significant depression of lysis was also observed with 0.5 mmol/L deferoxamine (~35% inhibition). By chelating iron, deferoxamine would prevent formation of hydroxyl radicals by the iron-catalyzed Fenton reaction. In contrast, deferoxamine saturated with iron had less of an inhibitory effect (Table 1). Table 1 also suggests that the iron required for hydroxyl radical generation was derived from the targets rather than from PMNs. Pretreatment of no. 222 with deferoxamine inhibited subsequent PMN lysis, whereas pretreatment of PMNs had no effect.

Oxidative lysis in cell-free systems. A similar increase in tumor lysis in 18-hour assays was observed when no. 204 was exposed to preformed or enzymatically generated H$_2$O$_2$ and no. 222 was exposed to enzymatically generated hydroxyl radicals in cell-free systems. Lysis of no. 204 by increasing concentrations of reagent grade H$_2$O$_2$ revealed an LD$_{50}$ of 7.3 × 10$^{-3}$ mol/L when the assay duration was six hours. When the assay was prolonged to 18 hours, the shape of the curve was identical but was significantly shifted to the left. The resulting LD$_{50}$ of 1.2 × 10$^{-4}$ mol/L indicated a more than tenfold increase in the sensitivity of no. 204 to H$_2$O$_2$. Catalase (5,000 U/mL) present during the 18-hour assay completely abrogated lysis by H$_2$O$_2$. However, addition of the same concentration of catalase at three or six hours of the assay had no effect on the enhanced lysis detected at 18 hours. When H$_2$O$_2$ was generated by adding xanthine (1 mmol/L) to different concentrations of XO, enhanced lysis of no. 204 was also demonstrated with the longer assay. As shown (Table 2), there was <10% with enzyme concentrations of 0.005 to 0.025 U/mL after six hours. In contrast, significant lysis (46% to 86%) was present after 18 hours incubation with these concentrations of XO. In all cases, catalase, but not heat-inactivated catalase, abrogated lysis. In addition, xanthine or XO was not toxic to targets when used alone at these concentrations, and DMSO, mannnitol, thiourea, methimazole, and aminotriazole had no effect on lysis (data not shown).

Table 1. Inhibition of Lysis by Deferoxamine

<table>
<thead>
<tr>
<th>Use of Deferoxamine</th>
<th>Lysis (%)*</th>
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<tbody>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>A. None</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>B. In assay</td>
<td>12 ± 0.9</td>
</tr>
<tr>
<td>C. In assay, iron saturated</td>
<td>19 ± 1.9</td>
</tr>
<tr>
<td>D. Pretreatment of PMNs</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>E. Pretreatment of targets</td>
<td>18 ± 1.8</td>
</tr>
</tbody>
</table>

PMN-stimulated PMNs and chromated no. 222 targets incubated for 18 hours and percentage of lysis assayed. In group B, deferoxamine 1 mmol/L was present during the entire assay. In group C, deferoxamine had been previously saturated with iron as described. 15 In group D, PMNs were preincubated with deferoxamine 1 mmol/L for three hours, washed three times, and then added to targets in the presence of PMA. In group E, no. 222 cells were chromated, preincubated with deferoxamine 1 mmol/L for three hours, washed three times, and then added to PMN-stimulated PMNs.

*Percentage of lysis determined at E:T ratios of 25, 5, and 1:1. Data are mean ± SD of quadruplicate samples. This experiment repeated with identical results.

Table 2. Oxidative Lysis in Cell-Free Systems

<table>
<thead>
<tr>
<th>Target No. 204</th>
<th>Incubation Time (h)</th>
<th>XO Concentration (U/mL)</th>
<th>Lysis (%)*</th>
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<tbody>
<tr>
<td></td>
<td>Catalase</td>
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<td>0.01</td>
</tr>
<tr>
<td>6</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>18</td>
<td>Hi†</td>
<td></td>
<td></td>
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</tbody>
</table>

