Stimulation of the Respiratory Burst of Murine Peritoneal Inflammatory Neutrophils by Conjugation with Tumor Cells

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ABSTRACT

Murine peritoneal neutrophils (PMNs), elicited by i.p. injection of formalin-killed Corynebacterium parvum, spontaneously lyse teratocarcinoma targets through the secretion of reactive oxygen intermediates. Examination of effector-target interactions at the single cell level revealed that PMNs conjugated to tumor cells were 3-fold more frequently stained by nitroblue tetrazolium compared to nonconjugating PMNs suggesting that tumor targets stimulated a potent tumor-lytic respiratory burst. This notion was confirmed by the detection of superoxide and hydrogen peroxide generation from PMNs as well as a luminescent response following conjugation with viable tumor targets. Generation of superoxide was dependent upon the presence of dihydrocytochalasin B. In addition to teratocarcinoma cells, comparable stimulation was achieved by conjugation with YAC and P815 targets but not thymocytes. Reactive oxygen intermediate release was also achieved by mixing peritoneal PMNs with heat-killed tumor cells. In contrast to bacteria-induced effectors, PMNs elicited by i.p. injection of thioglycollate were incapable of responding following conjugation with tumor targets although they were competent for reactive oxygen intermediate release when stimulated by phorbol myristate acetate. Teratocarcinoma targets were sensitive to concentrations of H2O2 that could be achieved by PMNs following contact. These data indicate that Corynebacteria-elicited inflammatory PMNs lyse their bound tumor targets by a mechanism similar to a stimulus-secretion model.

INTRODUCTION

The secretion of ROIs by PMNs mediates their potent tumor-lytic effect in vitro (1–5). The relevance of this phenomenon, however, is in question since, in all in vitro studies, PMNs are exposed to exogenous activators which provide an artificially maximal stimulation of the respiratory burst. It is unknown whether the physiological conditions of tumor cell contact could stimulate a burst sufficient for cytolysis. Prior studies in a MOT model demonstrated that successful i.p. therapy with formalin-killed bacteria was, in part, due to the accumulation of tumor-lytic PMNs in the peritoneal cavity (6). Tumor lysis was confirmed in vitro with the use of an isotope release assay. Significant cytotoxicity occurred when 10^4 - 5 x 10^5 PMNs were mixed with 10^4 targets in 0.2 ml (effector:target ratio, 1:50:1). Lysis was abrogated by SOD and catalase confirming a role for superoxide and H2O2 (7). Although PMN lysis was due to the secretion of ROIs, the addition of extrinsic activators to these cells was not required.

In our prior studies (7, 8), we have used a single cell conjugate and cytotoxicity assay to evaluate PMN effects on tumor cells. Conjugation of tumor cells by bacteria-elicited PMNs was related to the E:T ratio used (8), was dependent upon magnesium ions (7), was temperature independent (8), and necessary for tumor lysis (7, 8). A clue to the nature of the signal which triggered peritoneal PMNs for a tumor-lysing respiratory burst was provided by experiments where NBT was added to the conjugate assay. The frequency of positive NBT staining was 3x higher in tumor-binding PMNs compared to free unbound PMNs suggesting that tumor cells stimulated a respiratory burst from effectors. In the current study, we tested this hypothesis by assaying the production of H2O2, superoxide anion, and the chemiluminescent response of PMNs following interaction with tumor cells. The results confirm that bacteria-elicited peritoneal PMNs can be stimulated by tumor cells for a significant oxidative burst which has the potential for damaging bound tumor targets.

MATERIALS AND METHODS

Mice. Female C3HeB/FeJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6–8 weeks of age.

Tumor Lines. The murine ovarian teratocarcinoma (MOT; 9) and P815 tumors were passaged i.p. in C3H and DBA/2 mice. The YAC tumor line was maintained in vitro in RPMI media supplemented with 10% FCS (Reheis, Phoenix, AZ). All three lines were tested and found to be free of mycoplasma contamination.

Drugs. Corynebacterium parvum was purchased from Burroughs-Wellcome (Research Triangle Park, NC). Thioglycollate broth was obtained from Difco (Detroit, MI). Sodium azide was obtained from J. T. Baker Chemicals (Phillipsburg, NJ). BCNU was purchased from Bristol Labs (Syracuse, NY). Luminol was obtained from Sigma (St. Louis, MO), dissolved in 100% dimethyl sulfoxide, aliquotted in small stock solutions, and stored at −70°C. All other compounds were obtained from Sigma.

