Antitumor Activity of Murine Neutrophils Demonstrated by Cytometric Analysis

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ABSTRACT

The cytostatic and cytolytic activities of activated polymorphonuclear neutrophils (PMNs) against YAC-1 lymphoma target cells were examined using multiparameter flow cytometric analysis. PMNs were resolved from tumor cells by 90% light scatter. The number of surviving tumor cells was determined by adding a known concentration of fluorescent latex particles to the fixed cell suspension immediately prior to analysis and counting the particles simultaneously with the cells. Cell cycle progression of the YAC-1 target was studied by dual parameter analysis of DNA content and bromodeoxyuridine incorporation into tumor cell DNA either prior to or following addition of PMNs. The results indicate that activated PMNs effectively kill tumor cells within the first 24 h of coculture. However, between 24 and 48 h, tumor cells which escape destruction resume growth and eventually reach a growth rate greater than control cells.

INTRODUCTION

Immunological mechanisms associated with tumor cell killing include antibody-dependent cellular cytotoxicity, cytotoxic T-cells, natural killer cells, activated macrophages, and lymphokine-activated killer cells (1, 2). More recently, several studies have indicated that, under appropriate circumstances, PMNs develop in vitro and in vivo tumoricidal activity (3–6). PMN-mediated tumor cell killing has been shown to occur following treatment with pharmacological agents such as PMA (3), during phagocytosis (7), in association with antibody-dependent cellular cytotoxicity (8, 9), and in the presence of lectins (9). The antitumor activity of PMNs in vitro has been demonstrated by a variety of assay systems ranging from visual quantitation of tumor cell destruction to the release (for cytotoxicity) or incorporation (for cytostasis) of radiolabeled materials (3, 4). The major disadvantage of these assay systems is that they do not examine regrowth kinetics of surviving tumor cells. In addition, assays monitoring cytostasis do not examine cell cycle-specific growth inhibition and cannot differentiate between reduced uptake of radiolabeled nucleotide precursors (e.g., [3H]dThd) due to cell death from decreased DNA synthesis.

In this study flow cytometry was utilized to demonstrate PMN-mediated cytotoxic and cytostatic activity. The data demonstrate that PMA-activated PMNs are highly cytolytic and cytostatic against YAC-1 tumor cells over a period of 24 h as demonstrated by a decrease in the number of target cells and a lack of cell cycling. Between 24 and 48 h, however, surviving YAC-1 tumor cells start to repopulate the culture medium, eventually growing at a rate faster than those in the absence or presence of unstimulated PMNs, as demonstrated by their increase in cell number and accelerated cell cycle kinetics.

MATERIALS AND METHODS

Mice. Specific-pathogen-free, 10- to 12-wk-old, female C57BL/6N x C3H (hereafter called B6C3F1) mice were obtained through the National Cancer Institute production contracts (Charles River, Portage, MI). The mice were housed 10 per cage and provided with sterile food (Zeigler Bros., Inc., Gardners, PA) and water ad libitum.

Target Cell Culture. The YAC-1 cell line, a lymphoma induced in strain A mice by Moloney murine leukemia virus, was used throughout the study and was obtained from Dr. Ronald Herberman. The cells were maintained in vitro in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Ogden, UT), 2 mM L-glutamine, and 50 µg/ml of gentamycin, which will subsequently be referred to as “medium.” YAC-1 tumor cells have been previously shown to be sensitive targets of PMN-mediated tumor cytotoxicity (3, 4).

Effectors Cells. PMNs were obtained from the peritoneum 2 h after a second i.p. injection of 3 ml of 3% sodium caseinate (Gibco Laboratories, Grand Island, NY) which was administered 16 h after a primary injection with 2 ml of the same material. PMNs were collected by lavage with 6 ml of calcium- and magnesium-free HBSS supplemented with 2 units/ml of preservative-free ammmonium heparin (Sigma, St. Louis, MO). The harvested cell population was washed 3 times with HBSS before being resuspended in medium. This population of cells contained between 88 and 99% PMNs, as assessed by differential counts on Wright-Giemsa-stained cytocentrifuge preparations, with the remaining cells being primarily lymphocytes and macrophages.

