ABSTRACT

In response to inflammatory stimuli, macrophages synthesize and secrete prostaglandins (PGE) along with other potent inflammatory mediators. We have studied the effect of hyperthermia on the production of PGE by murine mononuclear phagocytes. Exposure to high temperature-induced PGE production by cultured C3H/HeJ exudate macrophages in a time- and temperature-dependent manner. Increased in PGE production was detected when macrophages were treated at 41°C and above for 1 h with a much greater increase at 42 and 43°C. The secretion of PGE into culture supernatants by heat-treated macrophages reached a maximum approximately 24 h after heat treatment. The production of PGE by macrophages after hyperthermia was inhibited either by the addition of 5 × 10−5 M indomethacin or by the subsequent incubation at 4°C, suggesting that the elevated PGE production by macrophages is mediated through the activation of cyclooxygenase. Heat treatment under the same conditions failed to stimulate the production of PGE by either a human monocyte-like tumor cell line (U-937) or a mouse fibroblast cell line (L-929).

INTRODUCTION

Macrophages play a central role in the host's immune responses against tumors. Several lines of evidence indicate that macrophages, when properly activated, will kill neoplastic cells in vitro and possibly in vivo (1–4). Macrophages have been shown to be present in significant numbers in both human and animal tumors either by direct infiltration from circulating monocytes and/or by local proliferation (5–7). In response to inflammatory stimuli, macrophages synthesize and release prostaglandins PGEs, the main cyclooxygenase product along with several other potent biological mediators (8–10). Recent studies showed that PGEs released by macrophages appear to function primarily as a negative feedback inhibitor in suppressing a number of in vitro immunological responses including the production and functional activities of mononuclear phagocytes (10, 11).

Although hyperthermia is currently being used in treating localized or systemic cancers, the effect of heat treatment on cells of the mononuclear phagocytic system has not been carefully examined. Furthermore, the existing literature provides conflicting results on the effects of hyperthermia on the non-specific host immune response to malignant diseases. A number of studies have shown a heat-induced stimulation of the immune response in animals and humans (12, 13), while others have shown either no effect or a depression of response (13–15). Still others have demonstrated an increase in metastases following heat treatment under certain conditions (14, 16). Clearly, the interaction of hyperthermia with the host immune system and the overall effects on the control of tumor growth is complex.

The present study was undertaken as part of a larger project aimed at elucidating the biological effects of hyperthermia on tumor-associated macrophages and other immunocytes. While the accumulation of macrophages within tumors is obviously necessary for a local host-tumor interaction, however, equally important is the functional state of macrophages in determining the efficacy of any host immune antitumor effect. The functional state of macrophages is regulated by several inflammatory mediators many of which are produced by macrophages themselves. To better understand the overall effects of hyperthermia on the immune system, we examined the effect of heat treatment on the synthesis of PGEs by mononuclear phagocytes. We report here that the production of PGEs by peritoneal exudate macrophages was greatly enhanced by heat treatment.

MATERIALS AND METHODS

Mice. Male or female C3H/HeJ mice 8–12 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Cells obtained from this strain of mice are refractory to the stimulation of endotoxin in vitro. All mice were fed standard laboratory chow and water ad libitum.

Reagents. Fetal calf serum was obtained from Sterile Systems, Inc. (Logan, UT). The particular lot of serum used in this study contained extremely low concentrations of endotoxin (<0.1 ng/ml). α-MEM and LH (tissue culture grade) were obtained from GIBCO (Grand Island, NY). Anti-PGE2 antibodies are a product of Saragen (Boston, MA). Indomethacin was obtained from Sigma Chemical Co. (St. Louis, MO).