Treatment and Lavage of Mice. Mice were injected i.p. with a 0.2-ml solution containing 1400 μg of C. parvum or a 10% solution of thioglycollate. Twelve h later, peritoneal cavities were lavaged with 10 ml of cold phosphate buffered saline. PECs were washed at 200 x g which allowed cells to be recovered in the pellet while free bacteria were removed in the supernatant (10). Viability of PECs was always over 96%. PECs contained 93 ± 4% PMNs (mean ± SD of five experiments), 5 ± 0.4% lymphocytes and 2 ± 0.6% macrophages as determined by 200 cell differentials on stained smears.

Single Cell Conjugate Assay (SCCA). SCCA was a modification of the method of Grimm and Bonavida (11) as previously described (8, 12) with the addition of NBT to detect PMNs producing ROIs. Briefly, PECs were resuspended to 2 x 10^6/ml and MOT targets were resuspended to 10 x 10^6/ml in RPMI and 1% FCS. Effectors (0.15 ml) were mixed with targets (0.15 ml) in 10 x 75 mm borosilicate tubes and incubated at 30°C for 10 min. The tubes were centrifuged at 200 x g for 5 min and the supernatant was decanted. Fifty μl of 0.1% NBT in RPMI was added to the cell pellet. An equal volume of molten agarose was then added before placing the cell mixtures on slides to bring the final concentration of NBT to 0.05%. Slides were then immersed in media containing 0.05% NBT for a 1-h incubation at 37°C. All slides containing NBT were kept protected from light until examination. To identify effector cells, slides were flooded with safranin for 90 s and then immersed in water for 60 s to remove excess stain. Percentage of conjugation was calculated as number of conjugates/total number of PMNs (bound and unbound) x 100. At least 200 PMNs/slide were examined and enumerated to determine percentage of conjugation. The...
sides were run in triplicate and the SD was always less than 5% of the mean values. The data is recorded as mean ± SE of separate experiments. We have previously demonstrated (6, 8, 12) that all conjugates consist of either one target bound to one PMN (90%) or one target bound to two or more PMNs (10%). Any PMN containing dark blue formazan deposits was considered NBT-positive. Percentage of NBT-positive conjugating PMNs was calculated as follows: number of PMNs bound to tumor cells stained positively with NBT/total number of PMNs bound to tumor cells x 100.

Assay for Superoxide Production. Superoxide production was assayed spectrophotometrically at 550 nm at 37°C by the SOD-inhibitable cytochrome C reduction assay (13). Reaction mixtures [in 1 ml of a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered balanced salt solution] contained 2 x 10^6 PECs, 50 μM ferricytochrome C, and either 30 μg of SOD or no SOD. In some assays, cells were preincubated with DHCB (2.5 μg/ml) for 10 min. PMNs were either assayed alone, in the presence of 10^{-7} M PMA (added in 5 μl), or following conjugation with tumor targets. In the latter groups, PMNs were first mixed with targets in 10 x 75 mm borosilicate tubes and centrifuged for 3 min at 200 x g at 200 x g. The pellet was gently resuspended (by slowly drawing the mixture into a 50-μl MLA pipet) and transferred to the rest of the reaction mixture in 10-cm path-length semimicro quartz cuvets. Superoxide generation was recorded continuously for 20-40 min. Absolute rates of maximal superoxide generation were calculated from the linear portion of the response, using an extinction coefficient of 21.1 mM^-1 cm^-1. Control experiments demonstrated that centrifugation of PMNs alone (no targets present) did not stimulate the release of superoxide.

Chemiluminescence Assay. Luminol-enhanced chemiluminescence was assayed at room temperature in a Beckman liquid scintillation counter set in the "out-of-coincidence" mode. 5 x 10^{10}-10^{11} PECs were dark adapted for 30 min in 0.9 ml RPMI containing 2 x 10^{-6} M luminol (0.01% dimethyl sulfoxide) and 1% FCS in glass scintillation vials. The vials were then centrifuged (3 min at 200 x g) after addition of either 0.1 ml of media or 0.1 ml of targets. The pellets were gently resuspended and counts per min were recorded at 30-s intervals for a duration of 20-40 min. Data is presented as mean cpm at 30-s intervals. In some experiments, PECs were first dark adapted and then stimulated by the addition of varying concentrations of tumor membrane preparations. Luminol alone gave a recording of 35 x 10^3 cpm. In some experiments, chemiluminescence was compared between experimental groups by computing the area under the plotted curves for 30-min durations by weighing traced paper templates.

