Benzene and Its Metabolite, Hydroquinone, Induce Granulocytic Differentiation in Myeloblasts by Interacting with Cellular Signaling Pathways Activated by Granulocyte Colony-Stimulating Factor

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Abstract. Chronic exposure of humans to benzene (BZ) causes acute myelogenous leukemia. These studies determined whether BZ, or its reactive metabolite, hydroquinone (HQ), affect differentiation of myeloblasts. BZ or HQ administered to C57BL/6J mice specifically induced terminal granulocytic differentiation of myeloblasts. The ability of the compounds to induce differentiation of the myeloblast was tested directly using the murine interleukin 3 (IL-3)-dependent myeloblastic cell line, 32D.3 (G) and the human HL-60 promyelocytic leukemic cell line. Treatment of HL-60 myeloblasts with BZ activated protein kinase C and upregulated the 5-lipoxygenase (LPO) pathway for the production of leukotriene D_4 (LTD_4), an essential effector of granulocytic differentiation. Differentiation was prevented by sphinganine, a kinase C inhibitor, as well as by LPO inhibitors and LTD_4 receptor antagonists. BZ and HQ also induced differentiation in 32D.3 (G) myeloblasts. Both compounds interact with cellular signaling pathways activated by granulocyte colony-stimulating factor (G-CSF) and thus replace the requirement for G-CSF. IL-3 induces a growth response, whereas G-CSF provides both growth and differentiation signals. BZ does not induce growth in the absence of IL-3, but provides a differentiation signal. Both HQ and LTD_4 induce differentiation and synergize with IL-3 for growth, however, neither support growth in the absence of IL-3. BZ-induced 32D cells showed a gradual progression of progenitor differentiation to granulocytes similar to that seen with G-CSF or LTD_4. HQ blocks differentiation at the myelocyte stage; only a small percentage of progenitors proceed to granulocytes. BZ, like G-CSF, upregulates LTD_4 production, whereas HQ obviates the requirement for LTD_4 by activating the LTD_4 receptor.

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Introduction

Benzene (BZ), a widely used industrial chemical and ubiquitous environmental pollutant, is a Class I carcinogen that causes acute myelogenous leukemia in humans that are chronically exposed [1-4]. BZ hematotoxicity occurs when its hepatic metabolites [5, 6], phenol, catechol and hydroquinone (HQ), are transported to the bone marrow [7,8] and further oxidized in a peroxidase-mediated [9-11] reaction to biologically reactive intermediates that can potentially affect hematopoiesis.

Because of the association between BZ exposure and an increased incidence of acute myelogenous leukemia, it is important to determine whether BZ and/or a metabolite, such as HQ, directly affect the stem cell and/or progenitor cells of the myeloid lineage. The ability to alter cytokine-dependent growth and differentiation in hematopoietic progenitor cells appears to be a property of agents with leukemogenic potential for humans [12]. There have been several reports on the effects of BZ on hematopoietic stem and progenitor cells [13]. In one study [14], a dose-dependent depression of all stem cell compartments was observed in BDF1 mice exposed for 16 weeks to airborne concentrations of BZ as high as 99 ppm for 6 h per day, 5 days per week. However, the granulocyte-macrophage colony-forming unit (GM-CFU) was much less

sensitive than the erythroid CFUs at higher doses of BZ. ***Dempster*** and ***C.A. Snyder*** [15] reported that short-term exposure of mice to BZ induced a shift toward granulocytic differentiation, and a growth advantage for granulocytic progenitor cells in the marrow and spleen which increased the total number of granulocytes. These results suggest that BZ and/or HQ is acting on the myeloid stem or progenitor cells. We report here that the administration of BZ or HQ specifically stimulates granulopoiesis in mice and induces granulocytic differentiation in myeloblasts of the human promyelocytic leukemic cell line, HL-60, and the normal murine interleukin 3 (IL-3)-dependent myeloblastic cell line, 32D.3 (G). They do this by replacing the requirement for G-CSF and leukotriene D_4 (LTD_4), respectively, for induction of differentiation.

Materials and Methods

Animals

C57BL/6J inbred male mice were obtained from Jackson Laboratories, Bar Harbor, ME at 6 weeks of age (18-20 g). Animals were housed at an AAALAC-approved central animal facility (73" ± 3°F; humidity 53%; 12 h light cycle) in polycarbonate cages (four mice per cage) with hardwood bedding and fed Purina Rodent Chow and water ad libitum. Animals were acclimated for 1 week and weighed between 22 and 25 g when used. The experiments reported were carried out under a protocol approved by the Institutional Animal Care and Use Committee.

fetal bovine serum **(FBS)**. Cells used in the experiments were from passages 18-42. The IL-3-dependent myeloblastic cell line, 32D.3, was derived from normal bone marrow of C3H/HeJ mice by ***Greenberger*** [16]. It has a normal karyotype and is nonleukemic [16]. The clone was further characterized for IL-3 dependence by ***Metcalf*** [17], and for G-CSF induction of differentiation by ***Valtieri et al.*** [18] who gave the clone the designation (G). We are indebted to ***Dr. Giovanni Rovera*** for the kind gift of the 32D.3 (G) clone. The cells were maintained in Iscove's modified Dulbecco's medium (IMDM) in the presence of 10% FBS and 3 u/ml recombinant murine IL-3 (rMuIL-3) and supplemented with penicillin (50IU/ml) and streptomycin (50mg/ml). Cells were cultured at 37°C in a 5% CO_2 atmosphere with biweekly replacement of medium and adjustment of cell concentration to 10^5 cells/ml for optimal growth.

All cell cultures were demonstrated to be free of mycoplasma contamination by periodically testing the culture supernatant with a radiolabeled mycoplasma cDNA probe.

Reagents

IMDM, RPMI 1640, Dulbecco's phosphate buffered saline (PBS) minus Ca^{2+} and Mg^{2+}, Tyrodes buffered salt solution (TBBS) and Pen/Strep were obtained from Mediatech, Washington, D.C. BZ (spectroanalyzed) and HQ were purchased from Fisher Scientific Co., Pittsburgh, PA. FBS, the AS-D Chloroacetate Esterase Assay Kit, LTD_4, sphinganine, caffeic acid, pepstatin, leupeptin, phenylmethylsulonyl fluoride (PMSF), rhodamine-labeled antimouse immunoglobulin, nitroblue tetrazolium (NBT), May-Grunwald/Giemsa stain and acivicin (α-amino-3-chlor-4,5-dihydro-5-isoxazole acetic acid) were obtained from Sigma Chemical Co., St. Louis, MO. The 5-lipoxygenase inhibitor, AA-861, was a product of Waco Bioproducts, Richmond, **VA.** The LTD_4 receptor antagonists were gifts from Merck Frosst Canada, Inc., Quebec (MK571) and Eli Lily & Co., Indianapolis, IN (LY 163443). Monoclonal antibody against human L-12-2 granulocyte-specificsurface antigen was a kind gift from ***Dr. Giovani Rovera.*** Recombinant human G-CSF, (rHuG-CSF) and rMuIL-3 were obtained from R&D Systems, Minneapolis, MN. Giemsa blood stain, Azure Type B, was obtained from EM Diagnostics, Gibbstown, NJ and HemaQuick II from the Curtin Matheson Co., Houston, TX. Gen Probe, San Diego, CA was the source of the Mycoplasma Test Kit. ^{32}Pi was a product of Dupont NEN, Boston, MA.

