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Enhancement of Phorbol Diester-induced HL-60-mediated Cytotoxicity by Retinoic Acid, Dimethyl Sulfoxide, and 5-Azacytidine[1]

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ABSTRACT

Both peripheral blood monocytes and neutrophils are known to be capable of lysing a variety of extracellular tumor and non-tumor cell targets. The HL-60 human promyelocytic leukemia cell line has served as a useful model of human granulocyte and macrophage differentiation in studies from many laboratories. We have previously reported that phorbol diesters, which induce differentiation along the macrophage pathway, stimulate HL-60 cells to become strikingly cytotoxic to a variety of red cell targets. We now report that agents known to differentiate HL-60 along the granulocyte pathway (retinoic acid, dimethyl sulfoxide, 5-azacytidine) do not, in themselves, induce HL-60 to become cytotoxic. However, previous exposure (3–5 days) to these granulocyte pathway active agents markedly enhances phorbol diester-triggered killing. This enhancement is particularly striking at decreased effector:target ratios (as low as one effector per five targets) and is also demonstrated by a shift to lower concentrations of the phorbol diester dose-response curve. Retinoic acid is the most effective of the three agents tested, although priming (previous exposure) with dimethyl sulfoxide or 5-azacytidine also markedly enhances killing. These studies demonstrate that HL-60-mediated killing may be dissected pharmacologically into at least two distinct steps and further support the utility of this model system in studies of the development of macrophage-like cytotoxic cells. This system has also proven to be useful in the characterization of cytokines which mimic the differentiation effects of retinoic acid and dimethyl sulfoxide (J. A. Leftwich and R. E. Hall, manuscript in preparation).

INTRODUCTION

Mononuclear phagocytes are known to be an important system of host defense against tumor cells, bacteria, and other pathogens. A variety of *in vitro* systems have been developed for the detailed study of monocyte-mediated killing of extracellular targets (1–7). For example, human monocytes are capable of killing a variety of target cells in association with exogenous target-specific antibody (antibody-dependent cellular cytotoxicity) (3–5), lectins (mitogen-induced cellular cytotoxicity) (4, 5), certain complement components (synergistic cytotoxicity) (6, 7), and in some systems, in the absence of apparent exogenous factors. This latter response is considered to be analogous to lymphocyte models of "natural cytotoxicity" and is felt to represent a likely possibility for first-line host defense (8).

Improved understanding of the differentiation and development of noncytotoxic cells of the monocyte-macrophage lineage into cytotoxic cells has been hampered by the difficulty in obtaining sufficient numbers of purified mononuclear phagocytes and the known functional heterogeneity of cells of this lineage. For this reason, monocyte-like cell lines have been used by several laboratories to study the immunobiology of mononuclear phagocytes (9–11). Cells of the HL-60 human promyelocytic leukemia cell line are known to assume several characteristics of mononuclear phagocytes after exposure to phorbol diesters (11) and, in addition, become strikingly cytotoxic to a variety of red cell targets (9). This response appears to be dependent upon new protein synthesis, the generation of oxidative intermediates, and intact microfilament, but not microtubule function. Moreover, saccharide inhibition studies have suggested that, like other systems of "natural" cytotoxicity, sugars found on cell surface glycoproteins or glycolipids are important potential recognition units in this response (9). Time-lapse photography studies have indicated that such red cell targets are lysed extracellularly and appear to require contact with HL-60 effector cells prior to lysis (9). Thus this system, which has been shown to be a useful model of human monocyte-mediated cytotoxicity, is dependent upon the production of oxidative intermediates as well as a number of other cell surface and intracellular processes.

HL-60 cells are also known to express several features of cells of the granulocyte lineage after exposure to other agents such as RA[4] (12), DMSO (13–16), sodium butyrate (12, 13), and 5-AZ (17, 18). For example, RA and DMSO are known to enhance phorbol diester-triggered production of oxidative intermediates (12, 14, 15, 19–21). Since human neutrophils have been reported to lyse extracellular targets *in vitro* (5, 22), we tested several agents known to enhance differentiation of HL-60 along this pathway for the ability to generate cytotoxic cells. We found that such "granulocyte pathway" active agents did not induce HL-60 cells to become cytotoxic. However, previous exposure of HL-60 cells to such agents was found to markedly enhance the phorbol diester-induced killing response. This paper characterizes this response in detail and extends our previous initial characterization of the HL-60 system as it relates to the study of monocyte differentiation and the development of monocyte-mediated cytotoxicity.