Cytolysis Assay. A [3H]dUrd release assay was utilized to quantitate tumor cell cytolysis (4). Briefly, PMNs in the presence or absence of 10 ng/ml of PMA (Sigma) and at different effector:target ratios were added to YAC-1 cells (2 x 10⁴) in round bottomed microtiter plates in 0.2 ml volumes. The YAC-1 tumor target cells (5 x 10⁶) were labeled with 3 µCi/ml of [3H]dUrd (49.7 Ci/mM; DuPont, Boston, MA) for 3 h and washed 3 times prior to addition into the wells. The reaction mixture was incubated for 16 h, the plates were centrifuged for 20 min at 1500 rpm, and the radioactivity in supernatants was determined with a Minaxi Beta liquid scintillation counter (United Technologies, Downers Grove, IL). The percentage of specific lysis was determined using the formula

% of lysis = \[
\frac{\text{cpm experimental release} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100
\]

where experimental and spontaneous release represent radionuclide release from tumor cells in the presence and absence of PMNs. Maximum release was measured after lysis of the labeled tumor cells with 0.2% Triton X-100. PMA alone did not affect the release of [3H]dUrd from tumor cells.

A flow cytometric method adapted from Stevenson et al. (10) for macrophages was used to determine cytolytic activity of PMNs. Briefly, PMNs were cocultured with YAC-1 tumor target cells in the presence or absence of 10 ng/ml of PMA in 35-mm-diameter wells of 6-well cluster plates (Costar, Cambridge, MA) at an E:T ratio of 3:1 (3 x 10⁵ PMN and 1 x 10⁶ YAC cells in 1 ml of medium/well). At various time intervals, the cells were collected, pelleted by centrifugation, and resuspended in 0.5 ml of HBSS. While vortexing, the cells were fixed in 5 ml of 70% ethanol and stored at 4°C until analyzed. Immediately prior to flow analysis, 1.5 x 10⁶ fluorescent latex particles (10-µm diameter; Coulter Electronics, Hialeah, FL) were added to each sample. Single-laser flow analysis was performed on a FACS IV flow cytometer (Becton Dickinson, Sunnyvale, CA) using an argon-ion laser operating at 488 nm.

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The abbreviations used are: PMN, polymorphonuclear neutrophil; PMA, phorbol myristate acetate; [3H]dThd, thymidine; [3H]Urd, thymidine; HBSS, Hank’s balanced salt solution; BrUrd, bromodeoxyuridine; PI, propidium iodide; FITC, fluorescein isothiocyanate; E:T, effectortarget ratio.

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nm, with emission measured at 530 nm. The number of cells contained in each sample was determined by monitoring the amount of beads (usually 15,000) recovered during cell acquisition.

Cytostasis Assay. Cytostatic activity was measured via the inhibition of \(^{3}H\)dThd incorporation as previously described (4). Briefly, PMNs were cocultured with YAC-1 target cells (2 \times 10^5/well) in 200-µl volumes in wells of flat-bottomed microtiter plates in the presence or absence of 10 ng/ml of PMA. The cells were incubated for 24 h and labeled during the final 6 h of incubation by addition of 1 µCi/ml of \(^{3}H\)dThd (6.7 Ci/mmol; Du Pont, Boston, MA). At the end of the incubation, the cells were harvested with a multichannel cell harvester (Skatron, Sterling, VA) and the radioactivity of the filter paper was determined by liquid scintillation counting. The percentage of \(^{3}H\)dThd uptake inhibition was calculated by the formula

\[
\% \text{ of inhibition} = \left(1 - \frac{\text{cpm of experimental group}}{\text{cpm of control group}}\right) \times 100
\]

where the experimental and control groups represent YAC cells in the presence and absence of PMNs, respectively. PMA (10 ng/ml) alone did not affect \(^{3}H\)dThd incorporation into YAC-1 tumor cells.