Exposure of Cells to HQ, BZ, G-CSF, LTD_4 and Other Agents

HL-60 cells (5×10^5/ml) were incubated with or without BZ in RPMI 1640/10% **FBS** for 7 days at 37°C after which differentiation to granulocytes was assessed. 32D cells (2.5×10^5/ml)

were incubated in IMDM/10% FBS with or without BZ, and with or without 3 u/ml rMuIL-3. When HQ was used, the cells were pretreated with 2 μM HQ in PBS/2 mM glucose (PBS-A) for 30 min at 37°C after which the cells were collected, washed twice with PBS and placed into culture in IMDM/10% FBS with or without 3 u/ml rIL-3. G-CSF and LTD, were added directly to the culture medium. The lipoxygenase inhibitors and LTD_4 receptor antagonists were added directly to the culture medium in amounts indicated in the legends to the tables and figures 20 min before the addition of the inducing agent. After 3 days of incubation, a sample of each culture was removed for cell counting and the remainder of the culture was diluted to 2.5 x 10^5 cells/ml to insure optimal growing conditions. Unless indicated otherwise in the figure or table legend, incubation was continued for 4 days, after which the cells were analyzed for the granulocytic phenotype.

Assessment of Differentiation

Granulocytic differentiation was assessed by: 1) the acquisition of granulocytic morphology, specifically promyelocytes, metamyelocytes and segmented or mature cells, 2) the development of superoxide production as measured by the reduction of NBT, 3) the development of chloroacetate esterase activity, and 4) in the case of HL-60 cells, the appearance of L-12-2 granulocyte specific surface antigen.

Morphological assessment of differentiation was performed by cytospin preparation and May Grünwald/Giemsa staining. Percentages were based on the average obtained by counting 200 cells on each of triplicate slides. For NBT reduction, cells were incubated for 1 hour with 0.125 nmol/l TPA and 0.05% NBT. Cells were fixed on a slide, counterstained with safranin and the percentage of cells containing black granules of reduced NBT was determined. Chloroacetate esterase activity was determined by incubating fixed cells with a diazonium salt, AS-D chloroacetate and Red Violet at pH 6.3 for 5 min at 37°C, after which the percentage of cells containing red granules was determined. The presence of L-12-2 surface antigen was detected by fluorescence microscopy using rhodamine-labeled antimouse immunoglobulin against the monoclonal antibody.

Treatment of Animals with BZ and HQ

Mice received BZ (600 mg/kg body weight) in corn oil or HQ (25 or 50 mg/kg body weight) in PBS i.p. twice per day, 7 h apart, for 2 days. Controls received corn oil or PBS. Eighteen h after the final injection the animals were killed by cervical dislocation, their femurs were removed and the bone marrow cells were obtained as described under the determination of bone marrow cellularity and morphologic hematology. The concentrations of BZ and HQ were not cytotoxic and the viability of the cells flushed from the femurs was >98% as measured by Trypan Blue exclusion.

Determination of Bone Marrow Cellularity and Morphologic Hematology

The epiphysial plate on each end of the femur was removed and 1 ml of undiluted FBS was forced through the femur using a syringe with a 25-gauge needle. The marrow was collected in a tissue culture tube and a single cell suspension was prepared by passing the marrow through the syringe two additional times. A sample (100 μl) of the cell suspension was used to prepare a slide which was stained as described below and used for a differential cell count. The remainder of the cell suspension was diluted in cold lysing buffer (10 mM Tris-HCl, pH 7.4, 155 mmol/l NH_4Cl) to remove red blood cells. The number of nucleated cells was determined with a hemocytometer and viability was tested by Trypan Blue exclusion. Viability was > 98%.

Staining was carried out as follows: the slide was dipped in Wright-Giemsa stain (Hema Quik II) for 3 min, washed by dipping in tap water for 3 min and allowed to air-dry for at least 30 min. Counter staining was carried out by immersing the slide for 7 min in Giemsa (Azure B Type) prepared fresh as a 1:50 dilution in Hydrion buffer, pH 6.8. The slide was then washed for 30 sec with running tap water and allowed to air-dry. Differential cell counting was carried out using oil immersion at 100×. Five hundred cells per slide were counted and the percentage of each type of cell present was determined.

BZ-Induced Protein Kinase C Phosphorylation of HL-60 Cellular Proteins

HL-60 cells (3 x 10^6/ml) were twice washed in TBBS-Ca^{2+} to remove P_i from inside the cells and then preloaded with $^{32}P_i$ (1.5 mCi/ml; sp. act. 1 Ci/mmol) by incubation for 1 h at 22°C

in calcium and phosphate-free TBBS. The cells were washed three times with TBBS and incubated in TBBS-Ca^{2+} with 5 mM BZ and/or 40 µM sphinganine or TBBS for 5 min. When sphinganine was used it was added 10 min before the other reagents. The reaction was stopped by chilling the tubes on ice, the cells were recovered and lysed in 100 µl of a solution containing Tris-EDTA, pH 8.0, 2 mM PMSF, 0.1 mM pepstatin A, 0.1 mM leupeptin and 0.01% Triton X-100. Protein was determined by the method of *Bradford* [19].

Polyacrylamide Gel Electrophoresis

Lysate protein (8 µg/well) was loaded onto an 0.8 mm, 8% T vertical slab polyacrylamide gel. Electrophoretic separation of proteins was carried out at 150 V for 3.5 h. The gel was silver-stained, dried and exposed to X-OMAT AR film for 96 h at −70°C.

HPLC Analysis of LTD_4 Formation in HL-60 Cells

Cells (1.5 x 10^6/ml in PBS) were incubated with or without 5 mM BZ or with BZ and 1 µM AA861 for 24 h at 37°C. After removal of the cells, a 100 µl sample of the incubation medium was extracted with an equal volume of toluene and the organic phase evaporated to dryness. The residue was suspended in 100 µl of mobile phase (65:35:0.02 v/v/v methanol:water:acetic acid, pH 5.6) and 50 µl was injected into a reverse phase, 5 mm ODS HPLC column. An LTD_4 standard (1 pg) was run as a control.

Statistical Analysis

Data between groups were analyzed using the t-test or ANOVA followed by Dunnett's t-test. Results are expressed as mean values ± SD. A $p \leq 0.01$ was considered significant.

Results

Stimulation of Granulocytic Differentiation in Mice by BZ and HQ

As can be seen in Figure 1, the administration of BZ to mice stimulated the differentiation of myeloblasts as measured by an increased percentage of promyelocytes and intermediate granulocytic progenitors in the bone marrow one day after the last BZ injection (three days after the initiation of treatment). BZ had no effect on the

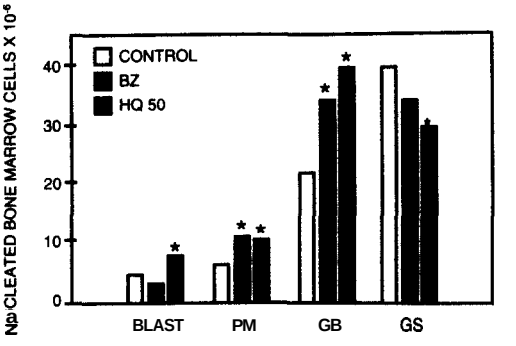

Fig. 1. The induction of granulocytic differentiation in C57BL/6J mice by BZ or HQ. Groups of mice (n = 4) were injected with BZ (600 mg/kg body weight) or HQ (25 or 50 mg/kg body weight) twice daily for 2 days. The control animals received corn oil and PBS-A. Eighteen h after the final injection the mice were killed, the bone marrow obtained and a morphological analysis of the bone marrow carried out as described under Methods. Data are presented as absolute numbers of myeloid cells per femur represented by blast cells and total granulocytic cells of 500 cells analyzed. Qualitatively similar results were obtained when the data were expressed as the percent of nucleated bone marrow cells. The results are expressed as the mean ± SD (n = 4); *$p \leq 0.001$ as compared with the control group. The data from various cell types from the individual marrows are extremely tight (they range from SD ± 0.001 to ± 0.03) and too small to be plotted by the printer/plotter so no error bars are visible. PM, promyelocytes; GBand, myelocytes and metamyelocytes (ring) forms; Gseg, segmented (mature granulocytes).