Target No. 222

<table>
<thead>
<tr>
<th>Incubation Time (h)</th>
<th>Reagents Added</th>
<th>Inhibitors</th>
<th>Lysis (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>3 ± 1</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td>3 ± 2</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td>24 ± 5†</td>
</tr>
<tr>
<td>18</td>
<td>H$_2$O$_2$ + FeSO$_4$</td>
<td>Catalase</td>
<td>3 ± 1†</td>
</tr>
<tr>
<td>18</td>
<td>H$_2$O$_2$ + FeSO$_4$</td>
<td>DMSO</td>
<td>8 ± 4‡</td>
</tr>
<tr>
<td>18</td>
<td>H$_2$O$_2$ + FeSO$_4$</td>
<td>Mannnnitol</td>
<td>7 ± 3‡</td>
</tr>
</tbody>
</table>

Cytotoxic assay run with designated chromated targets mixed with xanthine (1 mmol/L) and XO (0.005 to 0.025 U/mL) for no. 204 cells or H$_2$O$_2$ (1 × 10$^{-3}$ mol/L) + FeSO$_4$ (0.17 mmol/L) for no. 222 cells. Catalase was added at 5,000 U/mL; DMSO was added at 750 mmol/L, and mannnitol was added at 50 mmol/L.

*Specific lysis, mean ± SD of three to five experiments.
†Different from six-hour control, P < .05.
‡Different from corresponding control performed without inhibitor (catalase, DMSO, or mannnitol), P < .05.
§Hi†, heat-inactivated; enzyme boiled for 20 minutes.
To the no. 222 target, we added EDTA-chelated ferrous sulfate (0.17 mmol/L) and reagent grade H₂O₂ (10⁻³ mol/L). The ferrous sulfate and H₂O₂ in the concentrations used were not toxic when used individually (Table 2). However, the combination achieved lysis of 24% when no. 222 targets were exposed for 18 hours (Table 2). In contrast, only 1% lysis was present after a six-hour incubation. Cytotoxicity at 18 hours was probably due to hydroxyl radicals since (a) it was completely dependent on the presence of iron and, (b) DMSO and mannitol significantly inhibited the detected lysis (Table 2), whereas aminotriazole and methimazole had no effect (not shown). In addition, catalase (5,000 U/mL) present from time 0 prevented lysis but had no effect when added at three or six hours after initiation of the assay (not shown). Phorbol pretreated no. 204 and no. 222 targets (10⁻⁷ mol/L for one to four hours followed by washing) did not exhibit increased sensitivity to lysis by either reagent grade H₂O₂ or the combination of H₂O₂ and ferrous sulfate.

The sensitivities of nos. 204 and 222 to oxidants during 18-hour assays were directly compared in three separate experiments. The LD₉₀ for H₂O₂ was comparable (10⁻⁴ mol/L for no. 204 and 3.4 x 10⁻⁴ mol/L for no. 222). However, no. 204 targets could not be lysed by the combination of EDTA-chelated ferrous sulfate (0.17 mmol/L) and H₂O₂ (10⁻⁵ mol/L), whereas no. 222 targets were sensitive (Table 2).

**DISCUSSION**

Although previous investigators⁴⁻¹¹ have documented the ability of human PMNs to lyse continuously cultured hematopoietic human tumor lines, this study is the first indication that these effectors can kill freshly obtained human tumor cells. In contrast to our results, a recent report by O'Donnell-Tormey et al⁵ demonstrated a marked resistance of nonhematopoietic human tumor targets to PMA-stimulated human PMNs as well as to preformed or enzymatically generated ROIs. In our study, three of six OC targets remained resistant after 18-hour incubations, but three other specimens were sensitive to PMN lysis. Thus, complete resistance of human tumor cells may not be a generalized phenomenon. Whether the effective lysis of three of our targets depended on our use of freshly explanted tumor cells (O'Donnell-Tormey et al used continuously cultured established lines), a peculiar sensitivity of OC cells, or the longer duration of our cytotoxicity assay is unclear. Certainly, most other reports have studied PMN-induced or ROI-mediated cytolyis with incubations of six hours or less. Our data suggest these assays may underestimate the potential for PMNs in human tumor control. The degree of OC lysis we detected was comparable to that achieved by monocytes and macrophages⁶ and IL-2-activated lymphocytes.¹² Lysis depended on production of ROIs.