MATERIALS AND METHODS

Reagents. PMA and PDBU were purchased from Sigma Chemical Company, St. Louis, MO, and stored at −20°C as a stock solution (1 mg/ml) in DMSO. Prior to use, PMA and PDBU were diluted in tissue culture medium. All-*trans*-retinoic acid (Sigma) was stored as a 1 mM stock solution in ethanol at −20°C and diluted in medium prior to use. 5-Azacytidine (Sigma) was stored at −20°C as a stock solution of 5 mM in water.

Cell Culture. The HL-60 line was maintained in RPMI-1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (Flow Labs, McLean, VA), 4 mM L-glutamine (Gibco, Grand Island, NY), penicillin (50 units/ml), and streptomycin (50 μg/ml) (F10-RPMI). HL-60 cells were collected from culture and washed 3 times with Hanks' balanced salt solution (Gibco) and then resuspended at 3×10^5/ml in 10 ml of F10-RPMI with the designated concentration of agent (DMSO, RA, 5-AZ) or control. After incubation

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[4] The abbreviations used are: RA, retinoic acid; CRBC, chicken red blood cells; DMSO, dimethyl sulfoxide; E:T, effector:target ratio, 5-AZ, 5-azacytidine; PDBU, phorbol dibutyrate; PMA, phorbol myristate acetate; F10-RPMI, RPMI medium containing 10% fetal calf serum, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

(37°C, 5% CO_2/95% air atmosphere) for designated periods of time, cells were washed 3 times with Hanks' balanced salt solution and resuspended in FIO-RPMI at 3×10^6/ml for cytotoxicity assays. Viability as determined by trypan blue exclusion was >90%.

Cytotoxicity Assay. Assays were performed in round-bottomed Linbro microtiter plates. Each well contained variable numbers (6×10^3–3×10^5) of HL-60 effector cells and 3×10^4 ^{51}Cr-labeled CRBC targets which were prepared as previously described (9). Stimulants (PMA, PDBU) were added in 50-μl aliquots at various concentrations according to the figure legends (final volume, 200 μl). Assays were done in triplicate and incubated 18–20 h at 37°C in a 5% CO_2/95% air humidified atmosphere. Plates were centrifuged ($250 \times g$, 10 min), and 150 μl of supernatant from each well were assayed for released ^{51}Cr in a gamma counter. Results (percentage of lysis) were expressed as the mean percentage of ^{51}Cr release (triplicate determinations; variability <5%) compared to total detergent-releasable (1% Triton X-100 in 0.87% NH_4Cl) radioactivity.

RESULTS

Dose-Response for PMA and PDBU-induced HL-60-mediated Cytotoxicity. We have previously reported that PMA induces HL-60 cells to become strikingly cytotoxic to a variety of red cell targets at doses between 2×10^{-9} and 2×10^{-6} M. In contrast, U937, another monocyte-like cell line, is not cytotoxic after exposure to doses of PMA up to 2×10^{-6} M (9). As previously noted, this killing response is independent of exogenous antibodies, lectin, or other factors. Because PMA contains a lipophilic component and interacts significantly with cell membrane components apart from the extensively studied phorbol diester receptor (23, 24), we wished to examine other nonlipophilic phorbol diesters such as PDBU. Fig. 1 depicts dose-response curves for PMA and PDBU and indicates that, although higher doses of phorbol diester are required, the killing response is similar for PDBU and PMA. At an E:T cell ratio of 10:1, approximately 80–90% of targets are typically lysed under these conditions.