number of myeloblasts. The number of mature granulocytes (G seg) was not stimulated at Day 3, however, in experiments carried out for 7 days, BZ significantly increased the number of mature granulocytes compared to control values (data not shown). The experiment presented is representative of three such experiments which gave similar results. Identical results were obtained when the data were expressed as percentage of total cells counted. HQ administered to mice for 3 days at a dose of 50 mg/kg body weight also stimulated granulocytic differentiation as indicated by an increased percentage of promyelocytes and metamyelocytes (Fig. 1). In contrast to BZ, HQ doubled the number of myeloblasts and stimulated differentiation to band forms to a greater extent than BZ, and the terminal differentiation of intermediate progenitors (band forms) to mature (segmented) granulocytes was

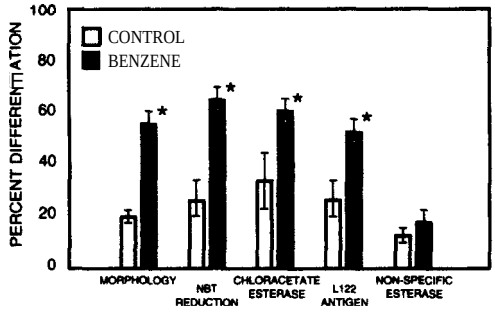

Fig. 2 BZ-induced granulocytic differentiation of HL-60 myeloblasts. Cells (5×10^5/ml) were cultured with and without 5 mM BZ as described under Methods for up to seven days. The development of the granulocytic phenotype was determined by measuring the characteristics presented in the figure. The values represent the mean ± SD of at least three experiments where each sample was carried out in triplicate.
*Significantly different from the control cells at $p \leq 0.001$.

limited. HQ administered at 25 mg/kg body weight caused a lesser, but significant stimulation of granulopoiesis (data not shown). At these doses of BZ or HQ, there was no loss of animals, no overt signs of toxicity and the viability of the cells flushed from the femurs remained at greater than 98%.

Induction of Granulocytic Differentiation in Myeloblasts by BZ and HQ

The results of the in vivo experiments suggested that BZ per se, or by metabolism to HQ, is capable of inducing granulocytic differentiation. However, they do not indicate whether either compound causes induction directly or indirectly in vivo. To answer this question and to have a system with which to study the mechanism of induction of differentiation, we turned to the use of myeloid cell lines. The well-studied human HL-60 promyelocytic leukemic cell line [20], which consists of approximately 35 to 45% myeloblasts and the remainder promyelocytes, was chosen because it has been used in many studies as a surrogate for the GM-CFU, and a number of agents have been shown to induce terminal granulocytic differentiation in these cells [20]. The normal mouse IL-3-dependent 32D myeloblastic cell line was used because it has been adapted to terminally differentiate to granulocytes in response to G-CSF [18], and because data obtained with this cell line can be compared with data obtained in the mouse. As can be seen from the data presented in Figure 2 and Table I, exposure of HL-60 myeloblasts to BZ followed by incubation for 7 days resulted in terminal granulocytic differentiation measured by morphology (determination of the percent of cells classified as promyelocytes or higher granulocytic forms) and the development of several characteristics of the granulocytic phenotype. No significant induction of nonspecific esterase activity indicative of monocytes was detected nor was monocyte morphology observed (Table I). The ability of BZ to induce granulocytic morphology as well as the appearance of other markers of the granulocytic phenotype was a function of the concentration of the BZ used over the range 0.1 mM to 5 mM (data not presented). As can be seen in Table I, the HL-60 cell population consists of about 45% promyelocytes and 35% myeloblasts. BZ caused a significant decrease in the number of myeloblasts as well as promyelocytes, and a corresponding shift into more intermediate progenitors and mature granulocytes. The majority of the differentiated progenitors appeared to be metamyelocytes and myelocytes, but a significant number

Table I. Morphological assessment of benzene-induced granulocytic differentiation of HL-60 cells

	Cell Type (%)					
System	Blast	Promyelocyte	Myelocyte	Metamyelocyte	"Mature" Granulocyte	Total Granulocyte
HL-60	36	45	2	15	2	19
HL-60 + 5 mM BZ	15	29	16	30	10	56

Cells were treated with benzene (BZ) as described in Figure 2. Morphological assessment was performed by Cytospin preparation and staining with May-Grünwald/Giemsa stain. Percentages were based on the average obtained by counting 200 cells on each of triplicate slides in six different experiments.

of terminally differentiated granulocytes was observed (Table I).

BZ-Induced Activation of Protein Kinase C and the Inhibition of Granulocytic Differentiation in HL-60 Cells by the Kinase C Inhibitor, Sphinganine

Activation of protein kinase C is involved in the induction of differentiation in HL-60 cells by tetraphorbol acetate (TPA) and other agents [20]. BZ has been shown to activate protein kinase C in intact rabbit platelets as well as the partially purified enzyme from mouse brain [21]. Consequently we tested whether BZ-induced differentiation of HL-60 myeloblasts to granulocytes was dependent on protein kinase C activation and therefore should be inhibited by sphinganine, a potent and specific inhibitor of protein kinase C in cell systems and in vitro [22]. In the representative experiment presented in Figure 3, HL-60 cells were pre-exposed to $^{32}P_i$ to label ATP and treated with 5 mM BZ under conditions that induce granulocytic differentiation. The activation of protein kinase C was measured by the phosphorylation of cellular proteins as monitored by SDS-PAGE analysis and autoradiography (Fig. 3). BZ induced the transfer of $^{32}P_i$ from AT[^{32}P] to a number of HL-60 proteins (lane a) that was prevented by preincubation of HL-60 cells with 40 μM sphinganine prior to BZ treatment (lane b). The phosphorylation observed with BZ (data not shown) was equally as great as that observed in other experiments which utilized HL-60 cells treated with 100 ng TPA as a positive control. Protein kinase C on plasma membranes from BZ-treated HL-60 cells was capable of lipid-dependent phosphorylation of the known protein kinase C substrate, histone III-S (data not shown). Sphinganine also prevented BZ-induced granulocytic differentiation (Fig. 4), but by itself did not affect differentiation (data not shown). Since both BZ-induced phosphorylation of cellular proteins and granulocytic differentiation were inhibited by the kinase C inhibitor, the results implicate BZ activation of protein kinase C in BZ-induced granulocytic differentiation in HL-60 myeloblasts.

