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Alteration by Flutamide of Neutrophil Response to Stimulation

IMPLICATIONS FOR TISSUE INJURY

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ABSTRACT. When activated, inflammatory cells such as polymorphonuclear leukocytes (PMNs) can damage isolated hepatocytes *in vitro*. These studies were performed to determine if flutamide activates PMNs. Flutamide (Eulexin) is an orally active, nonsteroidal antiandrogen that can cause liver injury associated with inflammation. Activation of PMNs was assessed from the production of superoxide anion and the release of myeloperoxidase in the presence or absence of flutamide and phorbol myristate acetate (PMA) or f-methionyl-leucyl-phenylalanine (fmlp). In addition, hepatocytes were cocultured with PMNs stimulated with PMA or fmlp in the presence or absence of flutamide, and cytotoxicity to hepatocytes was evaluated from the release of alanine aminotransferase into the medium. Flutamide alone did not stimulate the generation of superoxide anion by PMNs but potentiated its production in response to PMA. At lower concentrations of flutamide (i.e. 25 μM), there was a tendency toward increased release of myeloperoxidase, whereas at higher concentrations (i.e. 75–100 μM) flutamide inhibited degranulation in response to fmlp. In coculture with hepatocytes, PMNs exposed to either flutamide, fmlp, or PMA alone caused a significant increase in release of alanine aminotransferase. Hepatocellular toxicity caused by PMNs incubated with flutamide and PMA was additive and was not affected by the addition of superoxide dismutase and catalase. Flutamide had no significant effect on fmlp-induced injury in cocultures. These data indicate that flutamide alters the activation of PMNs by subsequent stimuli *in vitro*. In addition, in the presence of flutamide, minor PMN-mediated injury to isolated hepatocytes was observed. BIOCHEM PHARMACOL 53;8:1179–1185, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. nonsteroidal antiandrogen; superoxide; myeloperoxidase; PMA; fmlp; hepatotoxicity

Flutamide (Eulexin; 2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide) is a nonsteroidal, orally active antiandrogen. It exerts its antiandrogenic action by inhibiting androgen uptake and/or by inhibiting nuclear binding of androgen in target tissues. Flutamide is used in association with castration or luteinizing hormone releasing factor agonists for advanced prostate cancer. It is also used in combination with oral contraceptives for the treatment of hirsutism and benign prostatic hyperplasia. Its clinical use has resulted in the occurrence of hepatitis, with a reported incidence of 0.36% [1]. Flutamide-induced hepatitis is associated with cholestasis, and neutrophils and lymphocytes have been observed in areas of portal necrosis [1, 2]. Fulminant hepatitis as well as liver failure have occurred [3]. Although the mechanism of flutamide-induced liver damage is unknown, some cases have been associated with peripheral eosinophilia and neutropenia, suggesting an involvement of the immune system [4, 5].

A role for inflammatory cells such as neutrophils (PMNs[‖]) in some animal models of tissue injury has been established. For example, PMNs play a causal role in liver injury due to exposure to bacterial endotoxin [6] or to the cholestatic agent α-naphthylisothiocyanate [7]. The mechanisms by which PMNs contribute to liver injury in these models are poorly understood. One event likely to be common to all models of PMN-dependent damage is the activation of PMNs [8, 9]. Activated PMNs play an important role in host defense against pathogens in part through the generation of O_2^- and release of mediators such as proteolytic enzymes and arachidonic acid metabolites. Under some circumstances, however, these same functions may contribute to tissue injury. Activated PMNs are cytotoxic to isolated hepatic parenchymal cells [8, 9] and endothelial cells *in vitro* [10]. Since hepatic PMN accumulation is associated with liver lesions resulting from flutamide administration, the purpose of this study was to determine

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‖ *Abbreviations:* ALT, alanine aminotransferase; fmlp, f-methionyl-leucyl-phenylalanine; HC, hepatocyte; LDH, lactate dehydrogenase; MPO, myeloperoxidase; O_2^-, superoxide anion; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; and SOD, superoxide dismutase.

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if flutamide activates isolated PMNs and to test whether this activation leads to hepatocyte injury *in vitro*.

MATERIALS AND METHODS

All animals were used in accordance with the guidelines of the Michigan State All University Committee for Animal Use and Care.