Cell cycle progression of YAC-1 target cells was analyzed as previously described (11, 12). Briefly, PMNs (6 \times 10^5/well) were added to YAC-1 cells (2 \times 10^5/well) in 35-mm wells of 6-well cluster plates in the presence or absence of 10 ng/ml of PMA. The target cells (24-h culture) had been pulsed previously by addition of 10 µM BrdUrd for 30 min prior to the coculture. At various times the cells were harvested by centrifugation, fixed in 10% ethanol, and stored at 4°C until analysis. Prior to analysis, samples were acid hydrolyzed (4 N HCl) for 30 min, washed, and resuspended in 2.5 ml of 0.1 M Na$_2$B$_4$O$_7$. The samples were washed again and stored in 70% ethanol for 30 min, pelleted, and resuspended in 50 µl of 0.5% Tween 20 in phosphate-buffered saline. Five µl of FITC-conjugated anti-BrdUrd (Becton Dickinson Monoclonal Center, Mountain View, CA) were added to each sample for 30 min. The cells were washed and resuspended in 2 ml of phosphate-buffered saline containing 20 µg/ml of PI.

Cell cycle analysis of surviving tumor cells after culture with PMNs was performed by pulsing the cells for 30 min with BrdUrd immediately prior to fixation in 70% ethanol. The cells were then prepared for flow cytometry as described above. Single-laser flow analysis was performed using an argon-ion laser operating at 488 nm with emission measured at 530 nm (FITC) and 585 nm (PI) separated with a 560-nm short-pass dichroic mirror. Real time data acquisition, storage, and analysis were accomplished with a software-controlled Ciceria data acquisition system (Catalysis Corporation, Englewood, CA). The relative and absolute numbers of total tumor cells, as well as those in S-phase of their cell cycle, were determined by gating the area containing these cells and comparing the number of events within the gated area to the number of events outside the gated area.

Statistical Analysis. All statistics and figures are representative of replicate experiments. Statistical significance for Figs. 1 and 2 was determined by the RS/1 Multicompare procedure using the Wilkesshapiro test for normality, one-way analysis of variance, and Dunnett's test for multiple comparisons with a common group control.

RESULTS

Cytolytic and Cytostatic Activity of PMA-activated PMNs on YAC-1 Tumor Target Cells. To determine the optimal conditions for cytolytic and cytostatic activity of murine peritoneal PMNs, radiolabeled \(^{3}H\)dUrd release and radiolabeled \(^{3}H\)dThd uptake assays were used, respectively. As shown in Fig. 1, unstimulated PMNs or those treated with 1 ng/ml of PMA were not cytotoxic to YAC-1 target cells at any E:T ratio tested. At PMA concentrations of 5 ng/ml and above, marked killing of target cells was observed at E:T ratios as low as 1:1 (i.e., 30% killing at 50 ng/ml of PMA). Fig. 2 shows the cytostatic effect of unstimulated and PMA-activated PMNs as determined via \(^{3}H\)dThd incorporation. Cytostasis, measured as inhibition of \(^{3}H\)dThd incorporation, occurred in unstimulated PMNs at the 5:1 and 10:1 E:T ratios (Fig. 2). Following activation with 10 or 50 ng/ml of PMA, cytostatic activities of PMNs were enhanced even further at E:T ratios greater than 3:1. PMA, by itself, had no effect on either \(^{3}H\)dUrd release or \(^{3}H\)dThd incorporation of the YAC tumor target cells (data not shown). Furthermore, PMN cultures without tumor cells incorporated less than 0.2% of the radiolabeled nucleotide than control cultures containing tumor cells, indicating that PMNs did not contribute significantly to the counts obtained in cocultures. Since unstimulated PMNs did not exhibit cytolytic or cytostatic activity at an E:T ratio of 3:1 but could be activated by 10 ng/ml of PMA to demonstrate significant activity (Figs. 1 and 2), this ratio was used for all subsequent studies.
Flow Cytometric Demonstration of PMN-mediated Cytolysis and Cytostasis. Fig. 3 depicts tumor cell cytolysis and recovery in the presence of PMNs as determined by flow cytometry. PMNs and YAC-1 tumor cells were resolved on the basis of 90° light scatter. At the initiation of culture (time 0) there were no differences in the contour profile in the absence (Fig. 3A) or presence (Fig. 3B) of PMA-activated PMNs. After 24 h the initial 3:1 PMN:target ratio had reversed to a 1:4 ratio in the absence of PMA, while in the presence of the activator, the PMN:target ratio increased to 7:1, suggesting active cytolysis in addition to possible cytostasis (Fig. 3). However, between 24 and 48 h proliferation of surviving tumor cells was demonstrated by the reappearance of the cells in the contour profile, reaching a density similar to control cultures by 96 h (Fig. 3B). In the control cultures the rate of increase in tumor cell numbers plateaued between 48 and 96 h, while the repopulation rate in experimental cultures increased steadily over the same time (Fig. 3).