The Involvement of the 5-Lipoxygenase (LPO) Pathway in BZ-Induced Granulocytic Differentiation

An active LPO pathway of metabolism that converts arachidonic acid to the peptidoleukotriene, LTD_4, is essential for normal [23, 24] as well as leukemic [25, 26] myeloid progenitor

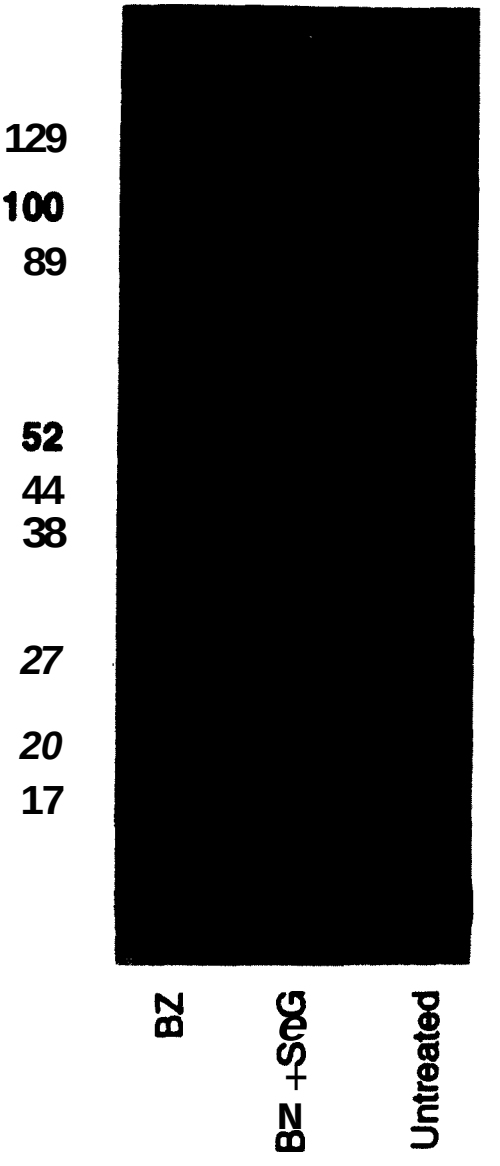

Fig. 3. Autoradiograph of an SDS-polyacrylamide gel separation of ^{32}P-labeled cellular proteins from BZ-treated HL-60 cells. Cells (3 x 10^6/ml) were preloaded with $^{32}P_i$ for 1 h, washed and exposed to 5 mM BZ (lane a), BZ plus 40 μM sphinganine (lane b) or buffer (lane c) for 5 min as described under Methods. Lysate protein (8 μg/lane) was added to each lane of an 0.8 mm, 8% T polyacryamide gel. Electrophoresis was carried out at 150 V for 3.5 h. The gel was silver stained, dried and exposed to x-ray film for 96 h at −70°C.

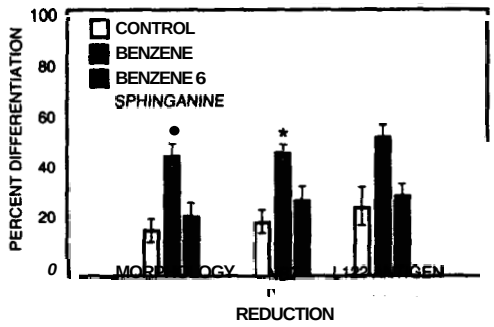

Fig. 4. Inhibition of BZ-induced granulocytic differentiation in HL-60 cells by sphinganine. Cells (5×10^5/ml) were preincubated with 40 μM sphinganine in RPMI 1640/10% FBS for 10 min at 37°C, 5% CO_2. The cells were exposed to 5 mM BZ and assessed for granulocytic differentiation after incubation for seven days as described under Methods. The values represent the mean ± SD of at least three experiments where each sample was carried out in triplicate. *Significantly different from the control cells at p 10.001.

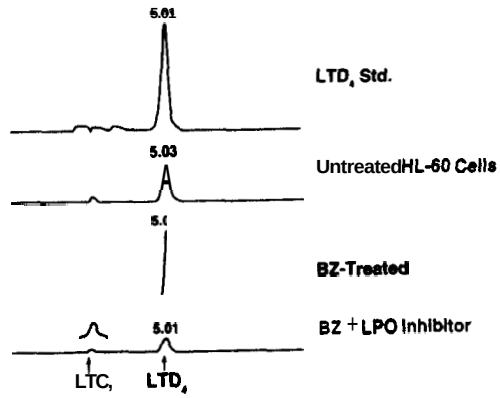

Fig. 5. BZ-induced formation of LTD, in HL-60 cells. Cells (1.5×10^6/ml in PBS) were incubated with or without 5 mM BZ (HPLC profiles 2 and 3) or with BZ and 1 μM AA861 (profile 4) for 24 h at 37°C. After removal of the cells, a 100 μl sample of the incubation medium was extracted with an equal volume of toluene and the organic phase evaporated to dryness. The residue was subjected to HPLC analysis for the presence of LTD, as described under Methods. Retention times (min) are printed above the peaks. An LTD_4 standard (1 pg) was added as a control (profile 1).

cell proliferation and differentiation; LTD_4 is an intermediate in CSF-induced clonal growth of GM-CFU [23, 24]. The ability of BZ to cause the production of LTD_4 and its immediate precursor, LTC_4, under conditions where BZ induced granulocytic differentiation in HL-60 cells was investigated. A lipid extract of the culture medium of differentiating HL-60 cells grown in the presence and absence of a 5-LPO inhibitor was subjected to reverse-phase HPLC. In several experiments, LTD, was produced in BZ-treated cells in amounts two- to four-fold greater than in untreated cells. Figure 5 presents an HPLC profile from a typical experiment in which BZ induced the formation of LTD_4 (two-fold increase over untreated cells) in HL-60 cells. LTD_4 is present in the untreated HL-60 cell population (Fig. 5) because approximately half of the cells are promyelocytes that have aborted their differentiation at that stage and thus contain LTD_4. The remainder are myeloblasts in which BZ should induce LTD, formation. The requirement for BZ-induced formation of LTD_4 for granulocytic differentiation was demonstrated by showing that 5-LPO inhibitors prevented both BZ-induced LTD_4 production and granulocytic differentiation in HL-60 myeloblasts (Table II). The highly specific 5-LPO inhibitor, AA-861 [27], prevented the formation of LTD_4 induced in myeloblasts by BZ and stopped its constitutive production in the promyelocytes (Fig. 5). AA-861 and another inhibitor, caffeic acid, prevented BZ-induced granulocytic differentiation (Table II). The inhibition was reversed by the addition of LTD_4 concomitantly with the inhibitor. A highly specific LTD_4 receptor antagonist, MK-571, [28] also completely inhibited BZ-induced granulocytic differentiation in HL-60 myeloblasts, and this inhibition was prevented by the concomitant addition of LTD_4 (Fig. 6). Similar results (data not shown) were obtained with another receptor antagonist, LY 163443 [29].

The conversion of the immediate precursor, LTC_4, to LTD_4 requires the removal of a glutamyl group from LTC_4 by γ-glutamyl transpeptidase [30], a reaction that is inhibited by the glutamine antagonist acivicin [23]. Data presented in Table III show that 1 μM acivicin prevented BZ-induced differentiation to granulocytes. This inhibition could be overcome by the addition of LTD_4 to the culture medium, further supporting a role for LTD_4 in BZ-induced differentiation of HL-60 cells to granulocytes. Taken together, these results suggest that BZ, via activation of protein

Table II. Effect of 5-lipoxygenase inhibitors on benzene-induced granulopoiesis

System	Morphology	NTB Reduction
	Percentage of cells counted	
Control	12.3 ± 5.5	16.7 ± 3.5
plus BZ	37.5 ± 3.8[a]	45.7 ± 3.3[a]
plus BZ/caffeic acid	17.5 ± 3.9	20.8 ± 3.7
plus BZ/caffeic acid/LTD_4	37.1 ± 3.7[b]	42.8 ± 8.3[b]
plus BZ/AA861	23.7 ± 3.3	18.3 ± 1.7
plus BZ/AA861/LTD_4	54.7 ± 10.5[b]	28.7 ± 4.1[b]

Cells (5×10^5/ml) were preincubated in culture with 5-lipoxygenase inhibitors caffeic acid (100 μM), AA861 (1 pm) and with LTD_4 (1 μM) in RPMI 1640/15% FBS for 10 min at 37°C, 5% CO_2. BZ (5 mM) was added directly to the culture and NBT reduction and granulocytic differentiation were determined after incubation for seven days. Values represent the mean ± SD of at least two experiments carried out in triplicate. ND, not determined. [a]Significantly different from control and BZ-treated cells pretreated with inhibitors. [b]Significantly different from cells treated with BZ and inhibitors, $p \leq 0.01$

kinase C, induces the formation of LTD_4 required for the initiation of granulocytic differentiation.