Isolation of PMNs

Glycogen-elicited PMNs were isolated from the peritoneum of male, Sprague–Dawley, retired breeder rats (Charles River Laboratories, Portage, MI) as described previously [11]. Briefly, 30 mL of 1% glycogen in sterile saline were injected into the peritoneum of rats anesthetized with diethyl ether. Four hours later, the rats were anesthetized again with diethyl ether and decapitated. The peritoneum was rinsed with 30 mL of 0.1 M PBS containing 1 U/mL heparin. The rinse solution was filtered through gauze and spun for 7 min at 500 *g*. Red blood cells were lysed with 0.15 M NH_4Cl, and the PMNs were washed twice with PBS. PMNs were resuspended in Hanks' balanced salt solution (HBSS) for studies of PMN function or in Williams' medium E (Gibco, Grand Island, NY) for coculture studies. The percentage of viable PMNs was routinely >95%.

Measurement of Oxygen Radical Production by PMNs

O_2^- generation by PMNs in the absence or presence of flutamide (Eulexin; a gift of the Schering Corp., Kenilworth, NJ) and/or PMA; (Sigma Chemical Co., St. Louis, MO) was measured spectrophotometrically as the SOD-sensitive reduction of ferricytochrome *c* [12]. PMNs (2×10^6) were incubated with various concentrations of flutamide (0, 5, 25, 50, 75, or 100 μM) or its vehicle, HBSS, at 37° for 5 min followed by incubation with various concentrations of PMA (0, 2, or 20 ng/mL) for an additional 10 min at 37°. For each condition, two samples were included: to one sample SOD (85 U/mL) was added before incubation, and to the other sample SOD was added after incubation. O_2^- produced during this 15-min period was determined from the difference in absorbance at 550 nm of the cell-free supernatant fluids from these two samples using an extinction coefficient of 18.5 mM^{-1} cm^{-1}.

Measurement of PMN Degranulation

Release of the azurophilic granular constituent MPO, used as a marker of PMN degranulation, in the absence or presence of flutamide was measured spectrophotometrically by the method of Henson *et al.* [13]. PMNs (2×10^6) were preincubated with cytochalasin B (5 μg/mL) for 10 min at room temperature, and then were incubated with various concentrations of flutamide (0, 5, 25, 50, 75, or 100 μM) in the absence or presence of 0, 10, 50, or 100 nM fmlp (Sigma) for 15 min at 37°. Cytochalasin B was used to promote release of granular constituents into the extracellular environment upon PMN stimulation [14, 15]. MPO released during this 15-min period was expressed as nanomoles per minute per 10^6 PMNs using an extinction coefficient of 7.5 mM^{-1} cm^{-1}.

Determination of Viability of PMNs

Viability of PMNs was determined from the release of LDH into the medium [16]. At the end of each experiment, PMNs were spun in a centrifuge, and the activity of LDH in the cell-free supernatant fluid was measured spectrophotometrically from the oxidation of NADH, using pyruvate as substrate. In addition, untreated PMNs (2×10^6) were lysed with Triton X-100 and sonication, then spun in a centrifuge, and the LDH activity in the cell-free supernatant fluid was used to estimate total cellular LDH. The activity of LDH in the medium was expressed as a percentage of the total releasable LDH.

Isolation of HCs

HCs were isolated from male, Sprague–Dawley rats (Crl:CD BR (SD) VAF/plus; Charles River Laboratories), weighing 125–150 g according to the method of Seglen [17] as modified by Klaunig *et al.* [18]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the portal vein was cannulated. The liver was perfused with approximately 200 mL of Ca^{2+}-, Mg^{2+}-free HBSS (39°) followed by perfusion with 250 mL of collagenase type B (0.5 mg/mL; Boehringer-Mannheim Biochemicals, Indianapolis, IN). The resulting digest was filtered through gauze and spun at 50 *g* for 2 min. The HCs were resuspended in Williams' medium E containing 10% fetal bovine serum (Intergen, Purchase, NY) and 0.1% gentamicin (Gibco), and cells were plated in 6-well plates at a density of 5×10^5 HCs per well. Using this isolation procedure, 98% of the cells in the final preparation are hepatic parenchymal cells; the remaining cells are lymphoid cells, macrophages, or PMNs [8]. Viability of cells as determined by the exclusion of trypan blue was routinely >90%. After an initial 3-hr attachment period, the medium and unattached cells were removed, and fresh medium was added.