Assay for H2O2 Production. H2O2 production was measured by the loss of fluorescence from scopoletin as previously described (14). H2O2 produced by PMNs assayed alone or following stimulation with PMA (10^{-7} M) or tumor targets was plotted against a standard curve of scopoletin reduction which was generated by sequential additions of 0.5 n mole of reagent grade H2O2 in the presence of 5 x 10^{-4} M scopoletin and 50 μg/ml of horseradish peroxidase. Cells were assayed in plastic disposable cuvets. To form close cell-to-cell contact between PECs and targets, these cuvets were centrifuged for 3 min at 200 x g and the pellets were gently resuspended. Centrifugation of PMNs alone (assayed without tumor cells) did not affect the base line (nonstimulated) production of H2O2.

Use of Pharmacological Agents. Interruption of the glutathione reduct cycle was achieved by treatment with BCNU. BCNU was dissolved in absolute ethanol and diluted with media before addition to tumor targets. MOT cells were incubated with 100 μg/ml BCNU (final ethanol concentration, 0.009%) for 10 min at 37°C as previously described (15). Following subsequent washing in RPMI, tumor cell viability was always over 94% and the glutathione reductase activity was decreased by 95% (relative to tumor cells identically treated with media and 0.09% ethanol).

Sodium azide was dissolved in media and added to MOT cells at 1 mM beginning 15 min prior to assay. Aminotriazole (3-amino-1,2,4-triazole) was added to cells at 50 mM beginning 30 min prior to assay.

Heat-Killing of Cells. Effector or tumor cells were killed by heating to 56°C for 30 min.

Determination of H2O2 Sensitivity of Target Cells. To determine sensitivity to lysis by H2O2, tumor cells were first radiolabeled by incubating 10^7 cells with 50 μCi of chromium-51 for 1 h at 37°C in a shaking water bath in RPMI and 10% FCS. After several washes, tumor cells (10^6 in 0.2 ml of RPMI with 1% FCS) were added to flat-bottomed microtiter plate wells containing increasing concentrations of reagent grade H2O2. After 3 h of incubation at 37°C on a 5% CO2 atmosphere, plates were centrifuged (1200 rpm for 10 min) and 0.1 ml of supernatant was removed and counted in a γ counter. Percentage of lysis was determined as:

\[
\frac{\text{cpm with H}_{2}\text{O}_2 - \text{cpm minimal release}}{\text{cpm maximal release - cpm minimal release}} \times 100 \quad (A)
\]

Minimal release was determined by incubating targets in medium alone.

RESULTS

Correlation of PMN-NBT Staining with Tumor Cell Binding. NBT was added to the conjugate assay to identify peritoneal inflammatory PMNs actively producing reduced oxygen moieties. Positive NBT staining was significantly more common in PMNs conjugated to tumor targets (78 ± 6%, mean ± SE, five experiments) than in free unconjugated PMNs (23 ± 5%). NBT-positive conjugating PMNs demonstrated coarse dark granules of formazan adjacent to the zone of contact with tumor targets. The presence of superoxide dismutase (200 U/ml) prevented this coarse staining. NBT-positive nonconjugating PMNs showed light-speckled staining in a symmetrical pattern throughout the cell.