Fig. 4 summarizes the cytostatic activity of PMNs as analyzed by flow cytometry. Initially, YAC cells exhibited similar BrdUrd contour profiles in the presence of unstimulated (Fig. 4A) or PMA-activated (Fig. 4B) PMNs, suggesting similar cell cycle distributions. However, rapid cell cycling of the tumor cells occurred during the first 16 h of incubation in the presence of unstimulated PMNs (Fig. 4A). This correlated with the rapid increase in cell number during this time period (Fig. 3). In the presence of PMA-activated PMNs, the cell cycle distribution profile of the tumor target cells remained identical over the same time period, suggesting rapid cytostasis and cell arrest in every phase of the cell cycle (Fig. 4B).

As mentioned above, between 24 and 48 h the surviving tumor cells started to proliferate, surpassing the growth rate of control cultures at the 96-h time period. It was, therefore, examined whether this increased growth rate could be expressed as an increase in the number of cells entering the S phase of the cell cycle. PMNs and tumor cells were cocultured in the absence or presence of PMA for up to 96 h. At the time points indicated in Fig. 5, the cultures were exposed to BrdUrd for 30 min prior to coculture. At various times, the cells were fixed in 70% ethanol and stored at 4°C. Samples were acid hydrolyzed before labeling with FITC-anti-BrdUrd (BrdU) and PI. Cytometric analysis was performed as described in “Materials and Methods.” As shown in Figs. 5 and 6, cytostasis was evident for up to 24 h as indicated by the inability of cells to incorporate BrdUrd in the presence of PMA-stimulated PMNs (Figs. 5B and 6). At the same time, 60% of the control tumor cells were able to incorporate BrdUrd, suggesting active cell cycling (Figs. 5B and 6). However, by 48 h, proliferation of surviving tumor cells occurred, as demonstrated by a 2-fold increase in the number of cells incorporating BrdUrd, as compared to 24-h cultures (Fig. 6). By 72 h and especially by 96 h, more cells had entered S phase of the cell cycle in experimental cultures than in the control cultures, with 57% of the cells in...
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DISCUSSION

The present studies examined the kinetics of PMN-mediated cytolytic and cytostatic activity against YAC-1 tumor target cells. In addition to radioisotope procedures, multiparameter flow cytometry was used to visualize tumor cell destruction, cytostasis, and repopulation kinetics. One advantage of this latter system lies in its capability to examine the recovery and repopulation kinetics of surviving tumor cells by simple cell enumeration and by the reappearance and quantitation of tumor cells undergoing active DNA synthesis. PMNs alone do not interfere with the quantitation of tumor cell activity since, due to their noncycling status, they do not incorporate BrdUrd (Fig. 5C).