Determination of HC Injury

PMNs (5×10^6/well) were added to adherent HCs in Williams' medium E containing gentamicin. Wells contained either HCs alone, HCs plus PMNs, or PMNs alone. After allowing PMNs to attach for 30 min, fmlp (50 or 100 nM) or PMA (20 or 100 ng/mL) was added, followed immediately by the addition of flutamide (5 or 25 μM) or its vehicle. Cultures were incubated for an additional 16 hr (37°; 92.5% O_2/7.5% CO_2), and the medium was collected. The cells remaining on the plate were lysed with 1% Triton X-100 and sonication. The medium and the cell lysates were spun at 600 *g* for 10 min, and the activity of ALT was

determined spectrophotometrically in the cell-free supernatant fluids using Sigma kit number 59-UV (Sigma). The apparent activity of ALT in naive medium incubated for 16 hr under the same conditions (approximately 6–7 U/mL) was determined and subtracted from all medium ALT values before further analysis. The ALT activity in the medium was expressed as a percentage of the total activity (medium + lysate). ALT is a sensitive and specific indicator of hepatocellular damage in rats [19, 20]. The ALT activity in PMNs is below the limit of detection, and ALT activity released into the medium was taken as an index of injury to HCs [8].

In some studies, SOD (300 U/mL) and catalase (3000 U/mL) were added to cell cultures as described previously [8] prior to the addition of PMA and flutamide, and experiments proceeded as described above.

Statistical Analysis

Results are presented as means ± SEM. For all results, N represents the number of repetitions of an experiment, each experiment using cells from different rats. Data were analyzed by two-way, repeated measures ANOVA using SigmaStat (Jandel Scientific, San Rafael, CA). Individual means were compared using the least significant difference (lsd) test or the Student–Newman–Keuls test. Data expressed as percentages were transformed (angular transformation) prior to ANOVA and further statistical manipulation. For all studies, the criterion for significance was $P \leq 0.05$.

RESULTS

Activation of PMNs in the Presence of Flutamide

In the absence of stimulation, PMNs did not produce O_2^- (Fig. 1). Flutamide alone at concentrations up to 100 μM did not cause significant generation of O_2^-. Two concentrations of PMA were used: 2 ng/mL of PMA alone did not cause O_2^- production, whereas 20 ng/mL stimulated significant generation of O_2^-. In PMNs exposed to 2 ng/mL PMA, the addition of flutamide at concentrations ≥50 μM resulted in significant superoxide production. This enhanced response occurred at lower concentrations of flutamide (25 μM) in the presence of 20 ng/mL PMA.

In the absence of stimulation, PMNs did not undergo degranulation, and little MPO was released (Fig. 2). Exposure to flutamide in concentrations up to 100 μM did not cause statistically significant release of MPO, although there was a trend toward increased activity in the medium of PMNs exposed to 25 or 50 μM flutamide. Activation of PMNs with 10, 50, or 100 nM fmlp caused significant release of MPO. Exposure to lower concentrations of flutamide in the presence of fmlp did not affect release of MPO, whereas at higher concentrations (>50 μM) flutamide inhibited degranulation in response to 50 or 100 nM fmlp. Release of LDH by PMNs was not affected by exposure to flutamide, fmlp, or the combination of these agents (Fig. 3).