Although experiments with CGD PMNs indicated that lysis was oxidative in nature, the identity of the ultimate cytotoxins is not entirely clear. We investigated this issue by using ROI inhibitors or scavengers, but their lack of specificity is a limitation of this approach. This is a particular problem with the no. 222 target and putative hydroxyl radical scavengers. At the concentrations we used, DMSO can also depress superoxide and H₂O₂ production¹³ and thiourea can prevent PMN binding to targets.¹⁴ The inhibitory effects of the iron-chelator deferoxamine may also be nonspecific. Of concern is the only modest reversal of inhibition when iron-saturated chelator was used (Table 1). A recent report¹⁵ documented deferoxamine's ability to inhibit MPO-catalyzed reactions. Although the lack of inhibition by aminotriazole or methimazole appears to mitigate against products of the H₂O₂-halide-MPO system, by increasing the accumulated H₂O₂ concentrations to lytic levels,¹⁶ the inhibitors may have masked a PMN-cytotoxic effect mediated by the MPO system. However, killing of no. 222 by the MPO system would be inconsistent with the effects of SOD. This enzyme inhibitor should either have little effect or should actually increase lysis by accelerating the rate of formation of H₂O₂. In contrast, SOD was quite inhibitory to lysis of no. 222 (Fig 2).

If deferoxamine indeed prevented lysis by chelating iron, Table 1 suggests the required iron was derived from the no. 222 tumor target itself. This is similar to other studies in which target-derived iron was required for the oxidative lysis of hepatocytes¹⁷ and endothelial cells.¹⁸ However, if target-derived iron is sufficient for oxidative lysis by intact PMNs (Table 1), why was exogenous iron required for lysis by 10⁻³ mol/L reagent-grade H₂O₂ in the cell-free system (Table 2)? One possible explanation is that the required target-derived iron is in the ferric state (Fe³⁺) and must be first reduced to ferrous iron (Fe²⁺) before it can serve as a substrate for H₂O₂ (Fenton reaction). Stimulated PMNs (used as shown in Table 1) can achieve this through secretion of superoxide which reduces Fe³⁺ to Fe²⁺, allowing it to react subsequently with H₂O₂ (Haber-Weiss reaction). The data in Table 2 suggest that the no. 222 target may be unable by itself to reduce the ferric iron critical for cytolyis. Thus, when exposed to reagent-grade H₂O₂ alone (Table 2, line 2), it is not injured unless an exogenous source of ferrous iron is also present (Table 2, line 4).

Prolonging our cytotoxicity assay to 18 hours increased the sensitivity of targets to ROIs presented in cell-free systems as well as to stimulated PMNs. These data suggest that detection of PMN-mediated lysis at 18 hours (in contrast to 3, 6, or 9 hours) is due to the increased susceptibility of targets to ROIs. The mechanism of increased sensitivity is unknown. One possibility is that the cellular defenses against ROIs, such as the glutathione redox cycle or target cell catalase, must be depleted before injury occurs. A second possibility is that successful lysis depends on a synergistic interaction between ROIs and products of nonoxidative metabolism such as granule proteins. We previously documented such an interaction between H₂O₂ and peptide defensins of primary granules.¹⁹ A slower release of such granule components or a slower mechanism of action might explain the successful lysis which occurs only with longer incubations. A third possibility is that a rapid H₂O₂-mediated insult is followed by target-dependent events that occur slowly and eventually result in disruption of plasma membrane integrity. The absence of protection when catalase is added at three or six...
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hours to stimulated PMNs or to cell-free ROIs indicates that the lethal lytic events are completed early, providing support for this latter hypothesis.

With reagent-grade H2O2, the sensitivities to lysis of nos. 204 and 222 were comparable (LD90 of 10^-4 and 3.4 x 10^-4 mol/L, respectively). In contrast, only no. 222 was susceptible to lysis by lower concentrations of H2O2 combined with iron. This suggests that both targets can be lysed by H2O2 generated by PMNs but only no. 222 can be lysed by hydroxyl radicals. The comparable H2O2 sensitivities might explain why optimal concentrations of the hydroxyl radical scavengers mannitol, DMSO, and the chelator deferoxamine can inhibit only ~50% of lysis (Fig 2). These inhibitors would have less of an effect on H2O2 which could then, by itself, lyse no. 222.

Two major questions remain concerning the role of PMNs in antitumor responses. First, whether our results can be generalized to other tumor types of nonovarian origin is unclear. Second, since our studies were performed with PMA, which is an optimal PMN stimulant, whether more physiologic stimulators can trigger production of sufficient quantities of ROIs to achieve lysis of these targets is unknown. These questions are currently under investigation.

REFERENCES

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