Retinoic Acid by Itself Does Not Induce HL-60-mediated Killing, but Primes HL-60 Cells for PDBU-induced Killing. RA at an optimum dose of approximately 10 nM has been reported by a number of laboratories to stimulate HL-60 to mature along the granulocyte pathway (12), as assessed by morphology and reduction of nitroblue tetrazolium. In order to test for the possibility that retinoic acid might also stimulate the development of cytotoxic HL-60, we initially exposed HL-60 cells to varying concentrations of retinoic acid in the presence of ^{51}Cr-labeled CRBC targets under conditions identical to those used to perform the phorbol diester-induced HL-60-mediated killing assay. As Fig. 2 indicates, at an E:T ratio of 10:1 under these conditions (18 h, 37°C), no appreciable lysis of targets occurs

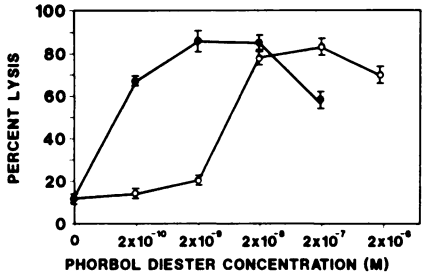

Fig. 1. Dose-response curves for PMA- and PDBU-induced cytotoxicity. HL-60 cells were cocultured with ^{51}Cr-labeled CRBC in the presence of varying concentrations of phorbol diester. Effector:target ratio was 10:1. Supernatants were harvested 18 h later, and the percentage of target lysis was measured as described in "Materials and Methods." ●, PMA; ○, PDBU. *Points*, mean; *bars*, SD.

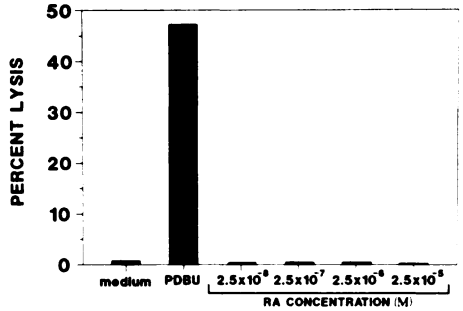

Fig. 2. Cytotoxic response of HL-60 cells to 18-h exposure of RA. HL-60 cells were incubated with ^{51}Cr-labeled CRBC targets (E:T ratio, 10:1) in the presence of either RA (25 nM–25 μM), PDBU (2.5×10^{-7} M), or medium (control). Target lysis was measured 18 h later.

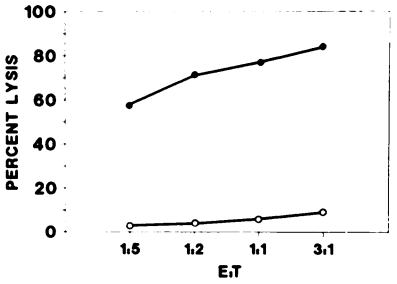

Fig. 3. Effect of RA on PDBU-induced cytotoxicity at various effector:target ratios. HL-60 cells were incubated 5 days with 10 nM RA (●), or medium control, (○), and washed, and cytotoxicity was measured in the presence and absence of 2.5×10^{-7} M PDBU at different E:T ratios. Background (non-PDBU stimulated) target lysis was less than 4%.

in the presence of up to 25 μM RA. In contrast, exposure of HL-60 to 2.5×10^{-7} M PDBU leads to marked lysis of CRBC targets. In addition, prolonged incubation (up to 5 days) in the presence of varying concentrations of RA similarly does not in itself cause development of cytotoxic cells (see Figs. 3 to 5).

However, when HL-60 cells are cultured in the presence of retinoic acid for 2–5 days followed by washing and exposure to PDBU, markedly enhanced cytotoxicity toward CRBC targets develops in a dose-dependent fashion (0.1–10 nM RA). This effect is characterized in Figs. 3 and 4 for 5-day cultures of HL-60 in the presence of RA. Fig. 3 depicts the E:T curve for HL-60 cells previously exposed to 10 nM RA for 5 days followed by triggering with PDBU. Appreciable cytotoxicity is demonstrated under these conditions at E:T ratios as low as 1:5. Control experiments in which HL-60 cells were previously cultured in the presence of an appropriate concentration of the diluent for retinoic acid (ethanol), followed by assay for PDBU-triggered killing, yielded no enhanced target lysis. As Fig. 3 indicates, enhancement of the HL-60-mediated killing response becomes particularly striking at low E:T ratios (*e.g.*, 1:1), since at low ratios PDBU-induced killing is minimal in the absence of previous exposure to RA. Fig. 4 depicts the RA dose-response at an E:T ratio of 1:1 and indicates that appreciable enhancement of the cytotoxic response develops when HL-60 cells are exposed to concentrations of RA as low as 0.1 nM. HL-60 cells cultured in the presence of RA followed by exposure to PDBU express nonspecific esterase and morphologically resemble cells of the monocyte-macrophage lineage rather than granulocytes (data not shown).