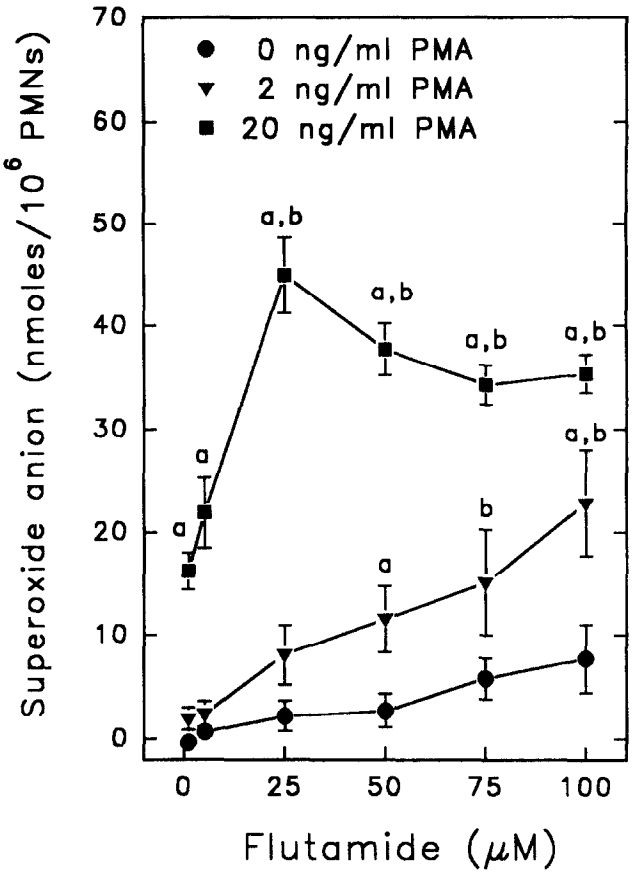

FIG. 1. Superoxide anion production by neutrophils in the presence of flutamide. PMNs were exposed to flutamide at the concentrations indicated for 5 min and then were exposed to PMA for 10 min at 37°. O_2^- released for the entire period was determined as described in Materials and Methods. Values are means ± SEM, N = 5. Key: (a), significantly different from 0 ng/mL PMA at the same concentration of flutamide; and (b), significantly different from 0 μM flutamide at the same concentration of PMA ($P \leq 0.05$).

PMN-Mediated Cytotoxicity to Hepatocytes in the Presence of Flutamide

In preliminary experiments, the effect of flutamide on hepatocyte viability was determined (data not shown). Release of ALT was concentration related, and significant cytotoxicity was observed at concentrations ≥50 μM. Concentrations of flutamide that were not cytotoxic to HCs were chosen for the remaining studies.

Experiments were performed to determine whether the effects of flutamide on PMN activity, as shown above, modulated PMN-mediated cytotoxicity to isolated hepatocytes. Incubation of HCs with flutamide and/or PMA did not produce statistically significant cytotoxicity (Fig. 4A). When HCs were cocultured with PMNs exposed to 5 or 25 μM flutamide in the absence of PMA, a statistically significant increase in the release of ALT was observed (Fig. 4B). As demonstrated previously [8], PMA-activated PMNs were cytotoxic to HCs. The cytotoxic effect in the presence of PMA and flutamide was approximately additive. Addi-