Induction of Granulocytic Differentiation in the Myeloblastic Cell Line 320.3 (G) by BZ and HQ

In order to compare the induction of granulocytic differentiation by BZ and HQ with that of the normal physiological inducer, G-CSF, we turned to the IL-3-dependent, diploid, myeloblastic cell line, 32D.3 (G) which was derived from normal mouse bone marrow and adapted to differentiate in the presence of G-CSF [18]. Table IV presents data from a representative of four experiments which gave similar results. There are few differentiated myeloid cells in the presence of IL-3 alone, which, although obligatory for proliferation and survival of the myeloblast, does not induce differentiation. rHuG-CSF that is fully capable of binding to the mouse G-CSF receptor induced terminal differentiation to granulocytes. Substitution of noncytotoxic concentrations of BZ or HQ in place of G-CSF also induced differentiation in 32D myeloblasts (Table IV). While both BZ and HQ replaced the requirement for G-CSF for differentiation to granulocytes, neither BZ (Fig. 8A) nor HQ (Fig. 8B) was able to obviate the dependence of the cells on IL-3 for survival and growth.

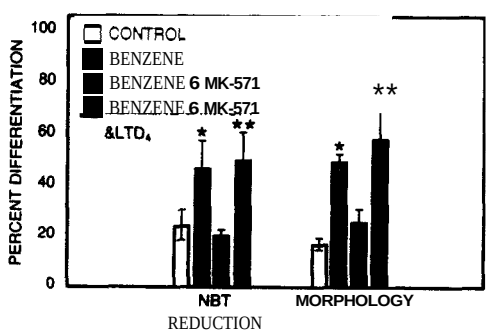

Fig. 6. Inhibition of BZ-induced granulocytic differentiation in HL-60 cells by a 5-LPO inhibitor. Cells (5×10^5/ml) were preincubated with 1 μM MK-571 in RPMI 1640/10% FBS for 10 min at 37°C, 5% CO_2. BZ (5 mM) was added and granulocytic morphology was assessed after incubation for seven days. Values represent the mean ± SD of two experiments where each sample was carried out in triplicate. *Significant difference from the control cells at the $p \leq 0.01$ level; **significant difference from cells treated with BZ and MK-571, but no significant difference from cells treated with BZ only $p \leq 0.01$.

The Ability of LTD_4 to Induce Granulocytic Differentiation in 32D Myeloblasts

Since LTD_4 is a downstream effector for G-CSF-induced signal transduction, and BZ has been shown to produce LTD_4 via activation of protein kinase C and thus arachidonic acid release, we tested the ability of LTD, to replace G-CSF, BZ and HQ in the induction of granulocytic differentiation in 32D myeloblasts. As can be seen in Figure 7, LTD_4 is capable of replacing these inducing agents in a concentration-dependent induction of terminal differentiation in 32D myeloblasts.

Table III. Effect of the γ-glutamyl transpeptidase inhibitor, acivicin on benzene-induced granulocytic differentiation

System	Morphology	NTB Reduction
	Percentage of cells counted	
Control	12.3 ± 5.5	16.7 ± 3.5
plus BZ	37.5 ± 3.8[a]	45.7 ± 3.3[a]
plus BZ/acivicin	22.0 ± 1.0	23.5 ± 4.2
plus BZ/acivicin/LTD_4	45.3 ± 11.9[b]	47.3 ± 9.1[b]

Cells (5×10^5/ml) were preincubated in culture with acivicin (1 μM) and LTD_4 in RPMI 1640/15% FBS for 10 min at 37°C, 5% CO_2. BZ (5 mM) was added directly to the culture and NBT reduction and granulocytic differentiation were determined after incubation for seven days. Values represent the mean ± SD of at least two experiments carried out in triplicate.
[a]Significantly different from control and BZ-treated cells pretreated with acivicin. [b]Significantly different from cells treated with BZ and acivicin. $p \leq 0.001$.

Effects of Inhibitors of LTD_4 Formation and of LTD_4 Binding to Its Receptor on the Ability of HQ to Induce Granulocytic Differentiation in 32D Myeloblasts

The induction of differentiation by G-CSF is inhibited by a specific 5-LPO inhibitor (Table V.A.), as would be expected since G-CSF causes the release of arachidonic acid, the substrate for 5-LPO, from plasma membranes [31]. The ability of HQ to induce differentiation is not prevented by a 5-LPO inhibitor suggesting that HQ, in contrast to G-CSF and BZ, does not induce the formation of LTD_4, but rather functions in some other way, perhaps by interacting with the LTD_4 receptor. An experiment was carried out to ascertain whether the ability of HQ to induce terminal granulocytic differentiation in 32D myeloblasts could be prevented by an LTD_4 receptor antagonist. The addition of the specific antagonist, MK571, completely blocked HQ-induced terminal granulocytic differentiation (Table V.B.). It is possible that HQ interacts with the ligand-binding domain of the receptor to activate its signal and initiate the cascade of events that result in granulocytic differentiation.

Table IV. Induction of granulocytic differentiation in the mouse myeloblastic cell line, 32D.3 (G) by benzene and hydroquinone

System[1]	Morphology[2]	NTB Reduction
	Percentage of cells counted	
IL-3 alone	7.5 ± 3.2	4.3 ± 0.6
IL-3 + G-CSF	30.8 ± 2.5	36.7 ± 1.7
IL-3 + BZ	53.0 ± 1.0	55.0 ± 0.7
IL-3 + HQ	67.8 ± 3.3	66.8 ± 1.4

[1]Cells (2.5×10^5/ml) were pretreated with HQ (2 μM in PBS-A) or PBS-A for 30 min at 37°C. The cells were harvested by centrifugation, suspended in IMDM (2.5×10^5 cells/ml) containing 3 u/ml rMuIL-3 and 10% FBS. rG-CSF (0.15 ng/ml) or BZ (5 mM) were added to the PBS-A-pretreated cells. Controls and HQ-pretreated cells received only PBS-A. After three days of culture at 37°C, 5% CO_2, the medium was changed and the cells diluted to the original concentration to insure optimal growth conditions. The incubation was continued for four more days at which time parameters indicative of granulocytic differentiation were assessed. The values listed represent the mean ± SD of the results in triplicate wells. Data is presented as the percentage of cells showing granulocytic differentiation out of a total of 200 cells counted.
[2]Includes cells from promyelocytic progenitors to terminally differentiated granulocytes.