Cells. Macrophages derived from endotoxin hyporesponsive C3H/HeJ mice were used throughout this study to minimize the effect of possible endotoxin contamination in culture medium and reagents (9). Peritoneal exudate cells were harvested by peritoneal lavage with 5 ml of α-MEM 3 days after one i.p. injection of 1.5 ml of thioglycollate medium (Difco Laboratories, Detroit, MI) (17). The yields of exudate cells varied from 10 to 25 × 106 cells/lavage. To enrich macrophages, appropriate number of exudate cells were seeded in Falcon T-25 tissue culture flask in α-MEM containing 10% fetal calf serum (Alpha-10) for 20 min at 37°C, after which another 10% fetal calf serum replaced the initial media. The adherent PEM that remained in the dishes were replenished with 3 ml of α-MEM containing 0.25% LH (α-LH) and cultured for an additional 20 h at 37°C to allow the cells to attach firmly to the flask. Typically in these studies, over 95% of the adherent cells are phagocytic and esterase stain positive.

Both U-937, a monocytic-like cell line derived from a patient with histiocytic lymphoma (18), and L-929, a mouse fibroblast cell line, were maintained in Alpha-10. For heat treatment, cells were removed from flasks by trypsinization. After extensive washings with phosphate-buffered saline. The adherent PEM that remained in the dishes were replenished with 3 ml of α-MEM containing 0.25% LH (α-LH) and cultured for an additional 20 h at 37°C to allow the cells to attach firmly to the flasks. Typically in these studies, over 95% of the adherent cells are phagocytic and esterase stain positive.

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3 The abbreviations used are: PGE, prostaglandin; PEM, peritoneal exudate macrophages; LH, lactalbumin hydrolysate; RIA, radioimmunoassay; a-MEM, α-minimal essential medium.
flasks were returned to a 37°C incubator with the caps loosely open.

PGE Assays. After heat treatment, macrophages were further cultured at 37°C for an additional 24 h or as indicated in the text. Culture supernatants were then collected, centrifuged (1000 x g, 20 min), and filtered (0.2 μm) to remove cell debris. The viability of macrophages remaining on tissue flasks was determined by dye exclusion. PGE2 levels in the culture media were measured by a RIA described by Wahl (20) with slight modification. PGE2 used for the standard curve was obtained from The Upjohn Co. (Kalamazoo, MI). Samples were measured directly without further extraction. We have shown previously that the presence of LH did not interfere with the assay. Bound and free ligand were separated by using dextran-coated charcoal. The sensitivity of this assay was about 0.5 ng/ml. Since the antibodies used in this study cross-react with both PGE1 and PGE2, the results were reported as PGE. The degrees of cross-reactivity at 50% inhibition of maximum binding for PGE2 and PGA1 were 6 and 3%, respectively. Medium alone (α-LH) without PEM was always included in each experiment to determine the possible interference of LH with RIA.

RESULTS

Induction of PGE Production by Hyperthermia. Cultured macrophages were treated at 37°, 39°, 40°, 41°, 42°, and 43°C for 60 min. Cells were then incubated at either 37°C or 4°C for an additional 24 h after which culture supernatants were collected, centrifuged, and filtered for PGE RIA. Heat treatment at or above 41°C for 1 h caused a temperature-dependent increase in the production of PGE (Fig. 1) with a much greater increase above 41°C. Compared to that at 37°C, the level of PGE in culture supernatants increased about 16-fold with heat treatment at 43°C for 1 h. When macrophages were incubated at 4°C instead of 37°C after heat treatment, the production of PGE induced by heat was, for the most part, inhibited. When culture medium without macrophages were subjected to similar heat treatment, no PGE was detected in the medium.

The effect of hyperthermia on cell viability was determined by dye exclusion method from parallel cultures. Heat treatment below 40°C was noncytotoxic for murine PEM. Heat treatment, no PGE was detected in the medium. Compared to that at 37°C, the level of PGE in culture supernatants increased about 16-fold with heat treatment at 43°C for 1 h. When macrophages were incubated at 4°C instead of 37°C after heat treatment, the production of PGE induced by heat was, for the most part, inhibited. When culture medium without macrophages were subjected to similar heat treatment, no PGE was detected in the medium.