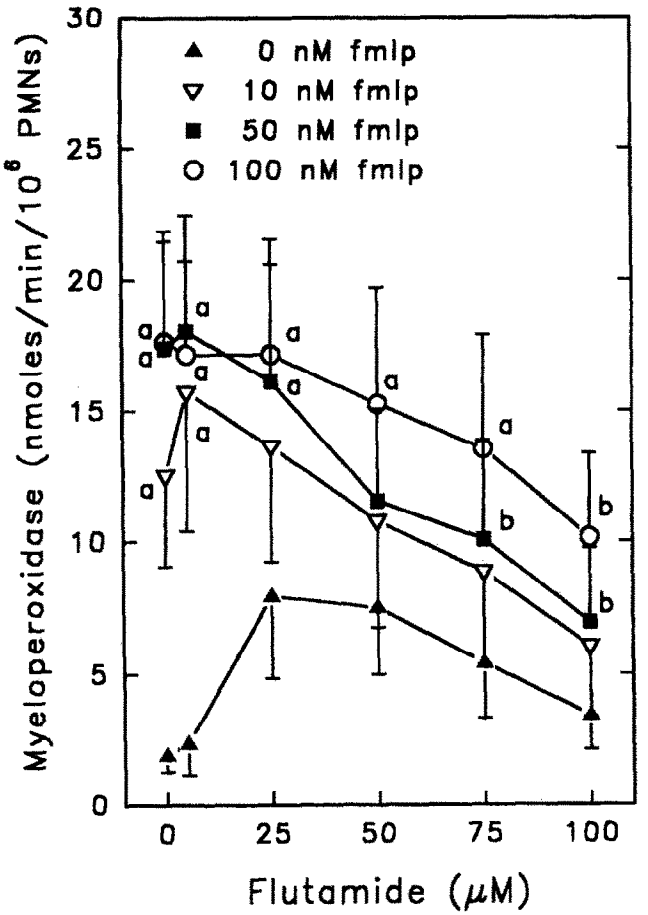

FIG. 2. PMN degranulation in the presence of flutamide. PMNs were pretreated with 5 μg/mL cytochalasin B for 10 min at room temperature. PMNs were then exposed to flutamide at the concentrations indicated in the presence or absence of fmlp for 15 min at 37°. Myeloperoxidase released into the medium was measured and reported as described in Materials and Methods. Values are means ± SEM, N = 5. Key: (a), significantly different from 0 nM fmlp at the same concentration of flutamide; and (b), significantly different from 0 μM flutamide at the same concentration of fmlp ($P \leq 0.05$).

tion of the antioxidants, SOD and catalase in combination, did not affect the release of ALT in the presence of PMA and flutamide (29.2 ± 4.5 and 31.4 ± 4.4% in the absence and presence, respectively, of SOD and catalase).

Flutamide alone or in the presence of fmlp was not cytotoxic to HCs (Fig. 5A). In cocultures of PMNs and HCs in the absence of fmlp, both 5 and 25 μM flutamide caused a significant increase in the release of ALT from HCs (Fig. 5B). As expected, activation of PMNs with fmlp caused increased release of ALT in cocultures. This cytotoxicity was not affected by the addition of either 5 or 25 μM flutamide.

DISCUSSION

Flutamide is hepatotoxic in some individuals, and the mechanism of hepatotoxicity is not well understood. Since

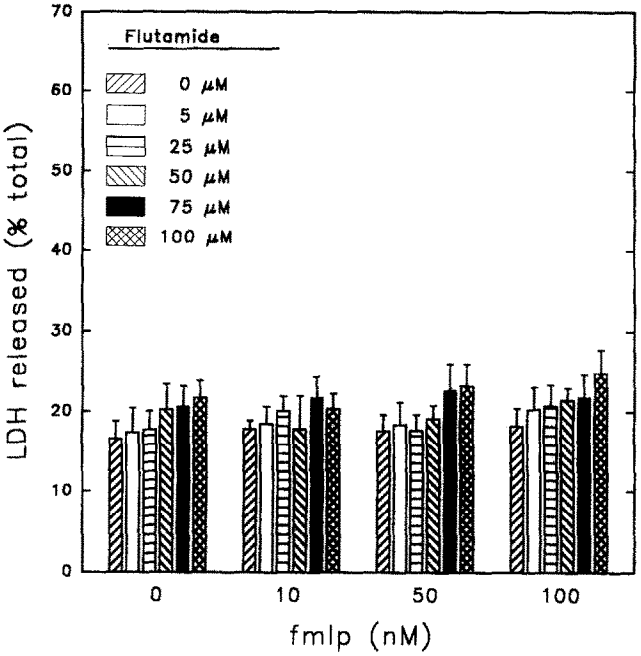

FIG. 3. Lack of cytotoxic effect of flutamide on PMNs. PMNs were treated as described in the legend to Fig. 2. LDH activity released into the medium was measured and reported as the percentage of total releasable LDH as described in Materials and Methods. Values are means ± SEM, N = 5. No significant differences were observed.

flutamide-induced liver injury is often associated with inflammation [1, 2], this study was performed to determine whether flutamide stimulates PMNs *in vitro*. Exposure of PMNs to flutamide alone did not induce production of O_2^-