The data demonstrate that, at effector:target ratios as low as 3:1, PMA-activated PMNs are very effective in destroying tumor cells during the first 24 h of coculture. This low E:T ratio suggests that, in contrast to lymphocytes, macrophages, and natural killer cells, which require considerably greater E:T ratios, activated PMNs are highly cytotoxic. However, surviving tumor cells were able to repopulate eventually reaching a faster growth rate than those in the absence or presence of unstimulated PMNs. This was probably due to a combination of exhaustion of PMN activity as well as to the more favorable culture conditions for the surviving tumor cells in the experimental cultures versus those in control cultures.

With regard to cytostasis, cell cycle arrest of YAC-1 cells began within 4 h after the addition of PMA-activated PMNs and continued for at least 24 h (Figs. 4 and 5). The lack of any observable cycling between 0 and 4 h after culture suggested an almost immediate cytostatic effect of PMA-activated PMNs on tumor cells. In addition, the lack of progress in cell cycling during the 16 h of observation suggested that cell cycle inhibition occurs nonspecifically in all phases of the cycle. However, cell cycling of surviving tumor cells resumed between 24 and 48 h, reaching proliferation rates greater than time-matched controls by 72 and 96 h. The reduction of cycling YAC-1 cells 72 and 96 h in the presence of unstimulated PMNs or in the absence of PMNs was not due to tumor cell death, since >98% of the YAC-1 cells were viable at that time point as determined by trypan blue staining. Rather, it may reflect the fact that the YAC cells are reaching a saturation density. This is also shown in the experimental cultures which seem to reach a plateau after 96 h, a time point when the number of tumor cells reaches that of control cultures. It is interesting to note that, in vitro, PMNs were effective for 24 h only. A similar temporal effect has been previously shown in vivo by Lichtenstein et al. (13) who found that murine peritoneal PMNs harvested 6 h after i.p. injection of Corynebacterium parvum demonstrated potent cytotoxicity, while those obtained at 24 h were much less effective.

Some of the events leading to tumor cell destruction by PMNs have recently been elucidated. According to these re-
ports, PMNs bind to tumor cells in the presence of magnesium, leading to killing (14). The actual lysis of tumor cells has been reported to be dependent upon the production of active oxygen products, lysosomal enzymes, and the presence of a functional myeloperoxidase-hydrogen peroxide-halide system (7). It should be noted that PMA is one of the most potent inducers of hydrogen peroxide in murine PMNs (15). More recently a spontaneously secreted proteinaceous factor(s) participating in tumor cell killing in the presence of wheat germ agglutinin or actinomycin D has also been reported (16, 17).

The biological significance of PMNs in resistance to tumors is not yet clear. Recently, den Otter (1) has suggested criteria to establish the biological relevance of antitumor mechanisms. PMNs fulfill at least some of these criteria, such as the requirement of low E:T ratios and presence in histological sections of neoplasms and cytological preparations of malignant tissues. In addition, they can be recruited in large numbers and are responsive to factors such as tumor necrosis factor and γ-interferon, which kill tumor cells either directly or by enhancing immunological antitumor mechanisms (18–20). Another hypothesis suggests that PMN antitumor activity, rather than being part of the natural defense system, represents a side effect during the attempt to eliminate the inflammatory stimulus after the administration of various biological response modifiers (5). The ability of PMNs to lyse tumor cells in vitro and induce tumor necrosis in vivo has been demonstrated after the administration of Bacillus Calmette-Guérin, pyran copolymers, levan, and C. parvum (6, 13, 15). Furthermore, PMNs, elicited 6 h but not 24 h after C. parvum administration, have been shown to activate other cells capable of destroying tumor cells, such as macrophages (21, 22).

In summary, PMNs may represent an important cell type involved in the early destruction of tumor cells. Flow cytometry offers a sensitive technique to examine and quantitate antitumor activity, through both cell enumeration and proliferation analysis.

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REFERENCES

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