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We examined the kinetics of PGE production by heat-treated PEM. The levels of PGE from culture supernatants increased after various time periods demonstrated a dramatic increase within 8 h following heat treatment and reached a maximum approximately 24 h after hyperthermia. Considerable levels of PGE, approximately 50% of the maximal level monitored at 24 h, were detected in 4-h culture supernatants of PEM after being exposed to either 42 or 43°C for 1 h. Constitutive PGE production by control cultures was minimal but detectable during the 24 h of culture (Fig. 2).

We examined the effect of exposure time on the production of PGE. Cultured PEM were heated at 41, 42, and 43°C for various periods of time. Cells were then further incubated for an additional 24 h after which PGE levels in culture supernatants were determined. The effect of hyperthermia on the stimulation of PGE production was dependent on thermal doses; treatment for 10–20 min at 42°C did not induce a significant increase in the production of PGE. A minimum of 40–60 min incubation at 42°C was necessary to trigger a measurable increase in the synthesis and production of PGE by macrophages (Fig. 3). Treatment for 90 min induced an even greater increase in PGE production. On the other hand, heating at 43°C for 30 min was sufficient to induce a considerable amount of PGE production by PEM.

Inhibition of PGE Production by Indomethacin. PGE is the main cyclooxygenase product released by macrophages in re-

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<th>Table 1 Cell viability as a function of time after heat treatment at various temperatures</th>
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Fig. 2. Kinetics of heat-induced PGE production by C3H/HeJ PEM. After exposure to 41, 42, and 43°C for 60 min, PEM (5 x 10⁶/flask) were further cultured at 37°C. After various time periods, supernatant media were harvested, centrifuged, filtered, and assayed for PGE. Data are means from duplicate determinations with a SD of 12% or less.

Fig. 3. Effect of thermal dose on the production of PGE by PEM. C3H/HeJ PEM (5 x 10⁶/flask) were heated at 41, 42, and 43°C for the time periods indicated. Cells were then transferred to a 37°C incubator and cultured for an additional 24 h after which PGE levels in the culture supernatants were determined. Data are means from duplicate determinations.

Production of PGE in Other Cell Lines. To study the specificity of PGE production, we examined whether the biosynthesis of PGE induced by hyperthermia also extended to established cell lines. One human monocytic cell line U-937 (18) and a mouse fibroblast cell line L-929 were used for this study. Previous studies by Kurland et al. (9) have shown that neither one can produce PGE either spontaneously or by the stimulation of endotoxin. Both tumor cells were cultured under the same conditions as those used for normal PEM and were heated at 42°C for 60 min in α-LH. Cells were then cultured for an additional 6, 24, and 48 h in the presence or absence of 5 x 10⁻⁷ M indomethacin after which PGE levels in culture supernatants were determined. Both U-937 and L-929 cells failed to produce significant amounts of PGE either spontaneously or after being subjected to hyperthermic treatment (Table 2). On the other hand, PEM were activated by heating at 42°C to secrete PGE which was inhibited by the addition of indomethacin.

DISCUSSION

It is well known that macrophages are good producers of PGEs in response to inflammatory stimuli including phagocytosis (8, 10, 11). In general, PGEs are produced via the cyclooxygenase pathway of arachidonic acid metabolism from membrane phospholipids subsequent to phospholipase activation (21). The present studies show that hyperthermia also stimulates the secretion of PGE by mouse PEM. The production of PGE by PEM is strictly dependent upon the temperature used; treatment above 41°C consistently stimulated the production of PGE at various levels; below 41°C, however, the effect was only minimal even after prolonged treatment (Figs. 1 and 3).

There are several possible mechanisms by which heat promotes PGE synthesis by macrophages. Heat is known to cause membrane protein unfolding or rearrangements as well as to induce plasma membrane damage (22) leading to the activation of phospholipase and liberation of arachidonate from mem-
brane-bound phospholipids or alternatively heat may activate cyclooxygenase directly to convert arachidonate to PGE precursors. Heat may also induce the release and secretion of cytoplasmic PGE by macrophages as a result of cell death and membrane leakage. Although hyperthermia is very cytotoxic at and above 42°C (Table 1), the fact that the increase of PGE in culture medium was inhibited by the addition of 5 x 10^{-7} M indomethacin or by the subsequent incubation of macrophages at 4°C strongly suggests that PGE is synthesized de novo, presumably through the activation of cyclooxygenase by hyperthermia and not due to the release of constitutive PGE through membrane “leakiness” as has been previously described (23). Since the addition of heat-killed PEM to fresh PEM cultures was not sufficient to trigger an elevated production of PGE, we concluded that the elevated production by PEM was due to the direct effect of hyperthermia rather than to phagocytosis of the dead cells by the surviving PEM.