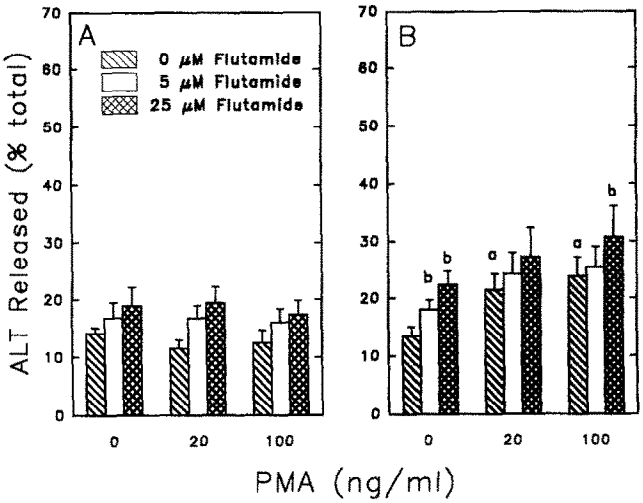

FIG. 4. Effect of flutamide on HCs alone (A) and in coculture with PMNs (B) in the absence or presence of PMA. HCs and PMNs were isolated and plated as described in Materials and Methods. Flutamide and PMA were added simultaneously at the indicated concentrations, and after 16 hr the activity of ALT released into the medium was determined as described in Materials and Methods. Values are means ± SEM, N = 5. Key: (a), significantly different from 0 ng/mL of PMA at the same concentration of flutamide; and (b), significantly different from 0 μM flutamide at the same concentration of PMA ($P \leq 0.05$).

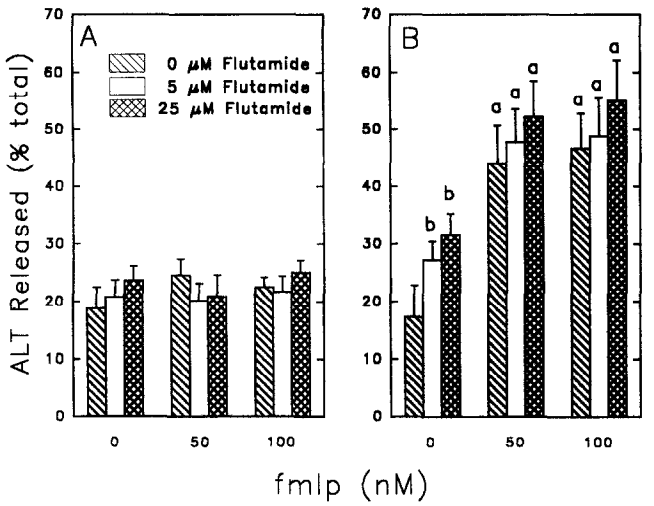

FIG. 5. Effect of flutamide on HCs alone (A) and in coculture with PMNs (B) in the absence or presence of fmlp. HCs and PMNs were isolated and plated as described in Materials and Methods. Flutamide and fmlp were added simultaneously at the indicated concentrations, and after 16 hr the activity of ALT released into the medium was determined as described in Materials and Methods. Values are means ± SEM, N = 4. Key: (a), significantly different from 0 nM fmlp at the same concentration of flutamide; (b), significantly different from 0 μM flutamide at the same concentration of fmlp ($P \leq 0.05$).

or secretion of MPO; however, flutamide did alter the response to other PMN stimuli. Exposure to flutamide potentiated the response to 2 ng/mL PMA. In contrast to effects on PMA-induced O_2^- production, larger concentrations of flutamide inhibited fmlp-stimulated degranulation. This inhibition was not a result of cytotoxicity, since flutamide did not increase release of LDH from PMNs in the presence or absence of fmlp (Fig. 3).

The observation that flutamide modulates activation of PMNs by these soluble stimuli raises the possibility that flutamide may influence activation of PMNs by endogenous stimuli, especially stimuli that activate PMNs by mechanisms similar to fmlp or PMA. Fmlp is a chemotactic peptide derived from gram negative bacteria [21], which activates PMNs through a mechanism involving phospholipase C and tyrosine kinases [22–24]. Activation of tyrosine kinases is common to the mechanism by which a number of mediators stimulate PMN function. For example, leukotriene B_4, complement factor C5a, platelet-activating factor, and cytokines including interleukin-8 and tumor necrosis factor-α increase tyrosine phosphorylation in PMNs, and inhibition of tyrosine kinase activity decreases O_2^- production [25, 26]. PMA activates PMNs by a mechanism dependent on protein kinase C (PKC) [27], and PKC-dependent and -independent pathways are involved in the mechanisms of chemoattraction of PMNs by C5a [26]. Thus, the possibility exists that flutamide could alter responses to these endogenous stimuli as was observed for fmlp and PMA in these studies.