Interaction of Differentiation-Inducing Agents with IL-3 for Growth of 32D Myeloblasts

In the hematopoietic system, cytokines, unlike growth factors in other systems, induce cell growth that is coupled to differentiation such that clonal expansion of a differentiating progenitor cell population is observed. As can be seen in Figure 8A, BZ does not provide a growth signal for 32D myeloblasts in the absence of IL-3, nor does it synergize with IL-3 to promote growth. G-CSF also does not synergize with IL-3 to promote growth, but is capable of inducing growth in the absence of IL-3 (Fig. 8A). Neither LTD_4 nor HQ can induce a signal for proliferation in 32D myeloblasts in the absence of IL-3 (Fig. 8B). In the presence of IL-3, both LTD_4 and HQ showed a significant stimulation of cell

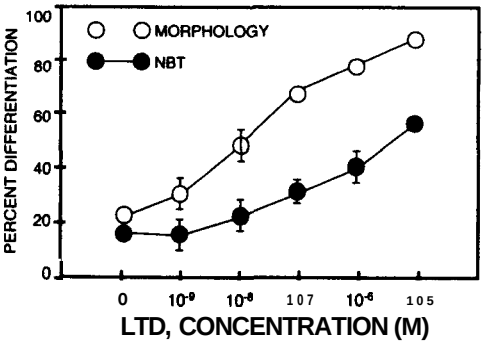

Fig. 7. LTD_4-induced granulocytic differentiation in 32D myeloblasts. Cells (2.5 x 10^5/ml) in IMDM (3u/ml rIL-3; 10%FBS) were incubated with or without LTD_4 for three days at 37°C, 5% CO_2. Cells were thinned to the original cell number/ml and incubated an additional 3 days without the further addition of LTD_4. Cytospin slides were stained for morphological analysis using May-Grünwald/Giemsa stain. Cells were monitored for superoxide production by following NBT reduction using TPA/NBT and counterstaining with Safranin.

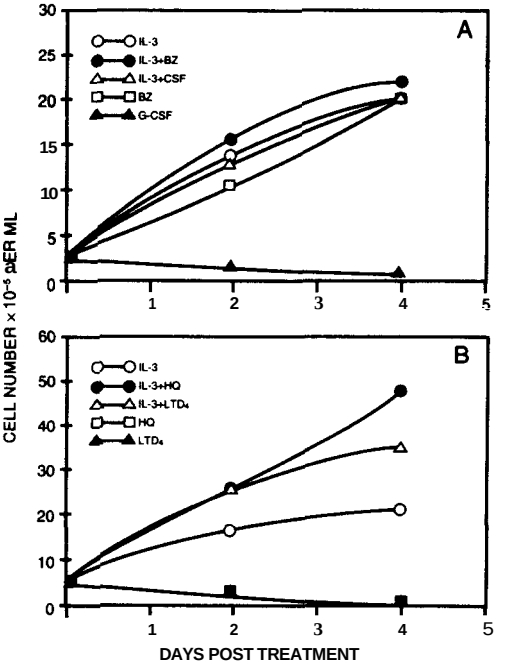

Fig. 8. Effects of differentiation-inducing agents on proliferation of 32D cells in the presence or absence of IL-3. A) Cells (2.5 x 10^5/ml) were incubated in IMDM/10% FBS with 5 mM BZ or rG-CSF (500 u/ml) with or without rIL-3 (3 u/ml) for eight days at 37°C, 5% CO_2. B) Cells were pretreated with 2 µM HQ/PBS-A or PBS-A for 30 min at 37°C, 5% CO_2, washed twice with PBS-A. PBS-A-treated cells were also treated with LTD_4 (3.8 µM). All cultures were incubated for eight days without further feeding. Cell viability and number were determined every second day. Data points represent the mean of triplicate samples which were within 5% of each other. Data were normalized to the controls.

growth which, in the case of HQ, was more than 100% at day 4.

A Comparison of the Kinetics of Stage-Specific Granulocytic Differentiation Induced by the Physiological Inducers G-CSF and LTD$_4$, and by BZ and HQ

These agents all transmit a signal for differentiation in the presence or absence of IL-3. In the presence of the minimal concentration (3 u/ml) of IL-3 required for survival and proliferation of 32D myeloblasts, each of the inducers of granulocytic differentiation caused 100% total differentiation, but the degree of differentiation was delayed in comparison with cells grown in the absence of IL-3 (Table VI). The proliferative signal provided by IL-3 competes with the differentiation signal from the inducer such that the degree of both the total and terminal differentiation is decreased at 6 days. In the absence of IL-3, G-CSF, in contrast to BZ, HQ or LTD_4, can provide signals for proliferation and differentiation. Consequently, G-CSF alone (Table VI, line 3) caused a level of terminal differentiation comparable to that observed with the other inducers in the absence of IL-3; however, the rate at which 100% total differentiation was achieved was delayed (Table VI). A comparison of the data presented in Table VI (total differentiation versus terminal differentiation on day 6 in the absence of IL-3) is presented in Figure 9. G-CSF induced predominantly, terminally differentiated granulocytes whereas HQ induced predominantly myelocytes. BZ and LTD_4 produced a significant number of terminally differentiated granulocytes as well as an increased number of intermediate progenitors. IL-3 alone induced virtually no differentiation.

An analysis of the kinetics of stage-specific granulocytic differentiation was performed on 32D myeloblasts induced by the physiological

Table V. The effects of a 5-lipoxygenase inhibitor (AA861) and an LTD_4 receptor antagonist (MK571) on G-CSF- and HQ-induced differentiation of 32D cells

System	Morphology	NTB Reduction
	Percentage of cells counted	
A.		
Control	17.5 ± 2.6	12.5 ± 3.6
+G-CSF	70.8 ± 3.5	48.2 ± 10.6
+G-CSF + 5-LPO inhibitor	31.9 ± 0.9	12.6 ± 2.4
+HQ	81.5 ± 1.3	48.6 ± 5.7
+HQ + 5-LPO inhibitor	66.1 ± 1.7	38.6 ± 2.0
B.		
Control	17.5 ± 2.6	12.5 ± 3.6
+G-CSF	70.8 ± 3.5	48.2 ± 10.6
+G-CSF + R antagonist	32.8 ± 8.8	26.8 ± 6.2
+HQ	81.5 ± 1.3	48.6 ± 5.7
+HQ + R antagonist	24.1 ± 1.8	15.2 ± 2.5

Cells (2.5×10^5/ml) were pretreated with a final concentration of 2 μM HQ as described in the legend to Table IV with the addition of the 5-LPO inhibitor, AA861, or the LTD_4 receptor antagonist, MK571, at 1 μM final concentration.
Morphological analysis was performed as described in the legend for Table IV.

and chemical inducers in the absence of IL-3. This allowed a study of the effects of inducer in the absence of any proliferative effects of IL-3. G-CSF caused a slight increase in each of the granulocytic progenitor stages at day 2 (Fig. 10A). Because of its ability to provide a proliferative signal as well as one for differentiation, G-CSF showed a marked stimulation of promyelocytes at day 4 as well as mature granulocytes. By day 6, the number of promyelocytes had decreased as was expected because of maturation and because the proliferative signal only permits two or three replicative cycles. The number of intermediate forms was comparable to the number at day 4 and the number of mature granulocytes was greater than 50%. By