Effect of Previous Exposure of HL-60 to Dimethyl Sulfoxide and 5-Azacytidine. Other agents, including DMSO and 5-AZ are known to enhance differentiation of HL-60 cells, along the myeloid pathway (13–15, 17, 18). We therefore tested for the possibility that these differentiation-active agents, like retinoic

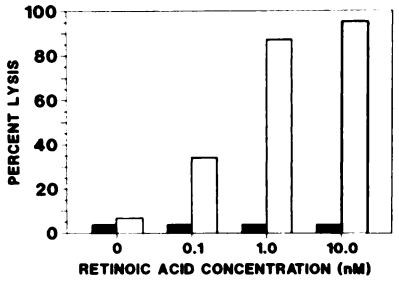

Fig. 4. RA enhancement of PDBU-induced cytotoxicity. HL-60 cells were exposed to varying concentrations (0.1–10 nM) of RA for 5 days and washed, and then cytotoxicity was measured in the presence of 2.5×10^{-7} M PDBU (□), or medium control (■) (CRBC targets, E:T ratio, 1:1).

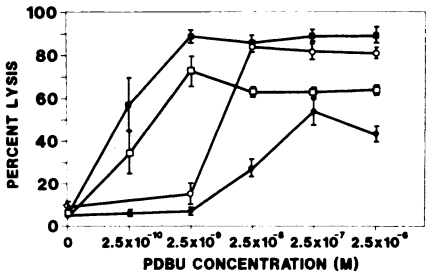

Fig. 5. Effect of RA, DMSO, and 5-AZ preincubation on PDBU-induced cytotoxicity. HL-60 cells were incubated 5 days with 10 nM RA (■), 5 μM 5-AZ (○), 1.15% DMSO (□), or control (●). The cells were then washed, and cytotoxicity was measured in the presence of varying concentrations of PDBU; E:T = 10:1. *Points*, mean; *bars*, SD.

acid, enhance killing mediated by HL-60 cells. Fig. 5 depicts the cytotoxicity dose-response curves for HL-60 cells primed (preincubated) in the presence of medium alone, 1.15% DMSO, 5 μM 5-AZ, and 10 nM RA, followed by washing and further exposure to varying concentrations of PDBU. The concentrations of priming agent used in these experiments are known to be optimal for induction of myeloid pathway differentiation (13–15, 17). As Fig. 5 indicates, the most striking effect is produced by RA, both in terms of enhancement of killing and shift of the PDBU dose-response curve to the left. Both DMSO and 5-AZ produce similar, although less striking, effects. Finally, prolonged (up to 5 days) incubation in the presence of RA, DMSO, or 5-AZ alone followed by washing does not lead to appreciable cytotoxic activity in the absence of further exposure to phorbol diester.