The physiological role of elevated PGE production by heat exposure is a matter of speculation. As one of the most important inflammatory mediators, PGEs are known to exert a variety of inhibitory effects upon mononuclear phagocytes. Thus, PGEs have been shown to inhibit the production of mononuclear phagocytes from both bone marrow and other tissue sources, to suppress plasminogen activator secretion, and to inhibit phagocytosis (9, 10, 24). Others have shown that PGEs also regulate the proliferation and secretion of lymphokines by lymphocytes. These observations together indicate that PGEs act as feedback-inhibitory mediators of mononuclear phagocytes and other immunocytes. Thus, the production of PGEs by macrophages in response to heat treatment (as well as immune stimuli) may help to suppress their own activation in an autoregulatory mode of action. This notion is also supported by the fact that the production of PGEs by macrophages is a slow process; the maximal level of PGEs occurred approximately 24 h after heat treatment (Fig. 2).

Recent in vitro studies also have shown that PGEs inhibit the activation of macrophages to kill tumor cells (11). Cameron et al. (25) have shown that indomethacin can convert noncytotoxic macrophages from cancer patients to become cytotoxic towards allogeneic and/or autologous target cells. Their results suggest that noncompetent macrophages from cancer patients may secrete abnormally high quantities of PGEs leading to the inhibition of macrophage activation. Our results demonstrate that hyperthermia greatly stimulates PGE production by cultured normal mononuclear phagocytes. Whether hyperthermia also induces PGE production by macrophages (and other cells) in vivo has not been determined. However, macrophages are known to be present in significant numbers in both human and animal tumors (4, 5, 7). Thus, if heat treatment also induces the synthesis of inhibitory PGEs by tumor-associated macrophages, the overall therapeutic effect of hyperthermia may be reduced.

Heat treatment failed to promote the production of PGE by two cell lines, U-939 and L-929, used in this study. Interestingly, previous studies have shown that both cell lines were unable to produce PGE even after endotoxin stimulation (9). Of relevance to this work, a recent study by Krag et al. (26) showed that a malignant melanoma cell line also produces higher levels of PGE when cultured at higher temperatures. Thus, the synthesis of PGE may be an intrinsic but not unique property of PEM that, when properly activated, synthesize and secrete PGE. It can be concluded from this study that hyperthermia, under proper conditions, may act as a stimulating signal for mononuclear phagocytes leading to the synthesis and production of PGE.

In the past decade, hyperthermia has become an important modality in treating various kinds of cancers. The existing literature provides conflicting reports on the effects of hyperthermia on nonspecific host immune responses to malignant diseases. While several lines of evidence suggest that heat induces stimulation of the immune response, others have shown that whole body heating may increase metastases (12-14, 16). Part of these conflicting observations may be accounted for by the production of either positive or negative mediators such as PGEs and interferon (27) by macrophages and other tissue cells during the course of heat treatment. In addition to its profound effects on the immune responses, high PGE levels have also been suggested to be linked to the tumors of metastatic potential (6). Indeed, using an in vitro metastasis model, Young and Newby (28) recently demonstrated that both tumor-associated macrophages and PEM enhanced tumor cell migration that appeared to be mediated through PGE; secreted by these macrophage populations. This suggests that macrophages may enhance tumor dissemination in vivo by increasing the motility of the tumor cells. A better understanding of the basic biological responses of macrophages to heat treatment is likely to have importance in optimizing clinical hyperthermia schedule and also should help us to define better methods for augmenting host antitumor effects.

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REFERENCES

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Induction of Prostaglandin Production by Hyperthermia in Murine Peritoneal Exudate Macrophages

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