Table VI. Effects of inducing agents on patterns of granulocytic differentiation

Agent	Percent of Total Differentiation				Percent of Terminal Differentiation
	Day 0	Day 2	Day 4	Day 6	Day 6
IL-3	0.5 ± 0.5	0.0 ± 0.0	0.7 ± 0.3	6.0 ± 0.0	1.0
IL-3 + G-CSF	0.5 ± 0.5	2.0 ± 0.0	24.0 ± 2.0	91.8 ± 1.6	16.7
G-CSF	0.5 ± 0.5	19.3 ± 0.4	71.5 ± 0.9	90.8 ± 1.6	51.2
IL-3 + BZ	0.5 ± 0.5	3.3 ± 0.3	22.3 ± 1.6	86.8 ± 2.3	18.0
BZ	0.5 ± 0.5	19.0 ± 2.2	89.8 ± 1.1	99.7 ± 0.6	39.8
IL-3 + LTD4	0.5 ± 0.5	9.2 ± 3.2	30.2 ± 2.8	92.8 ± 1.3	25.2
LTD4	0.5 ± 0.5	32.0 ± 0.0	97.0 ± 0.5	99.3 ± 0.3	46.8
IL-3 +HQ	0.5 ± 0.5	4.3 ± 1.4	41.8 ± 2.3	88.5 ± 2.8	14.8
HQ	0.5 ± 0.5	40.0 ± 0.5	91.5 ± 0.0	98.8 ± 0.3	17.2

Cells (2.5×10^5/ml) were treated as described in the legend for Figure 8. Samples of each culture were collected every second day and cytospin preparations were made and stained. Morphologic analysis and determination of the percentages for stage-specific differentiation were performed as described for Figure 9. Percentage of total differentiation was calculated by totaling the percentages of stage-specific differentiation for each sample. Terminal differentiation was calculated based on the number of segmented/banded granulocytes over the total number of cells counted for each sample on day 6.

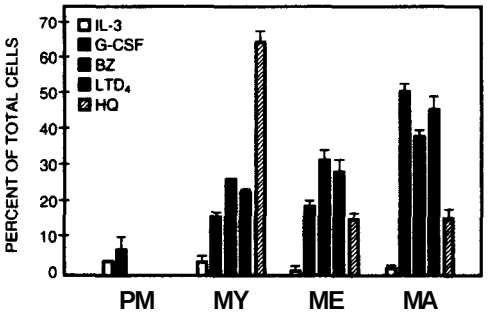

Fig. 9. Granulocytic differentiation in 32D cells 6 days post-treatment with inducing agents. Cells (2.5 x 10^5/ml) were treated with inducing agents as described in the legend for Figure **8.** Samples of each culture were taken on day 6 and Cytospin slides were prepared in triplicate for each culture and stained for morphological analysis with May-Grünwald/ Giemsa as described under Methods. Two hundred cells were counted and an average percent for each stage of differentiation was determined. PM, promyelocytes; MY, myelocytes; ME, metamyelocytes; MA, mature segmentedhanded granulocytes.

day 8, almost all of the early progenitor forms had terminally differentiated to granulocytes. In comparison, LTD_4, a downstream effector of G-CSF signaling, was incapable of providing a proliferative signal as evidenced by the lower number of promyelocytes detected over the six-day period (Fig. 10B). There were many more intermediate progenitors (myelocytes) and terminally differentiated granulocytes at day 2 in the presence of exogenous LTD_4, most probably because the cells do not have to wait for LTD_4 to be produced following the G-CSF signal. At day **4,** the promyelocyte burst seen with G-CSF is absent and the percentage of intermediate and mature forms **(45%)** is significantly increased because the effector was added directly and there was no proliferation signal. By day 6, the myelocytes have begun to mature into metamyelocytes and the number of terminally differentiated granulocytes has remained constant, probably because those that were mature at day **4** are beginning to die off. Because of the lack of a proliferation signal and the rapid induction of

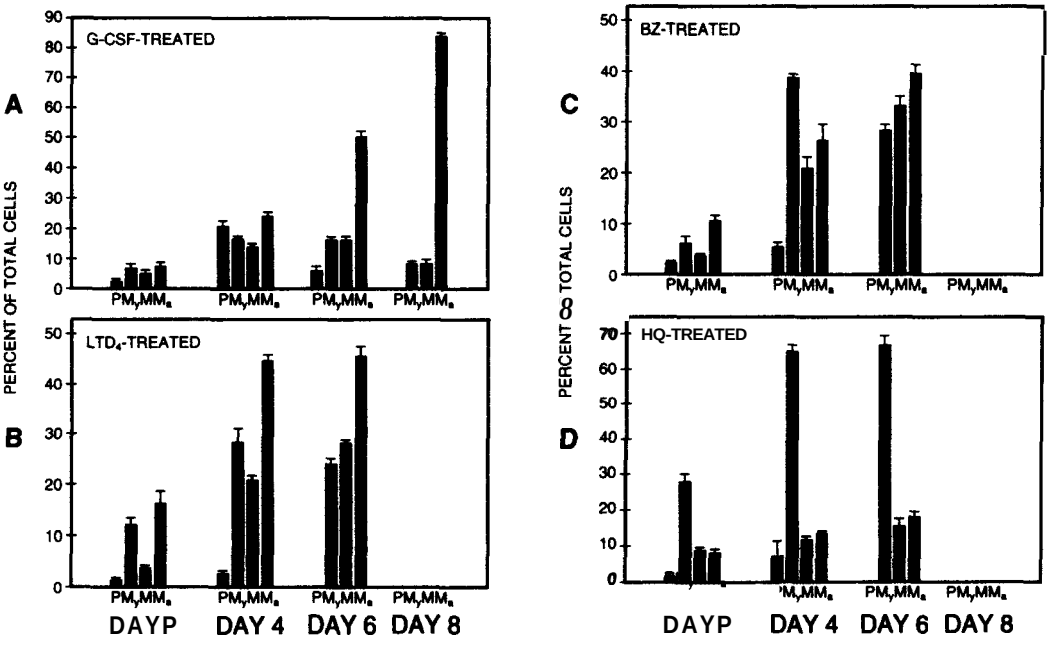

Fig. 10. Profile of stage-specific differentiation induced by G-CSF (A), LTD_4 (B), BZ (C) and HQ (D). Cells **(2.5** x 10^5**/ml)** were treated as described in the legend for Figure **8.** Samples of each culture were collected every second day and Cytospin preparations made and stained. Morphologic analysis and determination of the percentages for stage-specific differentiation were performed as described for Figure *9*. Percentage of total differentiation was calculated by totaling the percentages of stage-specific differentiation for each sample. P, promyelocyte; My, myelocyte; M, metamyelocyte; Ma, mature granulocyte.

differentiation in the presence of LTD_4, there were few viable cells by day 8; however, those that remained were 100% terminally differentiated (data not shown). Thus, while the pattern of differentiation with G-CSF or LTD_4 was different, they had in common the fact that at each day, the major progenitor type was the terminally differentiated granulocyte. BZ induced a pattern of differentiation at day 2 that was qualitatively similar to that seen with G-CSF or LTD_4 (Fig. 10C). At day 4, BZ showed a pattern of differentiation similar to LTD_4 except that the predominant progenitor type was the myelocyte rather than the terminally differentiated granulocyte. At day 6 the BZ-induced pattern continues to be similar to LTD, except that the numbers of myelocytes and metamyelocytes are higher. In contrast, HQ induced an incomplete differentiation pattern; at each day studied, HQ-induced differentiation appears to be partially arrested at the myelocyte stage (Fig. 10D). Thus, BZ appears to slow the progression of granulocytic differentiation in 32D myeloblasts beyond the myelocyte stage at day 4 which resolves itself at day 6 (Fig. 10C), whereas HQ appears unable to induce significant differentiation beyond the myelocyte stage (Fig. 10D). Here, as in the case of LTD_4-induced differentiation, the number of viable cells present at day 8 was very low, but those present were mature granulocytes in the case of BZ and with HQ, a mixture of predominantly intermediate progenitors and some mature granulocytes.