Superoxide Generation by Tumor-conjugating PMNs. Initial experiments tested the ability of MOT targets to interfere with the detection of superoxide production. Peritoneal PMNs stimulated with PMA (10^{-7} M) generated a peak value of 5.1 ± 0.4 nm/2 x 10^6 cells/min (mean ± SD of three experiments). The maximal rate of superoxide generation by PMA-stimulated PMNs fell to 0.2 ± 0.07 and 0.6 ± 0.2 nm/2 x 10^6 cells/min, respectively, when 10 x 10^6 or 5 x 10^6 viable or heat-killed MOT cells were added to the cuvet. In contrast, there was no decrease in cytotoxic C reduction when only 2 x 10^6 MOT cells were added. In addition, MOT targets never generated superoxide in the presence or absence of DHCB. Since 2 x 10^6 tumor cells did not prevent detection of superoxide generation, an E:T ratio of 1:1 (2 x 10^6 PMNs:2 x 10^6 MOT cells) was utilized to assess a possible PMN response following tumor conjugation. When peritoneal PMNs were mixed with MOT cells and cocentrifuged for 3 min, no significant superoxide generation was detected over the background samples of peritoneal PMNs assayed alone (Fig. 1). However, in the presence of DHCB, PMNs began to release superoxide approximately 6 min after centrifugation, generating 10 ± 1.2 nm/2 x 10^6 cells/5 min (mean ± SD of five experiments). The percentage of PMNs conjugating tumor cells was not affected by the addition of DHCB. PMNs assayed in the presence of DHCB but without tumor targets had no detectable response (Fig. 1). In addition, 2 x 10^6 PMNs mixed with thymocytes (E:T ratios, 5:1, 1:1, 1:5, and 1:10 tested) in the presence or absence of DHCB demonstrated no response. As expected, conjugating 2 x 10^6 PMNs with higher numbers of tumor targets (E:T ratios, 1:10, 1:5, and 1:2) produced less cytotoxic C reduction (0.4, 1.1, and 3.3 nm/2 x 10^6 cells/5 min) probably because excessive numbers of MOT cells prevented superoxide detection. An E:T ratio of 5:1 (2 x 10^6 PMNs:4 x 10^5 MOT cells) also produced
response of PMNs alone. As shown in Fig. 2, this enhanced response depended upon centrifugation of the effector-target cell mixture suggesting that intimate PMN-tumor cell contact is necessary for the enhanced chemiluminescent response. Centrifugation of PMNs alone (without targets) did not increase their response (Fig. 2). The base line response of PMNs and the enhanced response of PMN-MOT cell mixtures was abrogated by treatment of PMNs with aminotriazole or sodium azide (over 90% inhibited response) but SOD only had a modest effect (25% decrease). This is consistent with a prior report which indicated that luminol-dependent chemiluminescence was due to species resulting from a myeloperoxidase-catalyzed reaction (16).

Heat-killed peritoneal PMNs did not generate a chemiluminescent response when mixed with viable targets. However, the enhanced response was not diminished when heat-killed targets were mixed with viable PMNs (Table 2). Thus, similar to superoxide generation, chemiluminescence is produced by effector cells and not targets.

The chemiluminescent response of peritoneal PMNs is not specific for MOT tumor targets (Fig. 3). As shown, a significantly enhanced response above that of PMNs assayed alone is also realized when P815 or YAC targets are added to effectors. In contrast, the addition of murine thymocytes in a range of E:T ratios between 5:1 and 1:10 did not elicit a response. The chemiluminescent response correlated with the ability of PMNs to conjugate these respective targets (parentheses in Fig. 3).

Fig. 4 demonstrates that the effective stimulation of chemiluminescence by tumor conjugation is relatively specific for peritoneal PMNs elicited by i.p. injection of formalin-killed bacteria. Although thioglycollate-induced PMNs conjugated a comparable percentage of tumor targets (23 ± 2% at E:T ratio of 1:2, mean ± SD of three experiments) compared to bacteria-elicited PMNs (25 ± 2%), they were incapable of generating chemiluminescence following conjugation with tumor targets. In contrast, thioglycollate-elicited PMNs were competent for responding to PMA (Fig. 4). In control experiments not shown, the addition of thioglycollate (10% v/v) or thioglycollate-induced PMNs to bacteria-elicited PMNs did not suppress the chemiluminescent response. Thus, the lack of response of thioglycollate-elicited PMNs was not due to a quenching effect on chemiluminescence. These data correlate with our previous studies which demonstrated that thioglycollate-induced peritoneal PMNs were significantly less effective than bacteria-elicited PMNs in mediating oxygen-dependent tumor lysis (12).