Since treatment with flutamide has been associated with liver necrosis, and PMNs have been observed in necrotic lesions [1, 2], we tested the possibility that flutamide might influence PMN-dependent tissue injury in an *in vitro* model. We have demonstrated previously that activation of PMNs with PMA or fmlp causes hepatocellular injury *in vitro* [8], and these results were confirmed in this study (Figs. 4B and 5B). Neither production of O_2^- (Fig. 1) nor cytotoxicity (Fig. 4B) induced by PMA-stimulated PMNs was altered in the presence of 5 μM flutamide. Unlike O_2^- production, for which a greater-than-additive response was seen with 20 ng/mL PMA plus 25 μM flutamide, PMN-mediated cytotoxicity due to PMA and flutamide at these concentrations was approximately additive. Larger concentrations of flutamide increased O_2^- generation in response to 2 ng/mL PMA; however, these concentrations of flutamide were cytotoxic to HCs alone and, therefore, were unsuitable for coculture studies. At concentrations used in cultures, flutamide did not affect cytotoxicity to HCs induced by fmlp-activated PMNs (Fig. 5B) and did not affect degranulation of PMNs in response to fmlp (Fig. 2).

Flutamide increased O_2^- production in response to PMA; however, the addition of SOD plus catalase did not affect ALT released in the presence of PMA and flutamide in cocultures. These results are consistent with those reported previously for PMA alone [8], and suggest that production of reactive oxygen species is not the sole mechanism of cell injury under these conditions.

In HCs alone, concentrations of flutamide ≥50 μM were cytotoxic within 16 hr. Others have demonstrated that larger concentrations of flutamide (>500 μM) were cytotoxic to hepatocytes over 4 hr [28]. Thus, smaller concentrations of flutamide are directly cytotoxic over longer exposure periods, and it is not known if the mechanisms are the same under both conditions. Flutamide is oxidized by cytochrome P450 to electrophilic metabolites that bind covalently to protein [29], and this effect is thought to be involved in inhibition of mitochondrial respiration and ATP formation in flutamide-treated cells [28]. In addition, reduced glutathione concentration is diminished in isolated rat hepatocytes exposed to flutamide, potentially increasing the susceptibility to oxidant-mediated injury. The cytochrome P450-mediated formation of a toxic metabolite may explain why flutamide was toxic at smaller concentrations in HCs (i.e. 50 μM) than in PMNs (Fig. 3, concentrations up to 100 μM were not cytotoxic) because the latter are not well-equipped with cytochromes P450.

Concentrations of flutamide below 50 μM were not cytotoxic to HCs in any of our studies but did cause a small but significant increase in the release of ALT in cocultures of PMNs and HCs (Figs. 4B and 5B). This effect on HCs in the presence of PMNs is curious because at these concentrations flutamide did not affect the function of isolated PMNs. One possible explanation for these results is that the cytochrome P450-generated metabolite of flutamide produced by HCs is a more potent stimulus for activation of PMNs than flutamide itself. Alternatively, exposure to con-

centrations of flutamide or its metabolites which are not overtly toxic may alter hepatocellular membranes to make HCs targets of PMN attack.

Variability in response is observed in humans treated with flutamide: the incidence of liver injury is only about 0.36%, indicating that most patients do not experience liver toxicity. Furthermore, serum transaminases are elevated but return to normal in some patients without alteration of treatment, whereas for other patients reduction of flutamide dose is required [30, 31]. It is interesting to speculate that PMN-mediated injury may contribute to the variability in response. The concentrations of flutamide used in these studies were larger than would be achieved in plasma in humans undergoing treatment, but it is possible, for example, that in some individuals flutamide simultaneously renders HCs more susceptible to oxidant-mediated injury, initiates infiltration of PMNs into the liver, and increases PMN responsiveness to endogenous activators.

In summary, flutamide altered the response of PMNs to other stimuli but did not by itself activate PMNs. Despite the lack of effect of flutamide alone on PMN O_2^- production or degranulation, flutamide was cytotoxic to HCs in the presence of PMNs at concentrations that were not toxic to HCs alone. PMN-mediated toxicity to HCs in the presence of flutamide and PMA was additive, whereas flutamide did not alter cytotoxicity to HCs induced by fmlp-activated PMNs.

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