Discussion

Exposure of C57BL/6J mice to a dose of BZ known to depress other hematopoietic cell lineages [32] significantly stimulated granulocytic differentiation as well as the total number of granulocytes indicating that granulopoiesis was also occurring (Fig. 1). BZ did not provide a growth signal for myeloblasts, but stimulated their differentiation to promyelocytes and intermediate progenitors. It did not stimulate the production of mature granulocytes at day 3, however, when measured at day 7, it had increased the number of segmented, mature granulocytes (data not shown). The results showing a general increase in granulocytes after BZ exposure are consistent with those showing an increase in the number of granulocytes in bone marrow of DBA/2J mice after short-term exposure to BZ [15]. HQ, a major metabolite of BZ found in the bone marrow, also induced growth and differentiation of granulocytic progenitor cells measured at day 3 when administered to C57BL/6J mice (Fig. 1) as well as in mouse 32D myeloblasts in culture (Fig. 8B). In contrast to BZ, HQ provided both growth and differentiative signals for myeloblasts that increased the numbers of all progenitor forms, but appeared incapable of inducing terminal differentiation (Fig. 1) for reasons that are not clear at the present time.

In order to study the mechanism(s) whereby BZ and HQ stimulate granulopoiesis, we investigated whether the inductive effect of BZ or HQ on granulopoietic differentiation could be reproduced in myeloblasts in culture. BZ, at noncytotoxic concentrations, caused a dose-dependent specific induction of terminal granulocytic differentiation in HL-60 myeloblasts (Table I, Fig. 2), as measured by the determination of granulocytic morphology and functional parameters of granulocytic differentiation such as superoxide production, chloroacetate esterase activity and the appearance of the specific surface antigen, L-12-2. BZ induction produced a majority of intermediate progenitors (metamyelocytes and band forms), but a significant number of mature granulocytes were observed (Table I). This corresponds to what has been previously observed with HL-60 cells, that granulocytic differentiation induced by several agents is somewhat incomplete and defective [20].

BZ is known to activate protein kinase C [21]. Exposure of HL-60 cells containing ^{32}P-labeled ATP to BZ resulted in the ^{32}P-phosphorylation of cellular proteins (Fig. 2) under conditions where BZ induces granulocytic differentiation. The activation of kinase C appears to be involved in the induction of granulocytic differentiation of BZ-treated HL-60 myeloblasts, since the phosphorylation of cellular proteins and the induced differentiation were prevented by the concomitant presence of BZ with the specific kinase C inhibitor, sphinganine (Fig. 3).

Differentiation of HL-60 myeloblasts to granulocytes by BZ appears to involve the induction of a functioning 5-LPO pathway for the production of LTD_4 since the differentiation is inhibited by the 5-LPO inhibitors, caffeic acid and AA-861 (Table II), by the specific LTD, receptor antagonist, MK-571, (Fig. 6) and by interruption of the LPO anabolic pathway at

the LTC_4 step by acivicin, an inhibitor of γ-glutamyl transpeptidase (Table 111). Inhibition by all of these agents was prevented by the concomitant addition of LTD_4 with the agent.

These results together support the view that the role of BZ in inducing granulocytic differentiation in HL-60 myeloblasts is to produce LTD_4 that is a necessary, if not a sufficient, signal for granulocytic differentiation. BZ appears to function in a manner similar to the physiological inducer of granulocytic differentiation, G-CSF. Although G-CSF has not yet been reported to cause the production of LTD_4, it has been shown to cause the activation of phospholipase A_2 and the release of arachidonic acid from cell membranes [31].

BZ is also capable of inducing granulocytic differentiation in the diploid IL-3-dependent myeloblastic cell, 32D.3 (G) derived from normal mouse bone marrow (Table IV). While IL-3 induces only a growth response, rHuG-CSF in the absence of IL-3 provides both growth (Fig. 6) and differentiation (Table V) signals for 32D myeloblasts. BZ is unable to induce growth in the absence of IL-3, but can provide a differentiation signal. Neither G-CSF nor BZ synergizes with IL-3 in stimulating growth. HQ also induces granulocytic differentiation in 32D myeloblasts (Table V). BZ and HQ can replace the requirement for G-CSF for induction of differentiation. It should be pointed out that in the experiment reported in Table V, no effort was made to optimize the concentration of a given inducer so that the magnitude of the BZ-or HQ-induced differentiation cannot be quantitatively compared with that of the physiological inducer, G-CSF. The results, however, do indicate that BZ and HQ can induce granulopoiesis in 32D mouse myeloblasts since both the number of progenitor cells and terminally differentiated granulocytes increased, and they support our in vivo results on the induction of granulopoiesis by BZ and HQ in mice (Fig. 1). The ability of HQ to synergize with GM-CSF to stimulate GM-CFU has been studied by Irons *et* al. [33], who showed that pretreatment of nonadherent murine bone marrow or lineage-restricted hematopoietic cells with HQ in vitro, followed by culture in complete medium for 8 days, significantly enhanced the number of granulocyte-macrophage colonies induced by recombinant GM-CSF. Optimal enhancement was observed with 1 μM HQ, was largely independent of the concentration of GM-CSF, and was not observed with other marrow metabolites such as phenol or catechol.

The facts that BZ-induced granulocytic differentiation in myeloblasts in culture is prevented by inhibitors of protein kinase C and 5-lipoxygenase support the view that BZ-induced differentiation is due to BZ per se and not the result of its metabolism to HQ in the myeloblast. Consequently, not all of the granulopoietic activity of BZ in vivo can be attributed to its metabolism to HQ. During chronic BZ exposure, BZ and HQ are present in the bone marrow and consequently both can contribute to granulocytic differentiation. On the basis of results present here, BZ, like G-CSF, appears to activate the arachidonic acid cascade and upregulate the 5-LPO pathway for the production of LTD_4. HQ appears to obviate the requirement for LTD_4 by activating the LTD_4 receptor since specific LTD_4 receptor antagonists, but not 5-LPO inhibitors, prevent HQ induction of granulocytic differentiation in myeloblasts.

One can speculate about the roles of BZ and HQ in BZ-induced acute myeloid leukemia. As a known clastogen, HQ may cause a leukemogenic initiating event in a myeloblast such as a one of the translocations or deletions characteristic of acute myeloid leukemia [4]. At the same time it may covalently bind to the LTD_4 receptor to constitutively activate this signal-driven process and induce granulocytic differentiation which is incomplete and arrested at the myelocyte stage. Blocks in the developmental program of terminal hematopoietic cell differentiation appear to be a major step in tumor progression [34]. Concomitantly, BZ might provide a promotional effect on the initiated myeloblast via constitutive activation of protein kinase C causing overexpression of its activity with resultant pleiotropic effects on morphology and growth control. Indeed, such effects and susceptibility to malignant transformation have been observed when protein kinase C is overexpressed in fibroblast cell lines [35, 36], and increased protein kinase C activity is seen during induction of HL-60 myeloblasts to granulocytes by retinoic acid or dimethyl sulfoxide [37]. A recent report indicates that BZ, possibly by activation of protein kinase C, causes the hyperphosphorylation of *p53*, the growth suppressor that regulates entry into the cell cycle at the G_1 phase, and suggests that

tumor promotion by BZ may involve hyperphosphorylation of *p53* [38].

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