**H₂O₂ Production.** A standard curve of scopoletin reduction was generated by sequential additions of 0.5 nmol reagent grade H₂O₂ to scopoletin and horseradish peroxidase in the presence or absence of MOT targets (Fig. 5). MOT cells prevented the detection of scopoletin reduction when added in numbers of 1–8 × 10⁶/cc. In contrast, 2–16 × 10⁶/cc thymocytes did not alter the standard curve. When MOT tumor cells were treated with aminotriazole (50 μM), azide (1 μM), or BCNU (100 μg/ml), their interference with the assay was not diminished (Fig. 5). However, simple heat killing of targets (56°C for 30 min) prevented their quenching effect. Heat-killed targets were trypan-blue positive but were morphologically intact and were conjugated by bacteria-elicited PMNs (28 ± 3% at E:T ratio of 1:2, three experiments) as frequently as were viable targets (25 ± 2%). Additional experiments demonstrated that the detection of H₂O₂ production by PMA (10⁻⁷ M)-stimulated inflammatory PMNs (4.5 nmol/2 × 10⁶ cells/5 min) was markedly inhibited in the presence of 2 × 10⁶ viable MOT cells (0.4 nmol/2 × 10⁶ cells/5 min) but not by heat-killed MOT cells (4.3 nmol/2 × 10⁶ cells/5 min).
10^6 cells/5 min). Finally, both viable and heat-killed MOT cells did not produce H_2O_2 in the presence or absence of PMA. Because of these data, the following experiments utilized heat-killed MOT cells as potential stimulators for an H_2O_2 response from inflammatory PMNs.

The production of H_2O_2 by bacteria-elicited PMNs is shown in Table 3. They spontaneously produced a small amount of base line H_2O_2 accumulating 0.274 ± 0.008 nmol/2 × 10^6 cells over 5 min (mean ± SD of three experiments). When PMNs were conjugated with heat-killed MOT targets (2 × 10^6 PMN:8 × 10^6 MOT) and then assayed, the amount of H_2O_2 produced significantly increased to 1.11 ± 0.3 nmol/2 × 10^6 cells/5 min. For comparison, in the same experiments, 10^-7 M PMA stimulated the release of 4.51 ± 0.4 nmol/2 × 10^6 cells over 5 min. Scopoletin reduction caused by PMNs assayed alone or after conjugation with heat-killed MOT targets (2x10^6 PMN:8 × 10^6 in this experiment) gave no response. Data presented as average of duplicate samples in one experiment. Three additional experiments yielded identical results.

**DISCUSSION**

Previous work has confirmed that in vitro tumor lysis by bacteria-elicited peritoneal PMNs is due to the secretion of H_2O_2 and superoxide (7). Sufficient production of these lytic molecules apparently does not require exogenous activators of the respiratory burst. The results of this study indicate that stimulation from tumor cells is an important signal for generation of a tumor-lytic oxidative burst by peritoneal PMNs. Mixing viable and heat-killed tumor targets with PMNs stimulated the production of superoxide anion, H_2O_2 and luminol-dependent chemiluminescence. Tables 1 and 2 confirm that ROIs were produced by PMNs and not by tumor cells. Furthermore, since the P815 and YAC targets were continuously passed in vitro, it is unlikely that antibody bound to their membrane antigens stimulated PMNs through binding to the receptor for the Fc fragment of IgG.

Other trivial explanations for the data are also unlikely. First, although mycoplasma contamination of tumor lines can trigger production of ROIs in leukocytes (18), our tumor cell preparations were free from infection with this organism. Second, although others (19, 20) have described cytokines released by NK cells following tumor contact that activated oxidative metabolism in phagocytes and although there may be a few NK cells in our PEC population, this phenomenon cannot explain our results since NK-resistant (P815) targets were equally effective in stimulating PMNs whereas P815 targets were ineffective in the aforementioned studies (19, 20). In addition, a generalized increase in oxidative metabolism of PMNs could not explain the results of the SCCA where the frequency of NBT staining only increased in PMNs that conjugated targets.

The data suggest that actual contact with target membranes is required for a PMN respiratory burst. First, PMNs in contact with targets had a 3-fold greater frequency of NBT staining only increased in PMNs that conjugated targets. Sensitivity of MOT Cells to H_2O_2. Fig. 6 demonstrates that MOT targets are sensitive to lysis by H_2O_2. These values are within the range detected by Nathan et al. (17) for other murine tumor targets.
TUMOR-STIMULATED PMN OXIDATIVE BURST

Fig. 3. Chemiluminescent response of peritoneal PMNs following conjugation with different targets. PMNs assayed alone (O), or following conjugation with MOT (O), P815 (□), YAC (△), or thymocytes (□) targets (E:T ratio, 1:2). Thymocytes (O) and all three tumor lines (□) did not show any response. Data presented as average of duplicate samples in one experiment. Two other experiments yielded identical results. PMN-thymocyte mixtures also assayed at E:T ratios of 5:1, 1:1, 1:5, and 1:10 and never demonstrated a chemiluminescent response. Numbers in parentheses, percentage of conjugation of targets by PMNs at an E:T ratio of 1:2 (mean ± SE of three experiments).