DISCUSSION

The study of the development of cytotoxic cells of the human monocyte-macrophage lineage has been facilitated by the development of potentially cytotoxic cell lines such as HL-60. Exposing HL-60 cells to phorbol diesters produces cells which resemble mononuclear phagocytes as judged by morphology, enzyme activity (NADase, acid phosphatase, and nonspecific esterase), and functional characteristics (phagocytosis, cytotoxicity) (9, 11). Phorbol diester-induced HL-60 cells become strikingly cytotoxic toward a variety of red cell targets (9). As demonstrated in this paper, this system is capable of remarkable efficiency, with appreciable target lysis observed at effector:target ratios as low as 1:5 in the presence of HL-60 effectors previously incubated with retinoic acid. The efficiency of killing (which relates to the homogeneity of effector cells), the ability to manipulate cytotoxic activity with defined agents, and other features make this system a useful model of human monocyte-mediated killing (9). As shown in Fig. 1, PMA and the less-lipophilic PDBU induce HL-60 to become cytotoxic in a dose-dependent manner. The dose-response curve for PDBU is shifted to the right of the curve for PMA for unknown reasons, but this may in part be due to increased binding affinity of PMA to phorbol diester receptor (23, 25). Maximum response, however, is similar in magnitude for both phorbol diesters. We have therefore chosen to conduct further studies using PDBU because of the emerging evidence that this phorbol diester interacts more specifically with the well-characterized phorbol diester receptor (23, 25). Because human neutrophils have been shown in some circumstances to be capable of mediating extracellular lysis of target cells (5, 22), we decided to examine the effect of several "granulocyte-pathway" active agents on HL-60-mediated cytotoxicity. Exposure of HL-60 cells to DMSO, RA, or 5-AZ alone under conditions known to lead to differentiation of HL-60 along the granulocyte pathway (19, 20) leads to no appreciable cytotoxic activity against CRBC targets. This would suggest that these agents in themselves are unable to induce differentiation far enough along the myeloid pathway to allow cytotoxic activity. This is consistent with several other studies in which exposure of HL-60 to these agents leads to the preferential development of myelocytes and metamyelocytes and relatively few mature neutrophils, as assessed by morphology (12, 15, 21, 26). However, when HL-60 cells were exposed to these granulocyte-pathway active agents, they developed markedly enhanced responsiveness to PDBU-induced cytotoxic activity. This is most striking at low E:T ratios, in which PDBU alone leads to little measurable target lysis, whereas previous treatment of HL-60 with DMSO, RA, or 5-AZ followed by exposure to PDBU leads to considerable target lysis. In addition to a shift of the E:T ratio curve (Fig. 3), enhanced responsiveness to PDBU is also demonstrated by a shift (to lower doses) of the dose-response curve for phorbol diester (Fig. 5). These data suggest that the development of cytotoxic effector cells with characteristics of mononuclear phagocytes may be separated pharmacologically into at least two steps which are responsive to two different classes of agents. One class of agents (DMSO, RA, and 5-AZ) is insufficient in itself to produce a cytotoxic cell. The second class of agents (phorbol diesters) is capable of producing cytotoxic cells at high doses, but is markedly potentiated by previous exposure of cells to agents of the first class.

A recent report by Fontana *et al.* (27) has shown that exposure of HL-60 cells to dimethyl formamide (a granulocyte pathway active agent) followed by phorbol diester leads to cells which resemble mononuclear phagocytes rather than neutrophils. In agreement with this, we have found that exposure of HL-60 to other granulocyte pathway activators (RA, DMSO, and 5-AZ) followed by PDBU produces cells which are nonspecific esterase positive, a marker of cells of the monocyte-macrophage lineage, whereas exposure to RA, DMSO, or 5-AZ alone leads to cells which are negative for nonspecific esterase (data not shown). These data suggest that cells more mature than the promyelocyte are under some circumstances able to develop into cells of the monocyte-macrophage lineage. In agreement with this, as discussed above, are observations that at least some granulocyte pathway active agents (*e.g.*, RA and DMSO) do not terminally differentiate (as assessed by morphology) most cells in the HL-60 population (12, 15, 21, 26).

The molecular basis for enhanced phorbol diester sensitivity of HL-60 cells previously exposed to DMSO, RA, and 5-AZ is not known but is currently under further investigation. One possibility is that these agents lead to enhancement of a protein kinase C-mediated event, since several studies now suggest that

protein kinase C is a phorbol diester receptor (28–30). In this regard, Lane *et al.* have reported a 2–5-fold increase in phorbol diester receptors on DMSO-treated HL-60 (25). In addition, a similar increase in protein kinase C enzymatic activity has recently been reported in DMSO- and RA-treated HL-60 (31). These findings do not rule out enhancement of other steps of the protein kinase C transducing mechanism by DMSO, RA, and 5-AZ nor the possibility that these agents may enhance a step in the development of cytotoxic activity which is distinct from protein kinase C-mediated events. These latter possibilities are currently under investigation.

In conclusion, exposure of HL-60 cells to DMSO, RA, and 5-AZ does not in itself lead to the development of cytotoxic effector cells, but it does markedly enhance the phorbol diester-induced killing response. Further characterization of the HL-60 system of cytotoxicity should lead to improved understanding of the steps involved in the development of human monocyte-mediated killing. Furthermore, this system has proven to be useful in the study of cytokines which mimic the differentiation activity of DMSO, RA, and 5-AZ.[5]

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[5] J. Leftwich and R. Hall, manuscript in preparation.