Fig. 4. Chemiluminescent response of bacteria-elicited PMNs compared to thioglycolate-elicited PMNs. C. parvum-PMNs (1 x 10⁶) assayed alone (A) or following centrifugation with MOT targets (2 x 10⁶) (A). Thioglycollate-PMNs also assayed alone (□) or following centrifugation with target cells (O). Thioglycollate-PMNs stimulated with 10⁻⁶ M PMA (D). Numbers in parentheses, percentage of conjugation of targets by PMNs at an E:T ratio of 1:2 (mean ± SE of three experiments).

decreased. Third, centrifugation of the effector-target cell mixture was required for a chemiluminescent response. Since the formation of PMN-tumor conjugates during the first 30 min depends upon centrifugation of cell mixtures, these data suggest that tumor contact is necessary for a detectable response. Experiments with semipermeable filters are planned to further investigate this issue.

The selectivity of PMN triggering, in regard to type of targets and type of effector mimics the selectivity of tumor lysis. First, YAC and MOT targets effectively stimulated an oxidative burst and were sensitive to PMN-mediated lysis (12) while thymocytes were not stimulatory and were not lysed by PMNs (8). Second, in contrast to bacteria-elicited PMNs, peritoneal PMNs obtained following i.p. injection of thioglycollate are not stimulated by targets for an oxidative burst and are markedly less lytic to targets (12). Although the conjugation of tumor targets by thioglycollate-elicited PMNs is comparable to that of bacteria-elicited PMNs, their staining for NBT following conjugation is significantly less [35 ± 5% in conjugating PMNs, three experiments, versus 71 ± 5% for bacteria-elicited PMNs (1)] which is consistent with their inability to respond with chemiluminescence after tumor contact. This deficiency is unlikely to be due to an active suppression or scavenging of ROIs by thioglycollate. Although thioglycollate can scavenge H₂O₂ (21), it is difficult to imagine how this effect could suppress internal cellular chemiluminescence. More importantly, the addition of thioglycollate or thioglycollate-elicited PMNs did not suppress the augmented response of chemiluminescence in PMNs elicited by i.p. injection of Corynebacteria.

Thus, it appears that certain characteristics of bacteria-elici-
either alone, following conjugation with tumor cells (heat-killed MOT targets; respond to nonspecific membrane contact with a detectable specific chromium release was then assayed. Targets were incubated with increasing concentrations of reagent grade H2O2 and assayed for H2O2 production as previously described (14). PMNs were assayed in a 1:1 ratio, 1:4) or following stimulation with PMA, 10^{-7} M. Data presented as the maximal rate of H2O2 produced in nmol/2 x 10^6 cells/5 min, mean ± SD of three individual experiments.

Table 3 Production of H2O2 by bacteria-elicited PMNs

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Catalase (5000 U/ml)</th>
<th>H2O2 Production (nmol/2 x 10^6 cells/5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>0.274 ± 0.008</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>0.015 ± 0.002</td>
</tr>
<tr>
<td>Tumor cells</td>
<td>-</td>
<td>1.11 ± 0.3*</td>
</tr>
<tr>
<td>Tumor cells</td>
<td>+</td>
<td>0.063 ± 0.01</td>
</tr>
<tr>
<td>PMA, 10^{-7} M</td>
<td>-</td>
<td>4.51 ± 0.4*</td>
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* Significantly greater H2O2 release (P < 0.05) than unstimulated control group.

Fig. 5. Loss of scopoletin fluorescence produced by adding reagent grade H2O2 to scopoletin and peroxidase in the presence or absence of MOT tumor targets. The linear portions of the curves are displayed for cuvettes assayed without tumor cells (O), with untreated MOT tumor cells (6 x 10^6/ml in this representative experiment, @), with BCNU-treated tumor cells (•) or with aminotriazole-treated tumor cells (•) or with heat-killed tumor cells (6). Azide treatment (1 mM) did not affect the tumor cell quenching effect (data not shown). This experiment was repeated two more times using MOT cells in concentrations of 1 x 10^6 and 8 x 10^6/ml with identical results. Thymocytes (O) had no effect on the standard curve.

Fig. 6. Sensitivity of MOT tumor cells to reagent grade H2O2. Radiolabeled targets were incubated with increasing concentrations of reagent grade H2O2 and specific chromium release was then assayed.

The particular requirements of the assay systems may have caused an understimation of the amounts of ROI's generated. First, tumor cells interfered with the detection of H2O2 and this could not be counteracted by treatment with azide, aminotriazole, or BCNU suggesting this scavenging effect was not due to either catalase or the glutathione redox cycle. Targets had to be heat-killed to prevent their quenching effect. Although the percentage of conjugation of such targets and their ability to stimulate chemiluminescence was comparable to viable targets, it is still possible that they provided a suboptimal stimulus for H2O2 release. Second, high numbers of viable or heat-killed tumor cells interfered with the detection of superoxide generation. When using 4-10 x 10^6 tumor targets, the suspensions were cloudy which may have caused interference with the measurement of the spectral change that accompanies the reduction of cytochrome C. Thus, only low target:PMN ratios could be employed to detect superoxide generation. Since the percentage of PMNs conjugating tumor cells significantly increases as the target:PMN ratio increases (8), the stimulus in this assay may also have been suboptimal. Third, since it is likely that ROI's were released primarily at the site of contact with tumor targets (Fig. 1), the indicators may have gained only limited access to the sites of secretion. Because of its extremely short half-life, the detection of superoxide might be a particular problem. In this regard, the levels of superoxide detected when PMNs were contacted with antibody-coated tumor cells (23) and micropore filters (25, 28) or basement membrane (24) coated with aggregated IgG, are comparable to that recorded in the present study. Of note, in the study by Vissers et al. (24), the more marked stimulation of oxygen consumption, H2O2 production, and production of cerium perhydroxide in the contact zone between cells and basement membrane suggested that the detection of superoxide generation was significantly underestimated.

In our assay, superoxide generation could only be detected in the presence of DHCB. The results are reminiscent of the enhancing effect DHCB has on generation of superoxide when PMNs are stimulated by phagocytosable particles in suspension. In these latter studies, the prevention of pseudopod and phagocytic vacuole formation by DHCB is thought to allow more superoxide to be generated at the cell surface where it is more accessible to cytochrome C. In similar fashion, DHCB may prevent the PMN from increasing the length of its contact zone with its bound target preventing further trapping of generated superoxide between the cells and allowing it to become more accessible to cytochrome C.

The amount of H2O2 produced following tumor conjugation may be sufficient to account for tumor lysis in vitro. PMNs released H2O2 at a maximal rate (1.11 nm/2 x 10^6 cells/5 min) for at least 30 min. By comparison, our own studies and those of Nathan et al. (17) demonstrate a greater response of murine peritoneal PMNs when stimulated by PMA [4.5 nm/2 x 10^6 cells (this study) and 5.8 nm/10^6 cells/5 min (17), respectively]. In our chromium-release assay (12) where 5 x 10^6 PMNs (E:T ratio, 50:1) are present in 0.2 ml, the H2O2 concentration would be expected to reach 8.35 x 10^{-6} M during the first 30 min, an amount that causes approximately 20-40% specific lysis of MOT targets.
There are several unknowns, however, in these calculations. It may be inaccurate to extrapolate the amount of H₂O₂ produced in wells containing E:T ratios of 50:1 from data of experiments employing ratios of 1:1 to 1:4. Since a lower percentage of PMNs bind to targets at higher E:T ratios, the calculations may be an overestimation. In addition, reagent grade H₂O₂ was added as a pulse and targets may be better able to inactivate and detoxify the gradual increase in ROIs produced by PMNs over time. On the other hand, if most of the H₂O₂ is generated within the small contact zone between PMN and target, the critical concentration may be significantly higher than the calculated value.

In summary, these results demonstrate that simple membrane contact with tumor cells stimulates the production of ROIs by bacteria-elicited PMNs. The preceding interactions that allow these cells to respond to subsequent triggering with heightened ROI production as well as the nature and specificity of the membrane interactions are under investigation. The characteristics are clearly important in the generation of the spontaneous lytic potential of these antitumor effectors.

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